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

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ABSTRACT

HNL catalysis is usually carried out in a biphasic solvent and at low pH to suppress the non-enzymatic synthesis of racemic cyanohydrins. However, enzyme stability under these conditions remain a challenge. We have investigated the effect of different biocatalytic parameters, i.e., pH, temperature, buffer concentrations, presence of stabilizers, organic solvents, and chemical additives on the stability of *Baliospermum montanum* hydroxynitrile lyase (*Bm*HNL). Unexpectedly, glycerol (50 mg/mL) added *Bm*HNL biocatalysis had produced > 99% of (*S*)-mandelonitrile from benzaldehyde, while without glycerol it is 54% ee. Similarly, *Bm*HNL had converted 3-phenoxy benzaldehyde and 3,5-dimethoxy benzaldehyde, to their corresponding cyanohydrins in the presence of glycerol. Among the different stabilizers added to *Bm*HNL at low pH, 400 mg/mL of sucrose had increased enzyme's half-life more than fivefold. *Bm*HNL's stability study showed half-lives of 554, 686, and 690 h at its optimum pH 5.5, temperature 20 °C, buffer concentration, i.e., 100 mM citrate-phosphate pH 5.5. Addition of benzaldehyde as inhibitor, chemical additives, and the presence of organic solvents have decreased both the stability and activity of *Bm*HNL, compared to their absence. Secondary structural study by CD-spectrophotometer showed that *Bm*HNL's structure is least affected in the presence of different organic solvents and temperatures.

1. Introduction

Hydroxynitrile lyases (HNLs) in nature catalyze cyanogenesis, a process that involves cleavage of cyanohydrins to the 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 largely in the synthesis of chiral cyanohydrins. A few HNLs catalyze promiscuous nitroaldol reaction, retro Henry reaction, and some convert ester hydrolysis when engineered [7,8]. Enantiopure cyanohydrins are important molecules with pharmaceutical, agrochemical, and other industrial applications [9–13]. The demand for biocatalytic synthesis of chiral cyanohydrins has led to the discovery of several new HNLs [6,14–17]. 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 [18]. In order to minimize this background reaction, often HNL biocatalysis is carried out at (i) low pH and (ii) in the presence of organic solvents [8,19–23]. However, the stability of the enzyme in both the above conditions remains an issue. For example, *Hevea brasiliensis* HNL (*Hb*HNL) and *Arabidopsis thaliana* HNL (*At*HNL), two HNLs of α/β hydrolase fold superfamily have shown poor enzymatic stability at lower pH [18,19]. Optimization of HNL catalyzed synthesis of chiral cyanohydrin requires the investigation of the stability and other biophysical parameters of an enzyme, which may provide an appropriate reaction condition where the background reaction is minimum. Among the reported α/β hydrolase fold HNLs, the stability of *Hb*HNL and *Manihot esculenta* HNL (*Me*HNL) has been studied to help use them in biocatalysis [18,19,24,25]. *At*HNL has shown improved stability at a lower pH by protein engineering and immobilization of it with a flavin-based fluorescent protein [26,27]. *Bm*HNL, another member of α/β hydrolase fold family of HNLs catalyzes the synthesis of a broad range of chiral cyanohydrins [2,23,28,29]. *Bm*HNL is one of the rare (*S*)-stereospecific HNLs. The common problem of soluble protein expression in *E. coli* was observed with *Bm*HNL gene. The *Bm*HNLH103C variant created by protein engineering has improved the protein expression in *E. coli* [30]. The study of effect of pH, temperature, and additives on activity and stability of *Bm*HNL reveals that the enzyme has optimum activity at pH 5.0 and is stable in a broad pH range from 2.5 to 10.5 [2]. Further, *Bm*HNL shows optimum activity at 20 °C while it is stable at a broad range of temperature, i.e., 10 to 60 °C. However, the reported stability study is limited to only one h incubation and majorly focuses on finding optimal

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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 improves the enantioselectivity of *Bm*HNL in the formation of (*S*)-mandelonitrile up to > 99% ee compared to 75% ee in the absence of glycerol. Two previous reports show such increase in the % ee in the *Bm*HNL catalyzed synthesis of (*S*)-mandelonitrile. They are with (i) engineered enzyme, i.e., *Bm*HNL-H103C-N156 G [30] or immobilized *Bm*HNL [29]. Further, sucrose addition has improved *Bm*HNL's half-life at pH 3.5, more than five folds as compared to without stabilizer.

