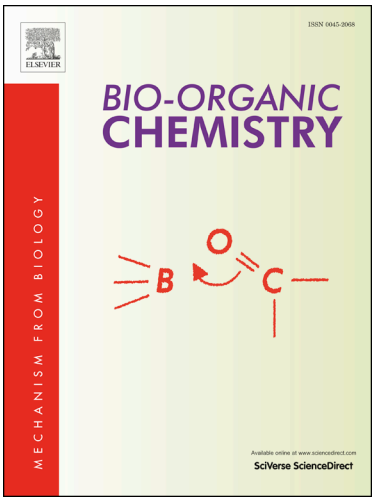

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Immobilized *Baliospermum montanum* hydroxynitrile lyase catalyzed synthesis of chiral cyanohydrins

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Abstract

Hydroxynitrile lyase (HNL) catalyzed enantioselective C-C bond formation is an efficient approach to synthesize chiral cyanohydrins which are important building blocks in the synthesis of a number of fine chemicals, agrochemicals and pharmaceuticals. Immobilization of HNL is known to provide robustness, reusability and in some cases also enhances activity and selectivity.

We optimized the preparation of immobilization of *Baliospermium montanum* HNL (*Bm*HNL) by cross linking enzyme aggregate (CLEA) method and characterized it by SEM. Optimization of biocatalytic parameters was performed to obtain highest % conversion and ee of (*S*)-mandelonitrile from benzaldehyde using CLEA-*Bm*HNL. The optimized reaction parameters were: 20 minutes of reaction time, 7 U of CLEA-*Bm*HNL, 1.2 mM substrate, and 300 mM citrate buffer pH 4.2, that synthesized (*S*)-mandelonitrile in ~99% ee and ~60% conversion. Addition of organic solvent in CLEA-*Bm*HNL biocatalysis did not improve in % ee or conversion of product unlike other CLEA-HNLs. CLEA-*Bm*HNL could be successfully reused for eight consecutive cycles without loss of conversion or product formation and five cycles with a little loss in enantioselectivity. Eleven different chiral cyanohydrins were synthesized under optimal biocatalytic conditions in up to 99% ee and 59% conversion, however the % conversion and ee varied for different products. CLEA-*Bm*HNL has improved the enantioselectivity of (*S*)-

mandelonitrile synthesis compared to the use of purified *Bm*HNL. Nine aldehydes not tested earlier with *Bm*HNL were converted into their corresponding (*S*)-cyanohydrins for the first time using CLEA-*Bm*HNL. Among the eleven (*S*)-cyanohydrins syntheses reported here, eight of them have not been synthesized by any CLEA-HNL. Overall, this study showed preparation, characterization of a stable, robust and recyclable biocatalyst i.e. CLEA-*Bm*HNL and its biocatalytic application in the synthesis of different (*S*)-aromatic cyanohydrins.

Keywords: Hydroxynitrile lyase, chiral cyanohydrins, cross-linked enzyme aggregates, asymmetric synthesis, biocatalysis, immobilization, recyclability.

1. Introduction

Hydroxynitrile lyase (HNL) are known to catalyze the synthesis of chiral cyanohydrins[1–3]. Optically pure cyanohydrins are useful molecules in the preparation of pharmaceutical intermediates, agrochemicals and fine chemicals [1,2,4]. The importance of these enantiopure cyanohydrins and the environment friendly method that the HNL catalysis provides, both enhances the significance of HNL research. Thus, there is a constant demand to discover new HNLs, [3,5–9] enzymatic methods to synthesize chiral cyanohydrins, improving enzyme's biocatalytic properties etc. Among the several α/β hydrolase fold HNLs reported, *Bm*HNL has shown unique biocatalytic features in terms of its substrate preference [10,11]. It prefers aromatic aldehydes as substrates 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.

One of the common problem in HNL biocatalysis is spontaneous formation of racemic cyanohydrin at pH 5 or above. To avoid the formation of racemic cyanohydrin which contributes in decreasing the enantiomeric excess (ee) of the biocatalytically produced chiral cyanohydrin,

usually HNL biocatalysis is carried out at (i) low pH, (ii) low temperature and (iii) in presence of organic solvent. Use of organic solvent helps in (i) lowering the substrate concentration in aqueous phase[12] which could be a reason for minimizing the racemic cyanohydrin formation, (ii) extraction of product, and (iii) may minimize degradation of product. However, use of organic solvent and low pH may reduce the stability of the enzyme. 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. Another important character of a biocatalyst is its reusability. Reusability 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.

2. Experimental

2.1. Chemicals and materials

*Bm*HNL (LOCUS: AB505969) synthetic gene cloned into pUC57 was procured from Gene Script, USA and sub-cloned into pCold1 plasmid at *BamH*I and *Sal*I. Culture media and ampicillin were purchased from HiMedia laboratory Pvt. Ltd, India. Isopropyl-β-D-1-thiogalactopyranoside (IPTG) was purchased from BR-BIOCHEM Pvt. Ltd, India. Glutaraldehyde was obtained from Molychem, India. Aldehydes and mandelonitrile were purchased from Sigma Aldrich, AVRA, SRL and Alfa-Aesar. HPLC grade solvents were obtained from RANKEM, Molychem, FINAR, and SRL.

2.2. Preparation of crude enzyme extract

A single colony of *E.coli* BL21 (DE3) containing the recombinant plasmid pCold1-*Bm*HNL was inoculated into 15 mL of LB broth containing 100 µg/mL ampicillin and incubated at 37°C, 200 rpm for 16 h. This primary culture was transferred into a 1 L of fresh culture medium in 1:100 ratio, incubated at 37 ºC until OD_{600} reached 0.5-0.6. Then cold-shock at 4°C for 2 h was performed followed by induction with IPTG to a final concentration of 1 mM and incubated at 18°C, 200 rpm for 24 h. Cells were harvested, the cell pellet was suspended in 80 mL of 20 mM potassium phosphate buffer (pH 7.0) and then disrupted by a sonicator (Sonics & materials INC (USA/VCX500) for 5x6 min at 25 KHz. The lysed cells were centrifuged at 12857*g*, 4°C for 45 minutes. The resulting supernatant was used as crude enzyme. The protein content was measured by a Nanodrop.

2.3. Preparation of *Bm*HNL aggregates

*Bm*HNL cell lysate (1 volume) was precipitated by adding 9 volumes of different precipitating agents e.g. ammonium sulfate (AS), *t*-butyl methyl ether (TBME), acetone, acetonitrile (AcN), methanol, isopropyl alcohol (IPA), dimethylformamide (DMF) and 1,2-dimethoxyethane (DME). Each of the nine mixtures were 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.

2.4. Optimization of ratio of cross-linking agent

Five best precipitating agents i.e. AS, TBME, AcN, IPA and DME of section 2.3 were selected and tested in the process of optimization of volume of cross linking agent. To the mixture of crude *Bm*HNL and precipitating agent (1:9 v/v), the cross-linker i.e. 25% solution of glutaraldehyde 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 these mixtures were shook 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.

2.4.1. 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 after shaking at 4°C for 6 h over a rocker, 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.

2.5. Characterization of CLEA-*Bm*HNL by scanning electron microscope

The CLEA-*Bm*HNL sample was air-dried over 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.

