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Expression, Purification and Biocatalytic Studies of a Plant (*Arabidopsis thaliana*) Hydroxynitrile Lyase

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Abstract: ***Hydroxynitrile lyases are the versatile group of enzymes, which play a significant defensive role in plant system against microbial attack by producing cyanide via cyanogenesis of Cyanogenic Glycoside. In chemical industries, HNL is employed as an important industrial biocatalyst for the synthesis of chiral cyanohydrins. Cyanohydrins are biologically active compounds used in production of β-amino alcohols, α-hydroxy ketones and α-hydroxy acids that have importance as fine chemicals, agrochemicals and pharmaceuticals. Enzymes are present in cellular environment. Cell consists of biomolecules like carbohydrates, lipids, nucleic acids along with proteins. To analyse the functions and applications of enzymes, protein purification is necessary. Important part of biotechnology research is to use protein engineering techniques to design proteins with optimized properties for specific industrial applications. Sonication and high-speed centrifugation separate out protein from the non-protein component in the cell. Isolation of protein is achieved by precipitation with ammonium sulphate $(NH_4)_2SO_4$. Different proteins precipitate in different concentrations of $(NH_4)_2SO_4$. Proteins of higher molecular weight precipitate in lower concentrations of $(NH_4)_2SO_4$. Affinity chromatography is a vital technique for protein purification. Beads in chromatography column in cross-linkage to ligands, bind specifically to the target proteins. Protein is then removed from the column by rinsing with a solution, free of ligands. This method gives the purest result and highest specific activity. Aim of protein purification is to study biocatalytic activity, enzyme kinetics, stereo selectivity, enzyme biophysical and bio chemical properties etc.***

Keywords: Hydroxynitrile lyase, Biocatalyst, Cyanohydrin, Affinity chromatography, Enzyme kinetics, Stereo – selectivity

1. Introduction

Hydroxynitrile lyases are the key enzymes found in various cyanogenic plants, bacteria, millipedes and are recently discovered in non-cyanogenic plants too. Hydroxynitrile lyases (HNLs) are the enzymes which mediate the release of hydrogen cyanide (HCN) and aldehyde or ketone from cyanohydrins and catalyses enantioselective synthesis of cyanohydrins. As a high-toxic compound, HCN is a good weapon for defense against microbial and herbivores attack in cyanogenic plants.

HNL can enantio selectively produce cyanohydrins. The cyanohydrins and their derivatives are finding wide applications in industries for production of pharmaceuticals, agrochemicals and cosmetics. These cyanohydrins can be readily converted into other synthetically relevant building blocks like α-hydroxy carboxylic acids, α-hydroxy ketones and β-amino acids. HNLs catalyze the decomposition of cyanohydrins into corresponding aldehydes or ketones and HCN for defense against predators and microorganisms. The reverse reaction, which is the synthesis of optically active cyanohydrins, has gained the attention of scientists and industry. Optically active cyanohydrins, containing a hydroxyl and cyanogroups attached to the same carbon atom, are versatile building blocks in the fine chemical and pharmaceutical industries. In addition to this, few of them are also reported for asymmetric synthesis using Nitro-Aldol reaction. Also called Henry reaction synthesizes enantiopure β-nitro-alcohols, by forming a C-C bond combining a nucleophilic nitro alkane with an electrophilic aldehyde or ketone. The Henry products or Nitro-Aldol products are chiral intermediates for biosynthesis in pharmaceutical, agricultural and chemical industries.

HNLs are commonly found in Cyanogenic plant. Cyanogenic plants belong to families of Rosaceae, Clusiaceae, Linaceae, Euphorbiaceae, Gramineae, Olacaceae, Passifloraceae, and Filitaceae. HNLs are of two types-R-selective (*Pa*HNL, *At*HNL) and S-selective (*Me*HNL, *Hb*HNL, *Sb*HNL). *At*HNL is an exceptional HNL was recently discovered in a non-cyanogenic plant, scientifically known as *Arabidopsis thaliana (*Thale cress). All the HNLs having an α/β hydrolase fold, have the catalytic triad Ser-His-Asp in active sites. Ser acts as general base to deprotonate to cyanohydrin substrate. Earlier, only S selective HNLs were thought to have the α/β hydrolase fold. But discovery of AtHNL in the year 2007 changed the whole scenario. Homology model of *At*HNL was created based on the crystal structure of *Hb*HNL. *At*HNL was found to be structurally similar to S-selectivity of HbHNL with 45% identity and 67% sequence similarity. The purpose of selecting *At*HNL over *Hb*HNL is that *Hb*HNL suffers from long reaction time (approx.48 hours) and moderate enantiomeric excess is seen i. e., 92% enantiomeric excess in pH 7.