2. Materials and methods

*Bm*HNL (LOCUS: AB505969) synthetic gene cloned into pUC57 was synthesized and procured from Gene Script, USA. Culture media, ampicillin, and Dialysis membrane bags were purchased from HiMedia laboratory Pvt Ltd. Isopropyl-*β*-D-1-thiogalactopyranoside (IPTG) was purchased from BR-BIOCHEM Pvt Ltd. For protein purification, Ni-NTA agarose superflow resin was purchased from Qiagen. Imidazole, aldehydes, 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. Acetone cyanohydrin (AcCN) was purchased from amino organics (local vendor). 10,000 MWCO Amicon tubes for concentrating protein were obtained from Merck Millipore.

2.1. Expression and protein purification

*Bm*HNL enzyme with His-tag was produced in *E. coli* as per our previous reports. [23,29]. The crude extract obtained was purified using Ni-NTA agarose resin. The crude extract was loaded onto a 10 mL bed-volume of the resin pre-equilibrated with 20 mL of equilibration buffer (50 mM KPB pH 7.0, 10 mM imidazole, 300 mM NaCl). The resin was allowed to bind to the enzyme by shaking the column at 4 °C for 45 min, and the unbound protein was collected as flow-through (FT). The resin was washed twice with 20 mL of wash buffer (50 mM KPB pH 7.0, 20 mM imidazole, 300 mM NaCl) followed by protein eluted with elution buffer (50 mM KPB pH 7.0, 150 mM imidazole, 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, 300 mM NaCl). All steps were carried out at 4 °C. All the fractions were analyzed by SDS-PAGE (Fig. S1) and protein quantitated by Nanodrop. Removal of imidazole from eluted protein was done by dialysis by using pre-treated membrane (20 kDa MWCO). Subsequently, the dialyzed protein was concentrated using 10'000 MWCO Amicon tubes, and the enzyme was stored at 4 °C.

2.2. HNL assay via mandelonitrile cleavage

HNL activity was measured by monitoring the formation of benzaldehyde due to cleavage of racemic mandelonitrile, in a microtitre plate using Multiskan Go UV–vis spectrophotometer (Thermo Scientific). The reaction mixture contained 175 µL of 50 mM citrate-phosphate buffer pH 5.0, 20 µL of 70 mM (*RS*)-mandelonitrile pre-dissolved in 5 mM citrate-phosphate buffer pH 3.15. The reaction was initiated by the addition of 5 µL of *Bm*HNL (0.24 mg/mL), and the rate of benzaldehyde production was measured at 280 nm over a period of 5 min. A control experiment was carried out in an identical manner without using enzyme, and the corresponding absorbance was subtracted. One unit is defined as the amount of the enzyme which converts 1 µmol of racemic mandelonitrile to benzaldehyde in one minute under standard conditions. All the reactions were performed in triplicates.

2.3. Influence of biophysical parameters on BmHNL stability and activity

We have studied various biophysical parameters to determine the stability and activity of *Bm*HNL under different conditions. The enzyme was incubated in different conditions such as different pH, temperature, buffer concentration, additives, substrate concentrations (as inhibitor), and stabilizers and their effect on stability and activity of *Bm*HNL were studied. In the case of each of the above studies, the specific activity of *Bm*HNL was calculated by using (*RS*)-mandelonitrile cleavage assay (as given in 2.2). In case of control reactions, the corresponding buffer was used instead of the enzyme. All measurements were performed in triplicates.