2.6. HNL assay

HNL activity was monitored using a Multiskan GO UV-Visible spectrophotometer at 25 ^{o}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.

2.7. Synthesis of racemic cyanohydrins

Racemic cyanohydrins were synthesized in order to use them as analytical standards. More than one method was used to synthesize different racemic cyanohydrins that involved KCN, TMSCN and acetone cyanohydrin as a cyanide donor. Two racemic cyanohydrins i.e. cyanohydrins of 2,4-dimethoxybenzaldehyde and *trans*-cinnamaldehyde were prepared using KCN as a cyanide donor [20]. Five mmoles of an aldehyde was dissolved in 10 mL of glacial acetic acid in a 100 mL round bottom flask, 3 equivalents of KCN in 10 mL of water was added to the mixture. The reaction mixture was stirred at 4°C and reaction progress was monitored by TLC. Finally, 25 mL

of water was added to the reaction mixture and the acid was neutralized by the addition of saturated $NaHCO_3$ solution. The reaction mixture was extracted by diethyl ether with consecutive washes of water and saturated NaCl solution. The organic extract was dried over anhydrous Na_2SO_4 followed by evaporation of the solvent under reduced pressure. Purification of products was done by flash chromatography (hexane/ethyl acetate: 85/15). Racemic cyanohydrins of 2-phenyl acetaldehyde, 3-phenoxybenzaldehyde and 3-benzyloxybenzaldehyde were prepared by using trimethylsilyl cyanide (TMSCN) as a cyanide source and lithium chloride (LiCl) as a catalyst in solvent-free medium [21,22]. To a pre-cooled 50 mL round bottom flask containing 5 mmoles of an aldehyde, 3 equivalents of TMSCN (caution: low temperature is must to avoid vigorous reaction) was added followed by 2-3 drops of 100 mM LiCl pre-dissolved in THF. Reaction was continued by stirring at 25°C and monitored by TLC. After completion, 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 consecutive washes of saturated $NaHCO_3$ and brine. The organic extract was dried over anhydrous Na_2SO_4, evaporated the solvent under reduced pressure and products purified by flash chromatography (hexane/ethyl acetate: 85/15). Four aromatic aldehydes e.g. 3,5-dimethoxybenzaldehyde, 2,5-dimethoxybenzaldehyde, 4-benzyloxybenzaldehyde, and 4-hydroxybenzaldehyde were converted into corresponding racemic cyanohydrins using acetone cyanohydrin as a cyanide donor by a modified protocol [23]. The modification includes the addition of $NaHCO_3$ as a base catalyst, aldehydes pre-dissolved in dichloromethane (DCM) and using water instead of the aliphatic amine as a solvent. Ten mmol of an aldehyde and 100 mmol of acetone cyanohydrin (10 equivalents) were added to a 100 mL round bottom flask containing 10 mL of 5% w/v aqueous $NaHCO_3$ solution. A 20 mL of DCM was also added to minimize the

dissociation of the cyanohydrin product formed. The reaction mixture was stirred at 25°C and monitored by TLC. The reaction was stopped by acidifying the mixture with 1N HCl, extracted using DCM with consecutive washes of water. The organic extract was dried over anhydrous Na_2SO_4, solvents evaporated under reduced pressure and the mixture was purified by flash chromatography (eluent hexane/ethyl acetate: 85/15; acidified by dil. HCl). 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.

2.8. 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 for 45 minutes.

The % of ee of a product was calculated using formula

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

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

(*S*-MN)rxn and (*S*-MN)control represent the area of (*S*)-mandelonitrile peak in the biotransformation and control respectively. (*R*-MN)rxn and (*R*-MN)control represent similarly for the (*R*)-mandelonitrile peak. BA: peak area of benzaldehyde.

2.9. Optimization of substrate concentration

Optimum substrate concentration for the enantioselective synthesis of mandelonitrile was determined by varying the concentration of benzaldehyde 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, equivalent to a final concentration 0.4 mM 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 (table S1) to find the % conversion and ee of mandelonitrile synthesized.

2.10. 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 number of enzyme units against two substrate concentrations i.e. 0.4 mM and 1.2 mM. Two different sets of reactions were carried out for different substrate concentrations. The 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 768 µL of 300 mM citrate buffer pH 4.2. Biocatalysis conditions and HPLC analysis of products was as described earlier.

2.11. 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 same as described earlier.

2.12. Effect of ratio of organic solvents

The effect of ratio of organic solvent to buffer in the biotransformation mixture was studied in the CLEA-*Bm*HNL catalyzed enantioselective synthesis of (*S*)-mandelonitrile. Among the organic solvents used in 2.11, the one showed 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.

2.13. Effect of buffer pH

Optimum pH for the CLEA-*Bm*HNL catalyzed enantioselective synthesis of (*S*)-mandelonitrile was investigated by varying the pH of the reaction buffer. The reaction mixture composed of 7 U of CLEA-*Bm*HNL (7 U), 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.

2.14. 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 was performed. In each cycle the reaction composition was kept same while the CLEA-*Bm*HNL of previous cycle was used for each successive round without wash.

2.15. 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 were carried out for 20-120 minutes. Percentage ee and conversion of each biocatalysis at different time was determined by chiral HPLC. Detail of HPLC analysis with varied hexane: IPA and flow rate for different cyanohydrins is given in table S1.

3. Results and discussion

3.1. CLEA-*Bm*HNL preparation

3.1.1. Preparation of *Bm*HNL aggregates

The first step in the preparation of CLEA of an enzyme is to prepare enzyme aggregates. Selection of precipitant for enzyme aggregation is crucial because achieving active enzyme after precipitation is more important than getting just enzyme aggregates. The enzyme aggregates resulted from different precipitants do not show same amount of catalytic activity. We studied eight different precipitants and tested the corresponding aggregates for their HNL activity. Among the different precipitants i.e. AS, TBME, acetone, AcN, methanol, IPA, DMF and DME used, AS based enzyme aggregate has showed highest *Bm*HNL activity i.e. 1.51 U/mg (Fig 1).

Ammonium sulfate is considered as a common precipitant and often used during protein purification. *Bm*HNL aggregates resulted from other precipitating agents such as AcN, TBME, IPA, and DME showed specific activity 1.43, 1.41, 1.2 and 1.19 U/mg respectively (Fig 1).