2. Literature Survey

Discovery of HNL was first done in the year 1837 by two German chemists Justus Von Liebig and Friedrich Wohler. Although enzymes that are to be used as catalyst for preparation of pharmaceuticals and fine chemicals can be produced by Recombinant DNA Technology, the demand arises for environment friendly catalysts, which led the

researchers to discover this new Hydroxynitrile lyases, that have novel substrate specificity. Asano et al. had screened for HNLs in 163 plants of 73 families using HPLC. HNLs belong to aldehyde lyases.

Some of the HNLs that have been reported, purified and characterized from several plants are almond (*Prunus amygdalus*), flax (*Linum usitatissimum*), cherry (*Prunus serotina*), peach (*Prunus persica*), capulin (*Prunus capuli*), rosary pea (*Abrus precatorius*), rubber tree (*Hevea brasiliensis*), cherrylaurel (*Prunus laurocerasus*), cassava (*Manihotesculenta*), Japanese apricot (*Prunus mume*), *Prunus lyonii*, *Phlebodium aureum*, *Ximenia americana*, *Eriobotrya japonica*, *Passiflora edulis*, etc.

In trying to expand the synthetic applicability of the HNL methodology HCN has been replaced by other nucleophiles to be added to carbonyl compounds catalyzed by these enzymes. Grounded on the medium of this biotransformation, pivotal parameters for similar indispensable reagents would be the molecular size and the pKa of the CH-acidic portion, which should be similar to that of HCN (pKa~9). Nitroalkane is an important substance class that meets these criteria. Their reaction with carbonyl compounds that is termed to be Nitroaldol or Henry reaction, comprises carboligation, which is of high synthetic value. The Henry reaction furnishes vicinal nitroalcohols, which can easily be transformed to a series of valuable intermediates such as, for example, 1, 2-aminoalcohols and α-hydroxy carboxylic acids. First such enzymatic report came from the addition of nitromethane to aldehydes in the presence of the hydroxynitrile lyase from *Hevea brasiliensis (Hb*HNL*).* Based on this analysis, the main product of the *Hb*HNL catalyzed addition of nitroethane to benzaldehyde is (*1S, 2R*)-2-nitro-1-phenylpropanol.

Recently, numerous approaches were proclaimed to identify new HNLs for biocatalytic processes by screening different cyanogenic plant extracts for HNL activity, yielding some novel enzyme sources. Approaches to identify novel enzymes based on similarities of sequence to known HNLs, have not yet been proved to be successful. Several sequences similar to *Me*HNL and *Hb*HNL are found in the genome of the non-cyanogenic model plant *Arabidopsis thaliana*. In the course of studies on structure–function relationships of α/β-hydrolases Martina Pohl et al have cloned several genes encoding Arabidopsis proteins with high sequence similarity to *Me*HNL and *Hb*HNL and expressed them in *E. coli*. Unexpectedly, one of them shows high activity towards mandelonitrile and also catalyses the cleavage of some other cyanohydrins derived from cyclohexanone and m-phenoxybenzaldehyde, while acetaldehyde, propionaldehyde, and acetone cyanohydrins are poor substrates.

In the current study, we have investigated about this R-specific HNL from Arabidopsis thaliana and its application in biocatalytic processes. The enzyme is a good alternative to currently known R-selective HNLs, such as PaHNL, for the production of R-cyanohydrins as it is readily available in technically relevant amounts by over expression in E. coli. Its extensive substrate range takes into account both aliphatic and aromatic aldehydes as well as ketones. As a primary R-specific HNL based on an α/β-hydrolase fold, its structure will provide relevant information pertaining to the enzyme mechanism of α/β-hydrolase fold-based HNLs. Due to the wide applicability of this reaction, the development of new chiral catalysts has aroused the interest of many groups and various reports have been appearing in the literature on the development of metal and nonmetal-based catalysts. Although enzymes as practical catalysts have been increasingly exploited for organic synthesis due to their mild reaction conditions and high selectivity, to our knowledge, there have been only a few reports of enzyme-catalyzed Henry reactions. Among them, only the reaction catalyzed by S-HNL from Hevea brasiliensis (*Hb*HNL) showed enantioselectivity. We have studied an R-HNL containing an α/β-hydrolase fold from the non-cyanogenic plant Arabidopsis thaliana (*At*HNL) and found that it accepts nitromethane (MeNO2) as a donor in a reaction with aromatic aldehydes to yield optically active nitro alcohols (Henry reaction; nitro aldol reaction). In this study, we describe efforts to do an in vitro study of *At*HNL catalyzed synthesis of R-β-nitro alcohols using purified enzyme as described by Asano et al.