2.3.1. Effect of pH

2.3.1.1. 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, 4.0, 4.5, 5.0, 5.5, 6.0, and 6.5. The enzyme found in 20 mM KPB pH 7.0 on purification was subjected to buffer exchange with 50 mM citrate-phosphate buffer of the corresponding pH, i.e. 3.5–6.5 and was stored at room temperature. HNL activity of each of the buffer exchanged enzyme was determined. The activity found was considered as the initial activity of that enzyme stored at the given pH. Further activities of *Bm*HNL were measured at different time intervals until the specific activity reached half of the initial activity.

2.3.1.2. On activity. Specific activity of *Bm*HNL was measured as described in 2.2, except the assay buffer was replaced by 50 mM citrate-phosphate buffer of pH 3.5–6.5 in separate experiments

2.3.2. Effect of temperature

2.3.2.1. On stability. Influence of temperature on the stability of *Bm*HNL was studied by incubating the enzyme in 20 mM KPB pH 7.0 at different temperatures ranging from 10 to 80 °C in a thermoshaker. The initial activity was measured using HNL assay at room temperature (as given in 2.2). The activity of enzymes incubated at different temperatures was measured at multiple time points until each of them reached half of its corresponding initial activity. A control experiment was also performed under similar conditions where the enzyme was replaced by its corresponding buffer.

2.3.2.2. On activity. Specific activity of *Bm*HNL was measured as described in 2.2, except separate experiments were carried out for measuring the activity by incubating the reaction mixture in 50 mM citrate-phosphate buffer pH 5.5, at different temperatures, i.e., 10 to 80 °C in a thermomixer.

2.3.3. Effect of buffer concentrations

2.3.3.1. On stability. The study of the effect of buffer concentrations on the stability of *Bm*HNL was carried out by storing the purified enzyme in different concentrations of citrate-phosphate buffer pH 5.5, i.e., 50, 100, 200, 300 and 400 mM. This was performed by an exchange of the citrate-phosphate buffer of appropriate concentration with the buffer of the purified enzyme, as described in section 2.3.1. The initial activity was measured after buffer exchange. The enzyme was stored at room temperature, and the activity of each enzyme present in different concentrations of buffer was measured until it reached half of the initial activity.

2.3.3.2. On activity. The specific activity of *Bm*HNL was measured as per the protocol described in section 2.2 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.

2.3.4. Effect of stabilizers

2.3.4.1. On stability. The study of the effect of stabilizers on the stability of *Bm*HNL present at low pH, was performed. 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. The stabilizers were added to the enzyme solution (50 mM citrate-phosphate buffer pH 3.5) stored at room temperature. After proper mixing, the initial activity of each enzyme solution was measured as mentioned in section 2.2 and subsequently activities were measured at different time intervals until half-life has reached.

2.3.4.2. On activity. The specific activity of *Bm*HNL with different stabilizers was measured. Different stabilizers were added to the reaction mixture. The additives were properly mixed and the assay was carried out as described in 2.2 using 50 mM citrate-phosphate buffer pH 5.5.

2.3.5. Influence of benzaldehyde concentration

2.3.5.1. On stability. The effect of benzaldehyde concentrations on the stability of *Bm*HNL was investigated. Benzaldehyde was added to *Bm*HNL in 20 mM KPB pH 5.5 at room temperature to maintain its different concentrations, i.e., 0, 5, 10, 15, 20, 25, 30, 35, and 40 mM in the enzymatic solution. The initial activity of each enzyme solution with benzaldehyde, was measured using racemic mandelonitrile cleavage assay. Subsequent activities for each enzyme solution were measured at different time intervals until the activity has reached half of the initial activity. Control experiments also carried the respective benzaldehyde concentrations.