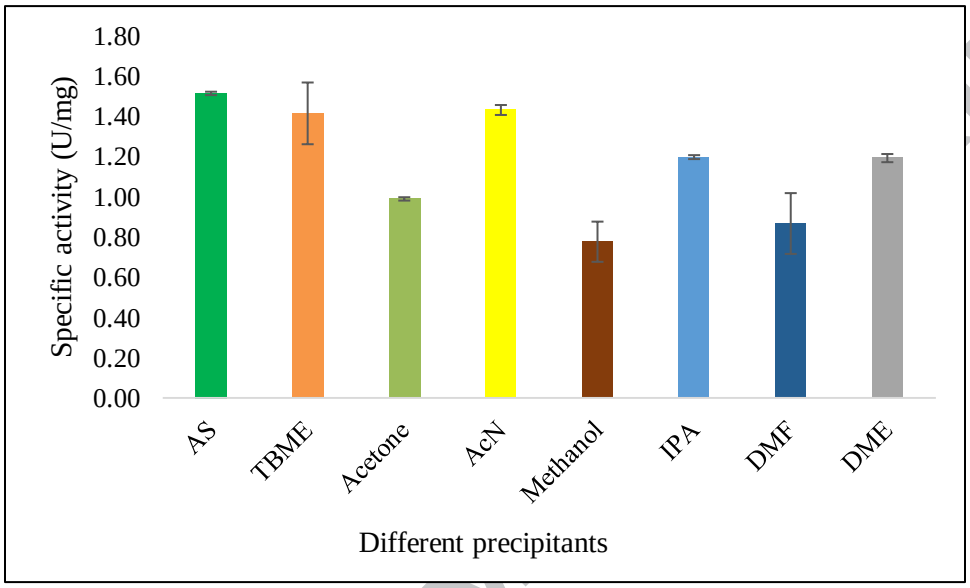

Figure 1: Effect of different precipitating agents

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

The second step is to cross-link the aggregated enzyme molecules. *Bm*HNL cross-linked enzyme aggregates have not been reported earlier. We optimized the preparation of *Bm*HNL-CLEA using the common cross-linker glutaraldehyde. The carbonyl groups of glutaraldehyde makes covalent linkage with amino group of a lysine residue present in the surface of an enzyme linking it to its neighbor, via Schiff base formation. Optimization of ratio of glutaraldehyde to protein is important in CLEA preparation to determine the best ratio that provides HNL activity to the CLEA. Use of high volume of glutaraldehyde can result in enzyme inactivation. Five best precipitants of section 3.1 were selected i.e. AS, TBME, AcN, IPA and DME for this study. In case of each of the five precipitants, to a total volume of 1 mL consisting of *B*mHNL cell lysate

and precipitant in 1:9 v/v, different volume of glutaraldehyde i.e. 0 to 600 µL was added (Fig 2). Among them, IPA has showed highest specific activity. Although IPA as a precipitant has showed less activity than AS in Fig 1, however after cross-linking, the corresponding CLEA has showed highest activity. The probable reason for this could be due to formation of some inactive CLEA or loss of some enzymatic activity in case of CLEA resulted from AS.

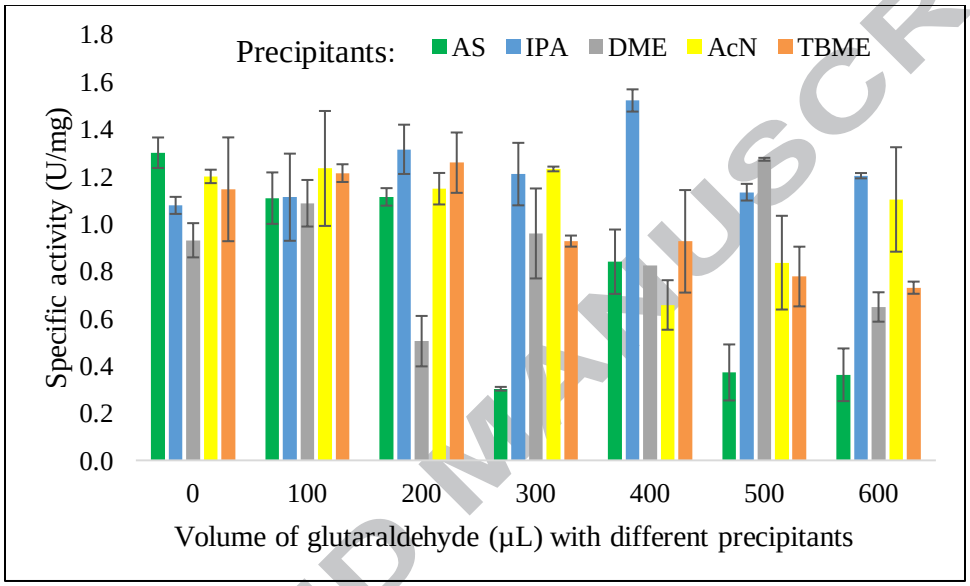

Figure 2: Effect of volume of glutaraldehyde along with different precipitants

Under optimal cross-linking conditions i.e. 1:9:4 v/v of *Bm*HNL cell lysate: IPA: glutaraldehyde, cross-linking time: 6 h and at 4°C CLEA-*Bm*HNL was prepared. CLEA-*Bm*HNL preparation was repeated several time. CLEA-*Bm*HNL with protein content 78.56 mg/mL and specific activity of 1.38 U/mg was used in successive experiments.

3.2. Characterization of CLEA-*Bm*HNL

3.2.1. Scanning electron microscope analysis of CLEA-*Bm*HNL:

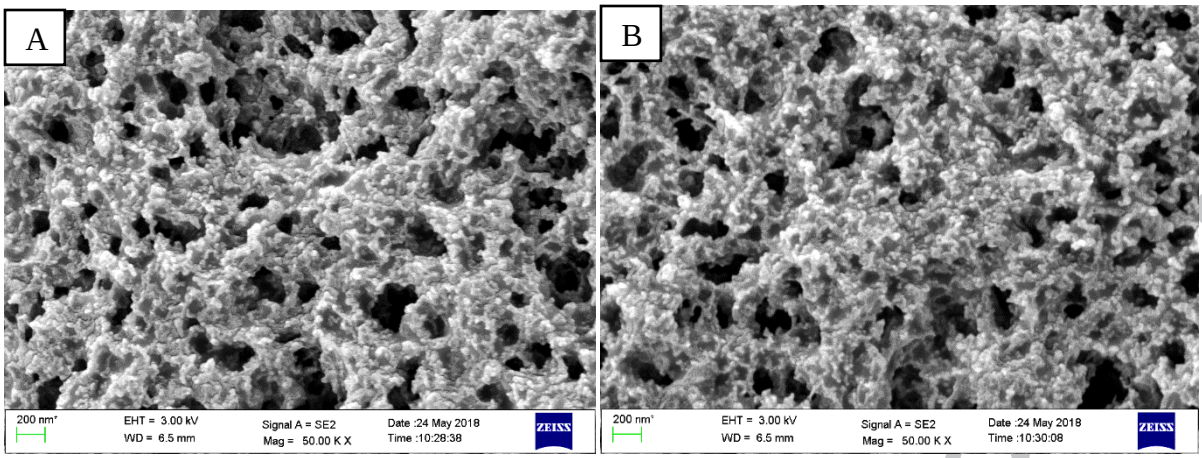

Figure 3 (A-B): SEM image of CLEA-*Bm*HNL prepared under optimized conditions

The FESEM images of CLEAs at different crosslinking times are shown in Figure 3A-B, which shows the aggregation of spherical CLEA particles. 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.

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

The success of preparation of immobilized enzyme can be described in terms of yield, efficiency and activity recovery as elaborated by Sheldon and Pelt [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, observed activity = CLEA activity

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%.