3. Background Information

Enzymes are present in cellular environment. Cell consists of biomolecules like carbohydrates, lipids, nucleic acids along with proteins. To study the functions of enzyme and applications of enzymes, protein purification is necessary. A crucial part of biotechnology research is to employ protein engineering techniques for designing and modifying proteins with optimized properties for specific industrial applications. To do this, scientists should be able to isolate and purify proteins of their target or interest, by which their conformations, substrate specificities, reactions with other ligands, and specific activities can be studied. After sonication of a whole cell or cell lysis, we can separate proteins from non-protein components like carbohydrates, lipids, nucleic acids & other cell components by centrifugation at high speed. Achievement of desired degree of protein purity is based on the intended end use of the protein. For some applications, a crude extract is enough.

However, for other uses such as in pharmaceuticals and food industry, a high level of purity of protein is required. In order to obtain this, several protein purification methods are generally used, in a series of graded purification steps. Each protein purification step usually results in product loss up to some extent. Achievement of highest level of purification in fewer steps is an ideal strategy for protein purification. The selection protein purification step is dependent on the size, charge, solubility and other properties of the target protein. The techniques mentioned here are suitable for purifying a single cytosolic protein. Purification of cytosolic protein complexes is more complicated and usually requires the use of more than one method.

The first step in purification of intracellular (inside the cell) proteins is preparation of a crude extract. The extract will comprise a complex mixture of all the proteins from the cell cytoplasm, some additional macromolecules, nutrients and cofactors. Crude extract may be employed for some applications in biotechnology.

However, subsequent purification steps must be followed if purity needs to be achieved. Crude protein is extracted by removal of cellular debris generated by cell lysis, which is accomplished by using chemicals and enzymes, sonication or a French press. The debris is discarded via the process of centrifugation and the supernatant is recovered. Crude preparations of extracellular proteins are collected by simply removing the cells via the process of centrifugation. For certain biotechnology applications, there are a demand for thermostable enzymes that can tolerate high temperatures without denaturing, while maintaining a high specific activity simultaneously. Organisms that produce them are called as extremophiles. An easy approach to purify a heat-resistant protein is to denature the other proteins in the mixture by heating, then cooling the solution (thus allowing the thermostable enzyme to reform or redissolve, if necessary). Centrifugation assists then in removing the denatured proteins.

In bulk protein purification, the first step to isolate proteins is precipitation with ammonium sulfate $(NH_4)_2SO_4$. Different proteins use to precipitate in different concentrations of ammonium sulfate. Generally, proteins of higher molecular weight precipitate in lower concentrations of ammonium sulfate $(NH_4)_2SO_4$. Salt precipitation does not work out to produce a highly purified protein but can assist in removing some unwanted proteins in a mixture and in concentrating the sample. This is performed by adding increasing amounts of ammonium sulfate and collecting different proteins, which will precipitate in different concentrations of ammonium sulfate. Ammonium sulfate can be removed by dialysis. One merit of this technique is that it can be carried out inexpensively with very large volumes. Acetone precipitation is also preferred for precipitation and concentration of proteins. Experiments are usually performed at 4°C. Ice-cold acetone of 4 volume, containing 20 mM DTT was added to 1 volume of protein sample. The mixture was vortexed and incubated at-20°C for 1 hour. This was followed by centrifugation at 10000 rpm for 15 min at 4°C. The pellet was air-dried following to the discard of the supernatant.

The development of methodologies for protein purification has been an important pre-requisite for many of the advancements implemented in biotechnology. Protein purification can vary from simple single step precipitation procedure to a large-scale validated production process. To reach the desired purity, often more than one purification step is essential. The pathway to attain efficient protein purification is to select the most efficient technique, optimize their performance in order to suit the requirements and combine them in a most appropriate manner to maximize yield. This can minimize the unnecessary steps. Most purification methodologies involve some form of chromatography. With respect to this, chromatography has become a vital tool in every laboratory, where protein purification is needed. The availability of different chromatography techniques with different selectivities provide a powerful combination for the purification of many biomolecules.