2.3.5.2. On activity. The specific activity of *Bm*HNL with different concentrations of benzaldehyde was measured. The above-mentioned benzaldehyde concentrations were added to the reaction mixture, and the assay was carried out as described in 2.2 using 50 mM citrate-phosphate buffer pH 5.5.

2.3.6. Effect of addition of organic solvents

2.3.6.1. 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 solvents in 10% v/v. After the addition of organic solvent to an enzyme solution, it was stored at room temperature. The initial activity of each enzyme solution was measured, and subsequent activities were determined until the half-life is reached.

2.3.6.2. On activity. The specific activity of *Bm*HNL with different organic solvents was measured. A 10% v/v of each organic solvent was added to the reaction mixture and the assay was carried out as described in 2.2 using 50 mM citrate-phosphate buffer pH 5.5.

2.3.7. Effect of addition of chemical additives

2.3.7.1. 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 [2]. $ZnSO_4$, $AgNO_3$, a 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 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 2.2.

2.3.7.2. On activity. The specific activity of *Bm*HNL in the presence of different inhibitors was measured by adding them separately into the reaction mixture. Mandelonitrile cleavage assay was carried out for each of the enzyme solutions in 50 mM citrate-phosphate buffer pH 5.5 to measure the activity. An enzyme solution without inhibitor was used as a control for the study.

2.4. Circular dichroism (CD) analysis

The study of the 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 temperatures ranging from 10 °C to 80 °C. The spectra recorded in 195 to 300 nm were analyzed by CDNN software. In the 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 °C.

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

The effect of benzaldehyde concentrations in the 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 °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 [29].

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

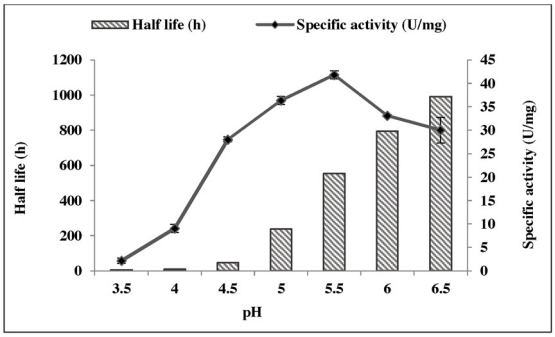
The effect of polyols in the enantioselective synthesis of mandelonitrile was studied. To pursue this, we have selected a condition where *Bm*HNL is less stable, so that any improvement in the enzyme's stability can be suitable studied. The enzyme stored in 50 mM citrate-phosphate buffer pH 3.5 was used in the biocatalysis. Glycerol and sorbitol of 50 and 200 mg/mL respectively were selected for the study. 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 1 M 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 were carried out as per 2.5 above. A control experiment was performed in the same manner without the addition of polyols.

We further studied the effect of polyols in the *Bm*HNL catalyzed cyanohydrin synthesis using the enzyme stored in 20 mM potassium phosphate buffer pH 7.0. The rest of the protocol remained the same as described above. Effect of glycerol was also studied in the *Bm*HNL catalyzed synthesis of (*S*)-2-hydroxy-2-(3-phenoxyphenyl) acetonitrile and (*S*)-2-hydroxy-2-(3,5-dimethoxyphenyl) acetonitrile using the enzyme stored in 20 mM potassium phosphate buffer pH 7.0.