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

3.3.1. Reaction time

Chiral HPLC analysis of CLEA-*Bm*HNL catalyzed enantioselective synthesis of mandelonitrile at different time intervals showed 46.68% conversion and 90% ee of product in 20 min, which found to be the highest among different time points (Fig 4). In 25 min, 41.57% conversion and 87.4% ee of (*S*)-mandelonitrile was observed from benzaldehyde. Beyond 25 min, % ee and conversion both were decreased. Decrease in % ee could be due to the cleavage of (*S*)-mandelonitrile to benzaldehyde i.e. the reverse reaction, with longer reaction time. Such cleavage could also be a possible reason for decrease in conversion observed in Fig 4. *Me*HNL-CLEA in a biphasic system is reported to show such decrease in % ee with increase in time [15]. Stereoselective synthesis of cyanohydrins by CLEA of other HNLs has been reported earlier. Reaction time of CLEA of different HNLs differ in the synthesis of chiral cyanohydrins. *Dt*HNL-CLEA has showed 99% conversion and 98% ee of (*R*)-mandelonitrile in 24 h [19]. *Pd*HNL-CLEA synthesized (*R*)-mandelonitrile in 99% yield and 99% ee in 72 h [24]. *Me*HNL-CLEA synthesized (*S*)-mandelonitrile in 97% ee and 96% conversion in 2 h while *Hb*HNL-CLEA in 72 h synthesized the same in 67% ee and 55% conversion [15].

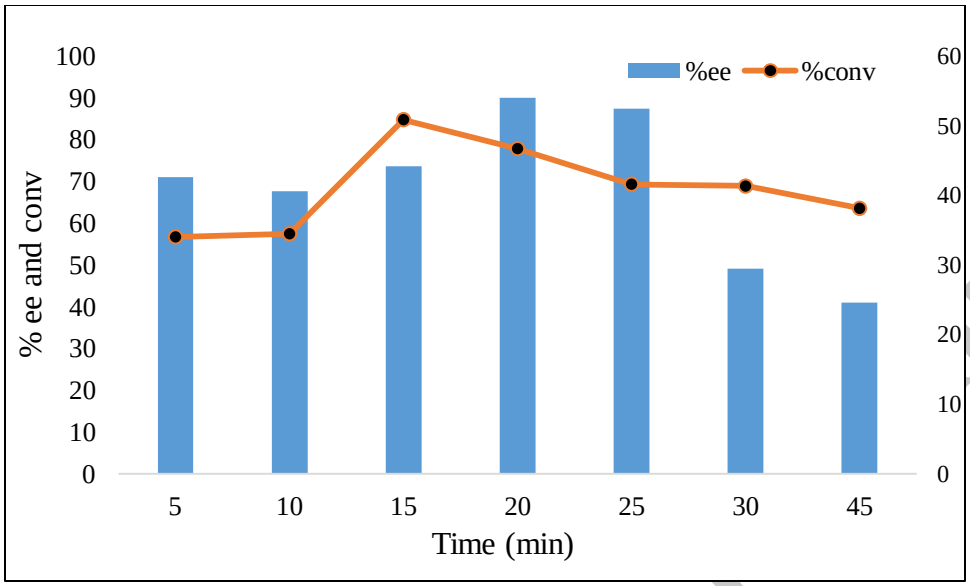

Figure 4: Time of biotransformation

3.3.2. Substrate concentration

Effect of different benzaldehyde concentrations in the CLEA-*Bm*HNL catalyzed synthesis of (*S*)-mandelonitrile was investigated (Fig 5) by performing the biotransformation for 20 minutes as per 3.3.1. Benzaldehyde concentration was varied from 0.4 to 2.2 mM in different biocatalysis. In case of 0.4 mM benzaldehyde, CLEA-*Bm*HNL showed highest i.e. ~93% ee and 42.8% conversion. With 1 and 1.2 mM concentrations, also high % ee was observed i.e. 91.2 and 90.2% respectively while % conversion was 42.7 and 45.5 respectively. Beyond 1.2 mM substrate concentration, % ee and conversion both decreased. However the reason for low % ee of product with 0.6 mM benzaldehyde is not clear.

Yildirim *et al* reported almost 0.056 M benzaldehyde (100 µL of 1 M benzaldehyde) for the synthesis of (*R*)-mandelonitrile using CLEA-*Pd*HNL [18]. Alagöz *et al* also reported the same concentration of benzaldehyde in CLEA-*Pd*HNL biocatalysis [24]. In CLEA-*Pa*HNL based biotransformations, 0.2 M aldehyde was used by Langen *et al* [25]. Torrelo *et al* reported the

recyclability of *Me*HNL-CLEA by using 0.5 M benzaldehyde [26]. Cabirol *et al* reported the synthesis of cyanohydrins catalyzed by CLEA-*Hb*HNL and CLEA-*Me*HNL by using 0.5 M substrate concentration [15]. However for these different CLEA-HNL biocatalysis, enzyme concentration also differs. Therefore, a comparison of optimal substrate concentration between different CLEA-HNLs would be difficult.

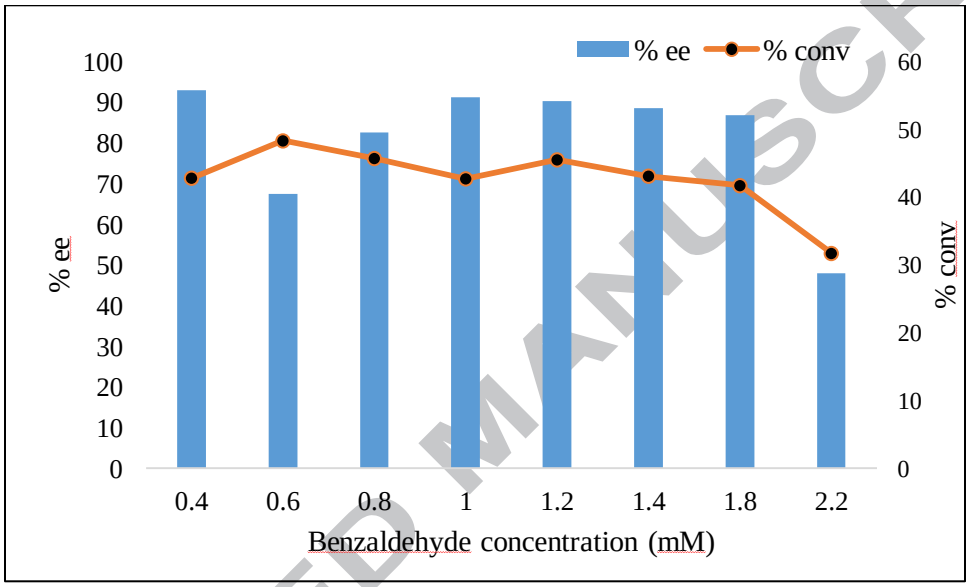

Figure 5: Effect of benzaldehyde concentration in the synthesis of (*S*)-mandelonitrile

3.3.3. Amount of enzyme

Two best substrate concentrations from the previous experiment i.e. 0.4 and 1.2 mM benzaldehyde were selected for the current study. Different amount of enzyme i.e. 5 to 15 U were used in the enantioselective synthesis of mandelonitrile (Fig 6). With 1.2 mM benzaldehyde, 92.7% ee and 60.5% conversion was observed in case of 7 U of CLEA-*Bm*HNL. The % ee decreased with increasing enzyme units such as with 15 U the % ee was 66 while the % conversion increased up to 71%. In case of 0.4 mM substrate concentration, the % ee and conversion was also found to be highest with 7 U of enzyme i.e. 88.2 and 35.4 respectively. Both

the substrate concentrations showed similar trend of decrease in % ee with increase in amount of enzyme. 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.