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4. Objective

*At*HNL is an important non-cyanogenic R-selective α/β hydrolase fold HNL. Study of biocatalytic properties contribute well towards synthesis of R-cyanohydrin. However, this enzyme catalyses production of R-β nitro alcohol. Objectives framed for the report is:

- Expression, Purification and Biocatalysis of *At*HNL

5. Materials and Methods

Materials

Luria Bertani broth medium**,** Glycerol stock [containing transformed cell, ***E. coli* BL21 (DE3)],** Kanamycin**,** Isopropyl β-D-1-thiogalactopyranoside (IPTG), KH_2PO_4 (Potassium dihydrogen phosphate), K_2HPO_4 (Potassium mono hydrogen phosphate), Mandelonitrile, Lysozyme

Apparatus

Conical flask, Autoclave, Incubator, Centrifuge, Spectrophotometer, Sonicator, Stirrer, Nanodrop, pH meter

Preparation of Primary culture

20 ml of primary media was prepared by adding 0.5 g of LB (Luria Bertani broth) in 20 ml of distilled water. Then it was subjected to autoclave at 121°C, 15 lbs. pressure for 20 mins. To the autoclaved media at room temperature, 15 µL of glycerol stock containing AtHNL transformed cells [*E. coli* BL21 (DE3)], 20 µL Kanamycin were added in sterilized conditions. Then it was kept in an incubator for 12 hrs.200 rpm at 37°C for incubation.

Preparation of Secondary culture

Two liters of secondary media were prepared by adding 50 g of LB (Luria Bertani broth) into two liters of distilled water. The media was autoclaved at 121°C, 15 lbs. pressure for 20 mins. After 12 hrs. of incubation of primary culture, 1% of it was added to the secondary media (10 ml in 1000 ml of LB media). To the secondary culture 0.05 mg/mL of kanamycin was added and kept for 6 hrs. of incubation at 37°C, 200 rpm. After 3 hours O. D was checked and when it showed ~0.8 value, then 0.5% (5 mL in 1000 mL) of IPTG was added to the media and further kept for 5-6 hours incubation at 200 rpm in 30°C.

Cell harvesting

After 5 hrs. of incubation, culture was centrifuged at 5000 rpm for 15 min at 4°C. The pellet was collected & weighed. This was followed by resuspension with 20mM KPB.

Sonication

It was sonicated at 4°C 30 KHz amplitude with 20 on and 40 off cycle (the 40 second off is done to avoid protein degradation that may result from continuous treatment) for 25 minutes for cell lysis. Lysed cells were then centrifuged at 10000 rpm for 50 minutes that gave the pellet and supernatant. The supernatant carried the crude protein that needs to be further purified.

Figure 1: Shows ultrasonication of sample to extract protein

Ammonium Sulphate Precipitation

To the supernatant 0-25% of ammonium sulphate was added and kept for stirring for 30 mins at 4°C. Then the solution was centrifuged at 10000 rpm for 10 mins at 4°C. After centrifugation pellet and supernatant were collected and the activity for cleavage assay was measured.25% Ammonium sulphate precipitated pellet was stored at 4°C. To the 25% Ammonium sulphate supernatant 21.239g of 25-75% Ammonium sulphate was added. [317g/1000mL (our 25% A. S supernatant volume is 67mL)]. This was kept on a stirrer at 4 °C for 1 hour 30 mins. Then, this solution was kept in a centrifuge at 4°C at 10000 rpm for 10 mins. After centrifugation pellet and supernatant collected separately and stored at 4°C.75% ammonium sulphate pellet and supernatant activity was checked by cleavage assay. Following to this, the protein concentration of pellet, supernatant (i. e.25%, 75%) was measured using Nano drop.