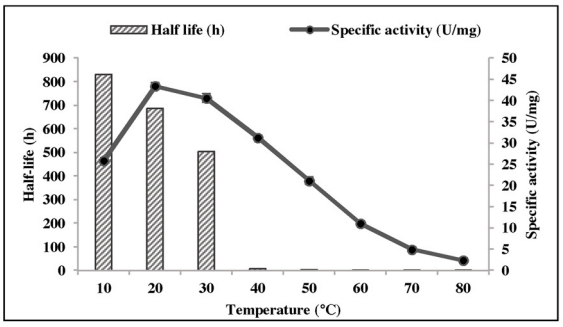
3. Results and discussions

3.1. Protein purification and HNL assay

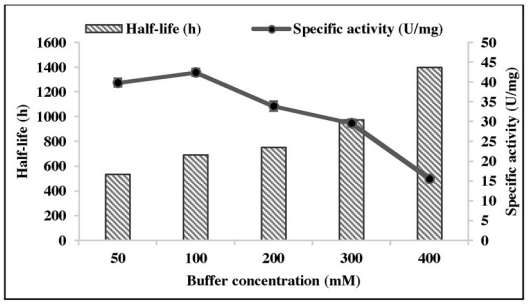
*Bm*HNL purified using Ni-NTA resin was analyzed by SDS-PAGE (Fig. S1). Presence of a clear band of ~29 kDa for the purified fraction, confirms the presence of *Bm*HNL. The specific activity of purified *Bm*HNL (as per 2.2) was found to be 42.4 U/mg in comparison to 49.3 U/mg reported earlier [2].



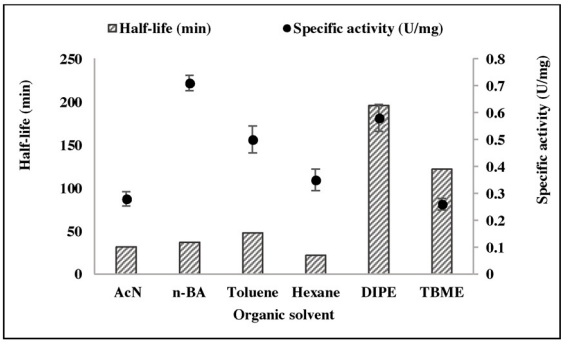
(a)



(b)



(c)



(d)

Fig. 1. Half-life and specific activity of *Baliospermum montanum* hydroxynitrile lyase (*Bm*HNL) in different (a) pH; (b) temperature; (c) buffer concentration; (d) organic solvents. (Bar): Half-life of enzyme; (●): specific activity of enzyme. Half-life refers pH, temperature, buffer concentration and organic solvent dependent stability of enzyme while specific activity (●) refers initial activity of enzyme in respective conditions. The substrate was racemic mandelonitrile.

3.2. Biophysical parameters

3.2.1. 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 a higher rate, which results in decreased enantiomeric excess of product. To overcome this limitation, HNL mediated reactions are usually carried out at lower pH. HNL biocatalysis, therefore, requires the knowledge of stability and activity of the enzyme. The stability and activity of *Bm*HNL were studied at 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 (Fig. 1a). The enzyme showed higher half-life and hence increased stability with an increase in pH from 4.5 to 6.5. *Bm*HNL's half-lives at pH 6.5, 6.0, and 5.5 were found to be 990, 794, and 554 h respectively. 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. At lower pH unfolding of 3D structure of enzyme occurs that leads to denaturation of the 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 [31]. 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 increased stability of the enzyme with an increase in pH [18]. The half-lives 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 [24]. In 20 mM citrate-phosphate buffer (CPB) at pH 4.5, 5.0 and 5.5, its half-lives were 3.3, 24.2 and 83.3 h respectively [24]. 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 83.3 h in pH 5.5. Guterl et al compared the effect of pH in two different HNLs i.e. *At*HNL and *Me*HNL [25]. The (*R*)-selective *At*HNL showed half-life up to 50 h in CPB 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 4.0. This suggests 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. 41.8 U/mg (Fig. 1a). 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 the enzyme [19]. At higher pH, spontaneous degradation of the substrate was more which resulted in lower activity. *Hb*HNL biocatalysis is carried out at pH 5.0, highest activity of *At*HNL was between pH 5.75 to 6.5 while *Me*HNL has in citrate-phosphate buffer pH 5.75 [25]. Among all α/β hydrolase fold hydroxynitrile lyases, *Bm*HNL showed the highest half-life of 554.5 h at its optimum 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.