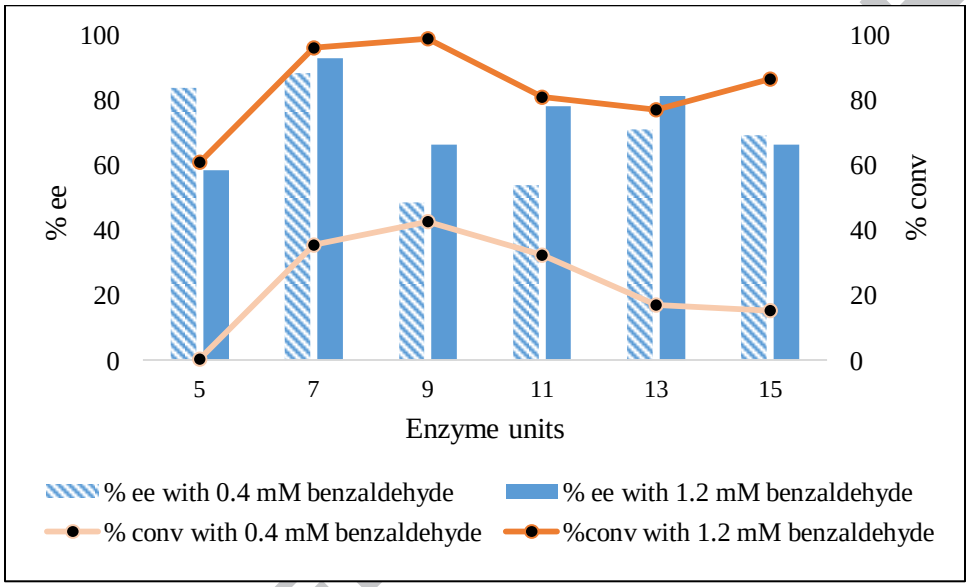

Figure 6: Effect of different CLEA-*Bm*HNL units in the synthesis of (*S*)-mandelonitrile

3.3.4. Different organic solvents

Application of biphasic system in HNL biocatalysis is well known. Use of organic solvent helps in minimization of spontaneous formation of racemic cyanohydrin and also in product extraction[27] [12,14,24]. In order to find out the effect of an organic solvent in the CLEA-*Bm*HNL biocatalysis, we have selected six different organic solvents which had been reported with other HNLs. They are hexane, toluene, n-butyl acetate, DIPE, TBME and AcN. The biocatalysis was performed using 1.2 mM benzaldehyde and 7 U of the enzyme (Fig 7). A 40% v/v of each of the mentioned organic solvents were used separately in biocatalysis. Another

biocatalysis without adding any organic solvent was also performed. None of the organic solvents improved % ee and % conversion of product compared to without organic solvent. Only in case of toluene, 96.9% ee of (*S*)-mandelonitrile was observed while in aqueous medium it was 96.8%. However, the % conversion was highest in the aqueous system i.e. ~38% compared to 9.3% in toluene. In case of DIPE, 32% conversion was seen but the % ee was 74 only. With n-butyl acetate, the % ee and % conversion were 82 and 12 respectively. Acetonitrile showed negative results in the CLEA-*Bm*HNL catalyzed the synthesis of (*S*)-mandelonitrile, as both the % ee and % conversion were less than 1. Compared to CLEA of other HNLs, this result was found to be different as it did not show any improve in % ee and conversion in synthesis of (*S*)-mandelonitrile.

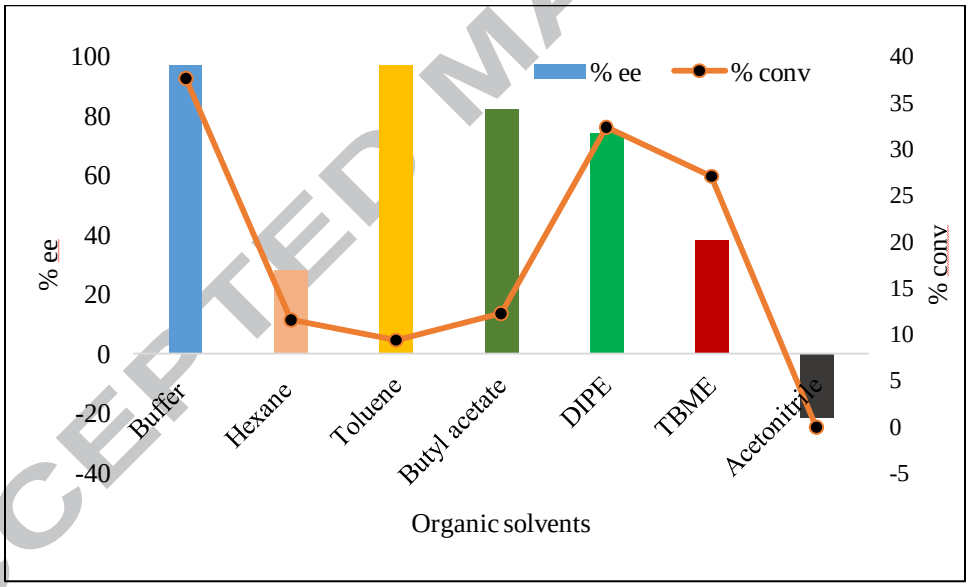

Figure 7: Effect of different organic solvents in the synthesis of (*S*)-mandelonitrile

3.3.5. Ratio of organic solvent to buffer

The effect of ratio of organic solvent in the biocatalysis was investigated. We have selected toluene as the best organic solvent among all (Fig 7) and different percentage by volume i.e. 30

to 78 of it was used in biocatalysis. With increasing percentage of toluene, decrease in % ee was observed (Fig 8). Although highest % ee i.e. 72.5 was observed with 40% toluene but the % conversion was only 14.6%. In contrary, highest % conversion i.e. 26.8, was observed with 60% of toluene, while the % ee was 0.9. With further increase in % of toluene resulted in decrease of both % ee and conversion.

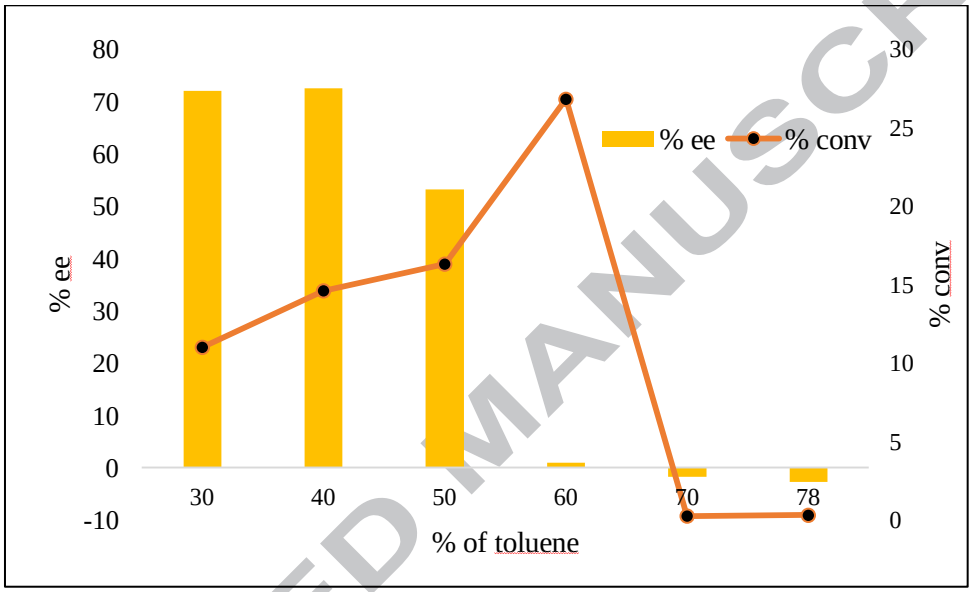

Figure 8: Effect of different ratio of toluene in the synthesis of (*S*)-mandelonitrile