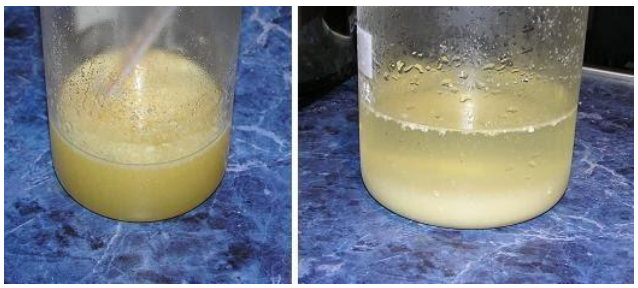

Figure 2: Shows the Ammonium sulphate precipitation

Protein Purification by Ni-NTA Chromatography

A chromatography column consisting of nickel-agarose beads is utilized for purifying proteins with histidine tags**.** The primary idea is to use a resin, containing a molecular fragment that specifically interacts with the species of interest. The species of interest gets attracted towards the affinity resin and binds with it while the other compounds present in the mixture gets passed through the column. Next to this, elution of species of interest can be performed. Affinity chromatography resin contains ligand or substrate that is attached covalently**.**

Table 1: Shows the components of all buffer required for protein purification

Buffers	Imidazole	NaCl	KPB	Distilled water
Binding Buffer	20 mM (1 ml)	300 mM (15 ml)	20 mM (1 mL)	33 mL
Washing Buffer	50 mM (2.5 ml)	300 mM (15 ml)	20 mM (1 mL)	31.50 mL
Elution Buffer	500 mM (25 ml)	300 mM (15 ml)	20 mM (1 mL)	9 mL

Note*: NaCl i. e., Sodium Chloride and KPB i. e., Potassium Phosphate Buffer*

First 10 mL of binding buffer was added and this was collected. Then again 10 mL of binding buffer was added and was collected outside. Then 10 mL dialyzed protein was added to binding column and placed on a rocker at 4°C for 30 mins**.** After 30 mins, column was placed in a stand and just drained by gravity. Then 10 mL of wash buffer was added twice and collected in a wash collecting falcon as washed protein**.1**0 mL of elution buffer was then added and it was collected in elution collecting falcon as elution protein. Elution protein contains our desired protein. These steps were repeated thrice**.** After column separation elution protein was collected and protein concentration was checked at Nano drop**.**

Dialysis

Eluted protein was subjected to dialysis to remove Imidazole. In dialysis a semipermeable membrane, eliminates proteins less than 10 kDa. This is used to separate small molecules and proteins based upon their size. A dialysis bag made of a semipermeable membrane (cellulose), having small pores is used. Diffusion of solutes and ultrafiltration of fluid across a semipermeable membrane until equilibrium state is reached. Before starting of dialysis, dialysis bags were continuously washed with distilled water for 5 mins. At one end, the bag was clipped first and then the protein sample was loaded. The other side of the bag was again clipped and this was kept in 2L of 20 mM KPB. Then it was left to be stirred by the magnetic stirrer for 3 hours at 4°C. After 3 hours, buffer was changed and the bags were again kept for stirring at 4°C for another 3 hours. Following to this, in another interval of 3 hours, the buffer was again changed and bags were kept on stirrer for overnight at 4°C. After completion of dialysis, dialyzed protein was taken in an Amicon tube and was centrifuged at 7000 rpm at 4°C for 15 mins. The protein was further stored at 4°C for various biocatalyst tests like Cyanohydrin cleavage and synthesis, Mandelonitrile cleavage and synthesis and HPLC analysis.

SDS PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis)

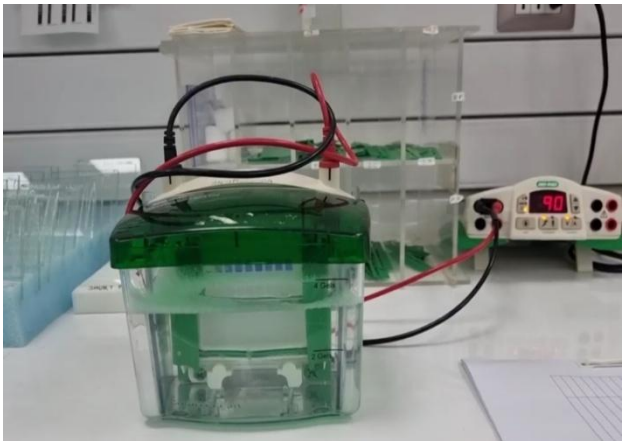

Figure 3: shows the SDS PAGE of protein sample

First separating gel was prepared & poured in to a SDS gel chamber then water was removed and stacking gel was added and comb was placed, allowed to solidify. Then 1x SDS buffer was added to SDS chamber and comb was removed. First ladder was loaded in to the well then samples were loaded. Before loading the samples in to the wells the samples were denatured at 100°C for 10 mins in dry bath and stained with SDS staining buffer. Separating gel was poured up to ¾ of SDS chamber then small amount of water was added, it was allowed to solidify separating gel. After loading the samples, electricity was supplied. After 3 hours, SDS gel was removed from the SDS chamber and was kept in a staining solution. It was then placed on a rocker for overnight. Next day, staining solution was removed and distaining solution I was added and kept on rocker for 1 hour. Following this, destaining solution II was added and kept on rocker for another 1 hour.

Table 2: Shows the components and their quantities of *(a) Stacking Gel (b) Resolving Gel*

(a)

H_2O	1.7 mL
30% Acrylamide	415 µL
1.5M Tris Cl pH 8.6	315 µL
10% SDS	25 µL
10% Ammonium per sulphate	25 µL
Tetramethyl ethylene diamine	2.5 µL

(b)

H_2O	1.65 mL
30% Acrylamide	2.05 mL
1.5M Tris Cl pH 6.8	1.25 mL
10% SDS	50 µL
10% Ammonium per sulphate	50 µL
Tetramethyl ethylene diamine	05 µL

Cleavage Assay

To measure the concentration of the purified protein, the Nano-Drop spectrophotometer was used. The enzyme-activity was checked by monitoring the cleavage of Mandelonitrile to Benzaldehyde at 280nm using *Skan it* software. The reaction comprises of 50mM CPB (pH5.5), 67mM Mandelonitrile and enzyme (for control the enzyme was replaced by 20mM KPB (pH7.0). The activity was calculated with the difference between the measured absorbance from the test and the control reaction using the following formula:

$$\frac{U}{ml} = \frac{diff * V_{rxn} * 1000}{\varepsilon_{product} * b * t * V_{enz}}$$

Note: $\varepsilon_{product}$ *= Molar attenuation coefficient of benzaldehyde = 1380 mM* $^{-1}cm^{-1}$, $\boldsymbol{b}$ *= path length = 0.57 cm,* $\boldsymbol{t}$*= Reaction time,* $\boldsymbol{V_{enz}}$*= Enzyme Volume,* $\boldsymbol{V_{rxn}}$*= Total Reaction Volume,* ***diff****= difference in absorbance*

Biphasic Biocatalysis

Figure 4: shows the operation of Thermal Shaker

Total Reaction volume for Pure Enzyme was 1 mL (500 µL organic + 500 µL aqueous)

The reactions containing Enzyme, Potassium Phosphate Buffer, n-Butyl Acetate, Nitro methane (CH4NO2), Aldehyde in vials were kept in thermal shaker for bio catalysis. In every interval of 1 hour, 100 µL of Aliquot was taken with 200 µL of Hexane: Isopropyl Alcohol (9: 1) in 1.5 mL vials, having a pinch of Sodium sulphate. And this was subjected to vortex for mixing properly. The sample was then being centrifuged at 13000-15000 rpm at 4°C for 5-10 mins. The supernatant (upper organic layer) was collected carefully to be analyzed in HPLC.

HPLC (High Performance Liquid Chromatography) Analysis

Samples were analysed using HPLC with run conditions: Chiral Pak IB, flow rate-1ml/min and flow ratio-9: 1 (Hex: IPA) and calculated the percentage of conversion and percentage of enantiomeric excess.

6. Results and Discussion

Protein concentration results of the culture:

Table 3.1 Illustrating the concentration of 1st batch sample

1st Batch Sample	Concentration (mg/ml)
After sonication pellet	138.696
After sonication supernatant	54.779
75% ammonium sulphate pellet	94.814
75% ammonium sulphate supernatant	25.870
Binding protein	3.276
Wash protein	2.197
Elution protein	0.817
Pure protein	40.723

Table 3.2: Illustrating the concentration of 2nd batch sample

2nd Batch Sample	Concentration (mg/ml)
After sonication pellet	33.6
After sonication supernatant	57.831
75% ammonium sulphate pellet	36.81
75% ammonium sulphate supernatant	13.772
Binding protein	3.078
Wash protein	1.650
Elution protein	1.817
Pure protein	22.1

Table 3.3: Illustrating the concentration of 3rd batch sample

3rd Batch Sample	Concentration (mg/ml)
After sonication pellet	32.620
After sonication supernatant	38.317
75% ammonium sulphate pellet	19.856
75% ammonium sulphate supernatant	13.048
Binding protein	0.178
Wash protein	1.817
Elution protein	1.314
Pure protein	9.390
Flow through protein	4.059

Enzyme Activity Results

Table 4: Illustrating total concentration and activity of pure protein

Batch number	Enzyme concentration (mg/ml)	Enzyme activity (U/ml)	Specific Activity (U/mg)
I	40.723	20.02	8.22
II	22.1	30.73	307.33
III	9.390	33.11	310.98