3.3.6. Buffer pH

pH is a key factor in the synthesis of enantioselective cyanohydrins. At higher pH spontaneous formation of racemic cyanohydrin occurs by chemical reaction that limits the enantiomeric yield of cyanohydrins [28]. To elucidate the effect of pH in the CLEA-*Bm*HNL catalyzed synthesis of (*S*)-mandelonitrile, pH of the aqueous system was varied from 3 to 6 (Fig 9). The biocatalysis was performed in aqueous system only, because no improvement in % conversion or ee was observed by using any of the organic solvents in a biphasic system. In pH 4.2, CLEA-*Bm*HNL showed highest % ee of (*S*)-mandelonitrile i.e. 98.76 while the % conversion was almost 50 (Fig

10). Further increase in pH resulted in decreased % ee and conversion because at high pH racemization takes place. At pH less than 4.0, decreased % ee was also observed. It could be due to enzyme instability at low pH. Optimum pH of *Bm*HNL was reported to be 5.0 [11]. While a lower pH is preferred for HNL catalysis, at pH 4.2 the HNL activity of *Bm*HNL was almost half of its maximum activity [11]. CLEA-*Bm*HNL in contrary has showed optimum pH at 4.2. Another important property observed by CLEA-*Bm*HNL is in the improvement of % ee of (*S*)-mandelonitrile i.e. 98.76% compared to 54% by purified *Bm*HNL [11]. Similar observation of change in pH optima by CLEA has been reported [29,30]. CLEA of *Pichia pastoris* alcohol oxidase [31] and *Roystonea regia* peroxidase [29] has showed higher activity at low pH compared to their corresponding free enzymes.

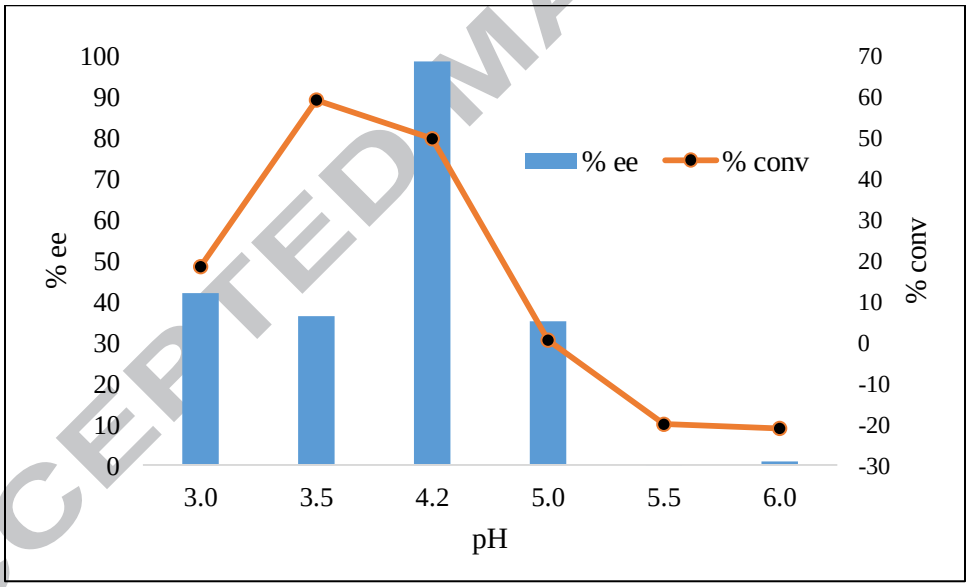

Figure 9: Effect of pH in the synthesis of (*S*)-mandelonitrile using CLEA-*Bm*HNL

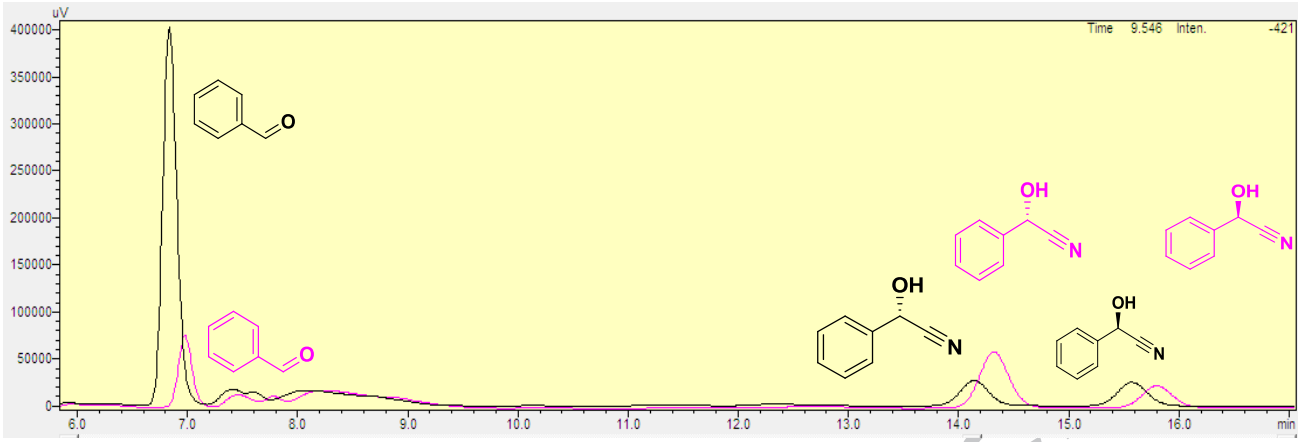

Figure 10: HPLC chromatogram of CLEA-*Bm*HNL catalyzed synthesis of (*S*)-mandelonitrile in 300 mM citrate-phosphate buffer pH 4.2. (Black: control; Pink: Reaction).