Analysis of Purified Enzyme by SDS-PAGE

The samples loaded at Lanes: *1) Ladder, 2) After sonication pellet, 3) After sonication supernatant, 4) Wash Protein, 5) $(NH_4)_2SO_4$ supernatant, 6) Binding Protein, 7) Elution Protein, 8) Pure Protein.*

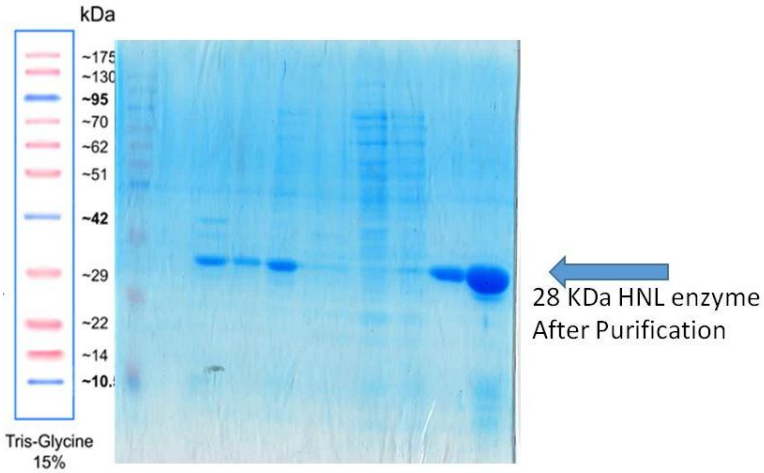

Figure 5: Illustrates the result of SDS PAGE denoting a 28 KDa band of pure protein

Analysis of biocatalytic product using HPLC

The retention times of benzaldehyde and the respective (*R)* and (*S)*-NPE were first seen on HPLC (IB-column, Flow rate was 1 ml/min) which tabulated below.

Table 5: Illustrating flow ratio and retention time of Benzaldehyde and the respective (*R)* and (*S)* – NPE

C. No.:	Samples	Flow Ratio	Dilution	Retention Times
1	Benzaldehyde, (*R)* and (*S)*-NPE	90: 10	-	4.760, 11.140, 12.807

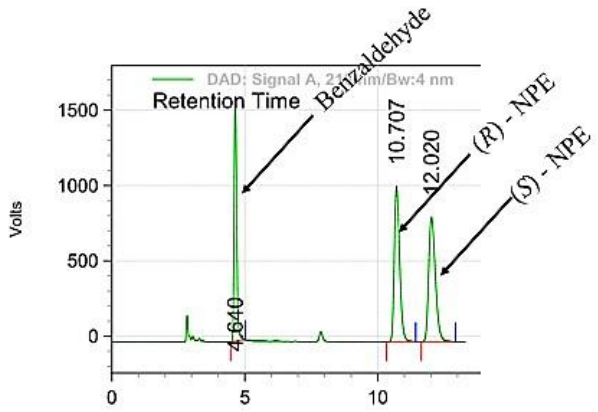

Figure 6: Illustrates the standard run on HPLC using Benzaldehyde + racemic NPE (*2-nitro-1-phenylethanol)*

After Standardization samples of *At*HNL catalysed synthesis reaction were run through HPLC. The % area of peaks were measured. Along with that, the % conversion (% con) and the % enantiomeric exchange (% ee) was calculated.

$$\%con = \frac{R+S}{R+S+BA*CF} * 100$$

$$\%ee = \frac{R-S}{R+S} * 100$$

R*= % Area of (R)-peak,* ***S****= % Area of (S)-peak,* ***BA****= % Area of benzaldehyde-peak,* ***CF****= conversion factor of benzaldehyde*

The % conversion shows how much of the reactant got transformed into product. The % enantiomeric exchange gives information about the enantiomeric specificity of the enzyme.

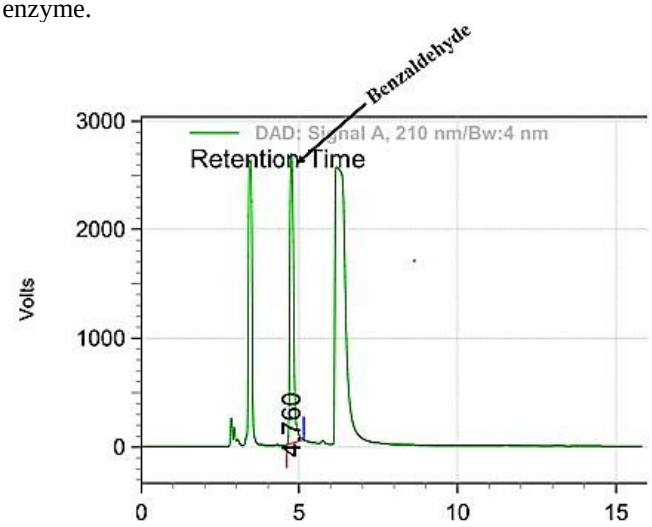

Figure 7: Illustrates the control reaction using KPB (Potassium Phosphate Buffer) replacing the enzyme

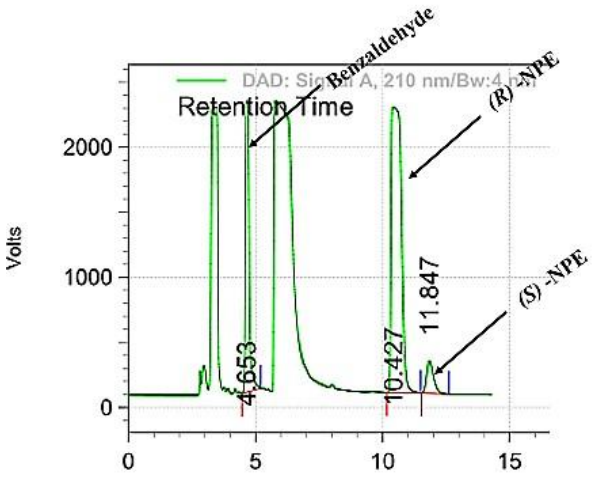

Figure 8: Illustrates the synthesis reaction using wild type enzyme

7. Interpretation and Analysis of Wild type *At*HNL enzyme

Wild type *At*HNL enzyme was expressed in *E. coli* BL21 (DE3) cells and purified by Ni-NTA affinity chromatography. The purified enzyme was concentrated to 19.586 mg/mL and total volume obtained after concentration was 4ml. The total amount of enzyme obtained from 2 L culture was 78 mg. The specific activity of the purified variant was 25.207 U/mg. The %conversion and % ee of the wild type towards the synthesis of (*R*)-2-nitrophenyl ethanol is been tabulated below:

Table 6: Illustrating % conversion and % enantiomeric excess

Substrates	Wild Type	
	% Conv	% ee
Benzaldehyde	44.86	96.27

8. Conclusion

HNLs are efficient biocatalysts in the synthesis of chiral cyanohydrins. In this study, we tried to check for the Nitro-Aldol activity of the wild type enzyme and calculated % conversion and % ee. We have performed this experiment about 3 times. The protein expression results clearly indicate that there was soluble plant HNL expression. Affinity based

purification was also successful in all the 3 experiments that are purified. We have further performed its biochemical characterization with respect to its protein concentration using Nano-drop, enzymatic purity by SDS-PAGE and enzymatic assay i. e., HNL assay. The purified enzymes have also showed their catalytic ability towards the stereoselective synthesis of Henry products. Our results showed that the purified *At*HNL has a specific activity of 310.98 U/mg and an enzymatic activity of 33 U/mL The purified enzymes have also shown their specific activity towards mandelonitrile cleavage and biocatalytic ability toward the stereo selective synthesis of Henry products. These studies accomplished toward the thesis have shown our effort to report the in-vitro biocatalytic potential of *At*HNL.

9. Future Scope

As the enantiomeric excess of the wild type enzyme is found to be 96.27 %, this would pave the pathway for the production of enantiopure drugs, which targets a single organ. Enhanced enantiomeric exchange may be achievable by experimenting with other variants of enzyme. In chemical industries, HNL can be employed as an important industrial biocatalyst for the synthesis of chiral Cyanohydrins by exploiting the reversible enzymatic reaction. Cyanohydrins are vital biologically active compounds used in production of β-amino alcohols, α-hydroxy ketone and α-hydroxy acids that have importance as fine chemicals, agrochemicals and pharmaceuticals. These components form a chief constituent of variety important drugs and can aid in the large-scale production of cardiovascular drugs, antihypertensive drugs, antimalarial drugs, etc. Production of single enantiomer drug, which is a recent trend can help in separating toxicity and efficacy.

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