3.4. Reusability of CLEA-*Bm*HNL

One of the important reason of doing enzyme immobilization is to reuse the biocatalyst. To investigate the reusability of CLEA-*Bm*HNL, it was used for 10 successive cycles in the synthesis of (*S*)-mandelonitrile (Fig 11). The biocatalysis was performed according to protocol mentioned in section 2.15. After each cycle, the reaction mixture and enzyme were separated by centrifugation followed by addition of freshly prepared reaction mixture into CLEA-*Bm*HNL to carry out the successive cycle. In the first three cycles, the % ee and conversion was almost similar. The ee was above 95% while the % conversion was from 59 to 66. From 4^{th} to 8^{th} cycle, there was almost no decrease in product formation. The % conversion was maintained between 58 and 72. The % ee of (*S*)-mandelonitrile has decreased a little in the 4^{th} and 5^{th} cycle; they are 88 and 81 respectively. However in case of 5^{th} to 8^{th} cycle, the % ee was from 66 to 68. In the last two cycles i.e. 9^{th} and 10^{th}, both % ee and conversion were decreased. They are ~55% ee and ~44-45% conversion. The possible reason for the gradual decrease in % ee in successive cycles could be due to the loss of catalyst during each cycle in the operational process or enzyme

inhibition by aldehyde. Nevertheless, this process has showed reusability of CLEA-*Bm*HNL for eight successive cycles without loss in conversion or product formation and five cycles with a little loss in enantioselectivity.

Torrelo *et al* reported that CLEA-*Me*HNL can be reused for seven cycles without wash. The % ee remained constant >98% but the % conversion was decreased from 98 to 95% in first three cycles. At the end of the 7th cycle, the % conversion decreased up to 55% [26]. While this is ~45% loss in % conversion to product at 7th cycle, in the present study CLEA-*Bm*HNL showed nearly no loss in % conversion until 8th cycle. CLEA-*Pa*HNL was used for 10 successive cycles without any loss in % conversion with substrate 2-methyl benzaldehyde. The enzyme was washed with water after each cycle [25]. The recyclability of CLEA-*Pd*HNL was tested for eight cycles towards the synthesis of (*R*)-mandelonitrile in a biphasic system at 5°C [24]. At the end of the 8th cycle, although the % of ee of product was 99 but activity has decreased to 29% of its original activity. CLEA-*Lu*HNL in the synthesis of (*R*)-2-butanone cyanohydrin at 30°C showed recyclability for four times [17]. Compared to the first cycle (81% ee and 84% conversion) at the end of 4th cycle it showed 78% ee and 56% conversion.

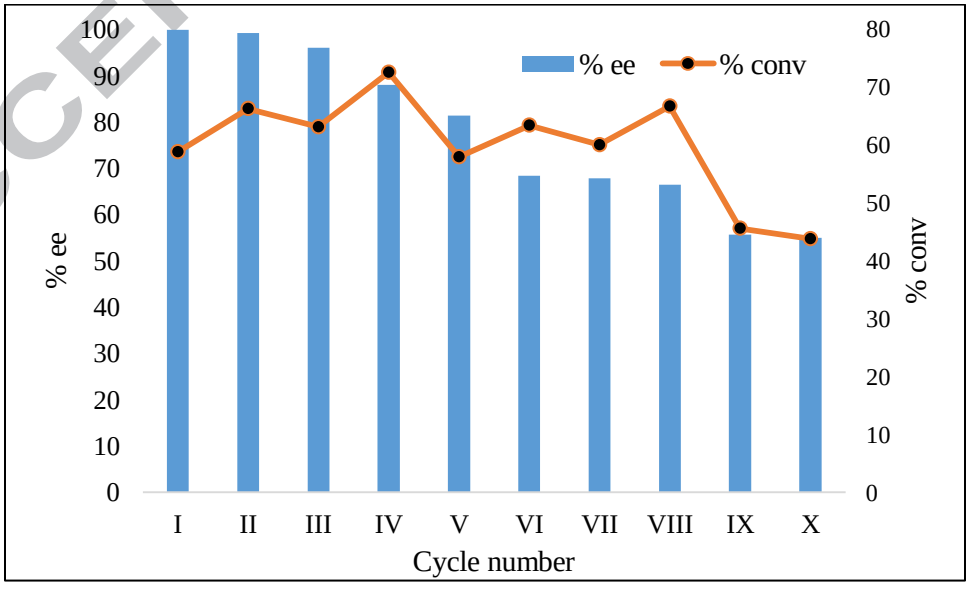

Figure 11: Recyclability of CLEA-*Bm*HNL towards the synthesis of (*S*)-mandelonitrile

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

CLEA-*Bm*HNL was used to synthesize different (*S*)-cyanohydrins using substrates other than benzaldehyde (Scheme 1). Eleven different aromatic aldehydes were used to synthesize the corresponding (*S*)-cyanohydrins using CLEA-*Bm*HNL under optimized reaction conditions. Selection of aromatic aldehydes is because of the preference of *Bm*HNL toward aromatic substrates. The amount of CLEA-*Bm*HNL used for all the biotransformations was only 7 U. The reaction time was different with respect to different substrates. The % ee and % conversion of the products obtained are summarized in Table 1.

Scheme1: CLEA *Bm*HNL catalyzed synthesis of (*S*)-cyanohydrins.

Table 1: CLEA-*Bm*HNL catalyzed synthesis of different chiral cyanohydrins

Substrate no	Aldehydes (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-$CH_2C_6H_4$	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

Using CLEA-*Bm*HNL, (*S*)-mandelonitrile was synthesized 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 enantioselectivity of this transformation. Kawahara and Asano have engineered *Bm*HNL to improve its enantioselectivity [32]. They reported *Bm*HNL-H103C-N156G catalyzed synthesis of (*S*)-mandelonitrile in 93% ee. 3,5-Dimethoxy benzaldehyde was converted to its corresponding (*S*)-cyanohydrin in 91.4% ee and 12.5% conversion by CLEA-*Bm*HNL in 100 minutes while with purified *Bm*HNL it's synthesis is reported with 85% ee [11]. The other nine substrates (No 3 to 11, Table 1) used in this study were not tested with *Bm*HNL earlier. Here we have converted these aldehydes into their corresponding (*S*)-cyanohydrins for the first time using CLEA-*Bm*HNL. Among the nine, five aldehydes i.e. 2,4-dimethoxybenzaldehyde, 4-allyloxybenzaldehyde, 3-phenoxybenzaldehyde, *trans*-cinnamaldehyde, and 3-benzyloxybenzaldehyde were converted to their corresponding (*S*)-cyanohydrins with very high % of ee i.e. 93 to 99 (No 3, 5, 8, 9 and 10, Table 1). Three among them have showed reasonable conversion i.e. 32 to 49% to their products. For example, 3-phenoxy benzaldehyde, 4-allyloxybenzaldehyde and 3-benzyloxybenzaldehyde (No 8, 5 and 10, Table 1) have produced their corresponding cyanohydrins in 48, 32.3, and 49% respectively. In case of 2,4-dimethoxybenzaldehyde and *trans*-cinnamaldehyde (No 3 and 9, Table 1) the % conversion was very less i.e. 1.3 and 3.5% respectively. CLEA-*Bm*HNL converted three other

aromatic aldehydes i.e. 2,5-dimethoxybenzaldehyde, 4-benzyloxybenzaldehyde and 2-phenyl acetaldehyde (No 4, 6 and 7, Table 1) to their respective (*S*)-cyanohydrins in 76.3, 88 and 75.6% of ee respectively. Although the % ee was moderate to high, the % conversion for these three substrates was very low i.e. 3 to 15% only. CLEA-*Bm*HNL catalyzed the synthesis of (*S*)-2-hydroxy-2-(4-hydroxyphenyl) acetonitrile from its corresponding aldehyde however with only 18.3% ee and 14.3% conversion. Among the eleven (*S*)-cyanohydrins syntheses reported here, eight of them (No, 2, 3, 4, 5, 6, 7, 10, and 11) have not been reported to be synthesized by any CLEA-HNL. The low % conversion observed in case of the synthesis of several mandelonitrile derivatives may be caused by product inhibition, however this is a speculation only and we do not have any evidence to explain the reasons behind it.

Synthesis of (*S*)-cyanohydrins has been reported by CLEA-*Hb*HNL [15] and CLEA-*Me*HNL [14,33]. The (*S*)-selective CLEA-*Hb*HNL is reported to synthesize (*S*)-mandelonitrile in 55% conversion and 67% ee in 72 h [15]. Cabirol et al synthesized (*S*)-*m*-phenoxybenzaldehyde 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 [16,17,24].

4. Conclusion

Preparation of CLEA-*Bm*HNL was optimized with different precipitants, and amount of cross linking agent i.e. glutaraldehyde. CLEA-*Bm*HNL was prepared using optimal conditions and characterized by SEM. Under optimized biocatalytic parameters i.e. 20 minutes of reaction time, 7 U of CLEA-*Bm*HNL, 1.2 mM substrate, and 300 mM citrate buffer pH 4.2, benzaldehyde was converted to (*S*)-mandelonitrile in high (~99%) ee and ~60% conversion. While CLEA of other HNLs show tolerance to organic solvent during their biocatalysis in a biphasic system, in case of CLEA-*Bm*HNL, addition of organic solvent did not improve in % ee or conversion of product. We have successfully demonstrated the re-usability of CLEA-*Bm*HNL for eight consecutive cycles without loss in conversion or product formation and five cycles with a little loss in enantioselectivity. CLEA of *Bm*HNL showed improved enantioselectivity in synthesis of (*S*)-mandelonitrile compared to the use of purified *Bm*HNL that showed only 54% ee. This observation also holds good with the second substrate i.e. 3,5-dimethoxy benzaldehyde, found common between our study and that reported with purified *Bm*HNL. However varied % conversion and ee was observed in the synthesis of different (*S*)-cyanohydrins, similar to the catalytic behavior of purified *Bm*HNL. We synthesized here eleven different chiral cyanohydrins using CLEA-*Bm*HNL, among them eight have not been reported to be synthesized by any CLEA-HNL and nine substrates were not tested earlier with *Bm*HNL. We showed here the preparation, characterization of a stable, robust and recyclable biocatalyst i.e. CLEA-*Bm*HNL and its biocatalytic application in the synthesis of different (*S*)-aromatic cyanohydrins. However preparative scale synthesis and practical application of CLEA-*Bm*HNL to synthesize enantiopure cyanohydrins still remains a challenge because of the low substrate concentration used here.

Acknowledgements

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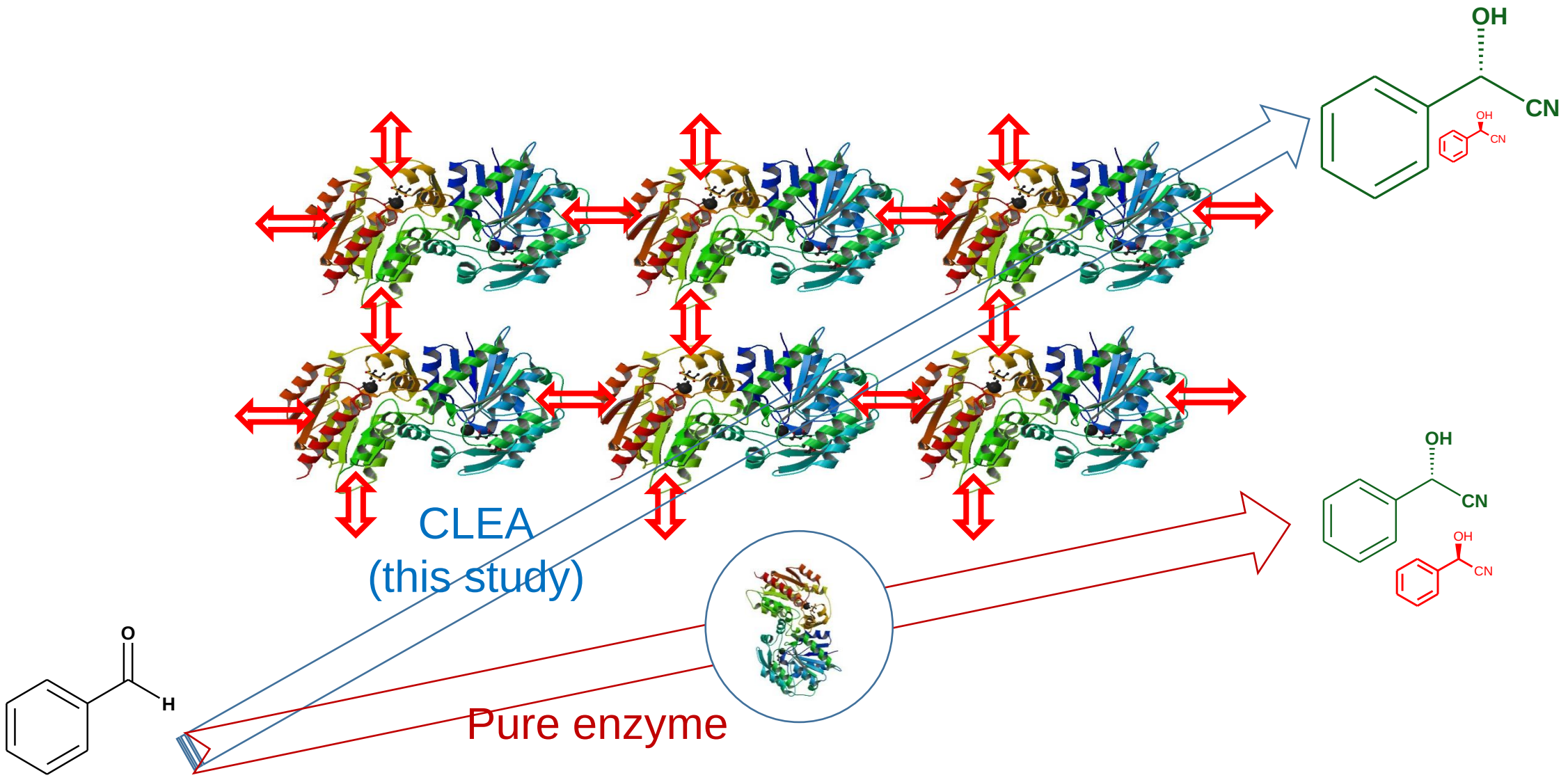
Highlights:

- Preparation and characterization of CLEA-*Bm*HNL reported first time.
- Reusability of CLEA-*Bm*HNL in (*S*)-cyanohydrin synthesis up to eight cycles without loss of conversion.
- CLEA has improved enantioselectivity of (*S*)-cyanohydrin synthesis compared to the use of purified enzyme.
- Eleven (*S*)-cyanohydrins synthesized by CLEA-*Bm*HNL, among which eight were not synthesized by any CLEA-HNL and nine substrates not tested by *Bm*HNL earlier.


OH
CN
OH
CN
CLEA
(this study)
OH
CN
OH
CN
O
H
Pure enzyme