3.2.2. Effect of reaction temperature

Study of stability and activity of *Bm*HNL at various temperatures (Fig. 1b), showed a decrease in half-life of *Bm*HNL with an increase in temperature. The enzyme exhibited high stablity with half-life of 831 h at lower temperature e.g., 10 °C. Its half-life decreased drastically at higher temperatures. At 40 and 50 °C *Bm*HNL's half-lives were ~7 and ~4 h respectively. The increased stability at low and decreased stability at high temperature could be due to slow or fast inactivation of enzyme respectively [32]. The specific activity of *Bm*HNL was also measured at different temperatures ranging from 10 to 80 °C. Maximum activity of 43.4 U/mg was found at 20 °C, which is similar to that reported [2]. *Bm*HNL's activity was reduced to 50% at 50 °C, while beyond that it

decreased further.

In the comparative study of stability and activity between *At*HNL and *Me*HNL at different temperatures, *At*HNL showed maximum activity at 35 °C while *Me*HNL at 60 °C [25]. *At*HNL was stable for 96 h in the temperature range of 0–10 °C, and its stability was low at a higher temperature. The half-life of *At*HNL at its temperature optima, i.e. 35 °C is not known, however at 30 and 37 °C it was 33 and 6.6 h respectively. Similarly *Me*HNL was stable up to 96 h in the temperature range of 0–20 °C, however at its temperature optima, i.e., 60 °C it was stable for only 30 min. The exact half-lives of both the enzymes are not known as it has been 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 min only. Bauer et al studied the influence of temperature on the stability of *Hb*HNL [18]. They observed its highest half-life of 2315 min, i.e., 38.6 h at 30 °C in 5 mM citrate buffer, and it decreased to 7 min at 70 °C [18]. Thus comparison of the half-lives of all the four α/β hydrolase fold HNLs at their corresponding temperature optima indicates that *Bm*HNL has highest among them.

3.2.3. 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 CPB showed increased enzyme stability with an increase in buffer concentrations (Fig. 1c). The enzyme showed maximum half-life of ~1399 h at room temperature in 400 mM of CPB. 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 [31]. The half-life of *Bm*HNL in 300 mM CPB was found to be ~973 h whereas in 50, 100 and 200 mM CPB, the half-lives were 534.5, 689.9, and 751 h respectively.

The specific activity of *Bm*HNL was also determined in different buffer concentrations. The highest activity was observed in 100 mM CPB, i.e., 42.4 U/mg. The specific activity decreased gradually with increased buffer concentration. *Bm*HNL's specific activity was found to be 33.9 U/mg in 200 mM CPB. It showed the lowest activity of 15.6 U/mg in 400 mM CPB. 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 the 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 [18]. The half-lives of *Hb*HNL determined at 40 °C, in 50 and 100 mM citrate buffer pH 6.5 were 600 and 700 min respectively. In contrary, in 50 and 100 mM buffer, the half-lives of *Bm*HNL were 534.5 and 689.9 h respectively. In the case of *Me*HNL and *At*HNL, a similar study is not found, although four different buffers, e.g. citrate phosphate, potassium phosphate, glutamate, and acetate buffer were used [25].

3.2.4. Effect of organic solvents

HNL-catalyzed cyanohydrin synthesis is usually carried out in a biphasic system to suppress the background reaction [23,33–39]. *Bm*HNL's stability in organic solvents has not been studied earlier. 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 report [23]. The enzyme showed lower stability in organic solvents compared to the aqueous system (Fig. 1d). Among the organic solvents, the highest half-life of 196 min was observed in DIPE, followed by 122 min in TBME. The half-lives of *Bm*HNL in hexane, AcN, *n*-BA, and toluene were 22, 32, 37 and 48 min respectively. Presence of organic solvent alters the hydrophobicity which could make conformational change of enzyme and may cause destabilization of its native structure or protein denaturation [40–42]. The aqueous environment is one of the dominant contributors to protein folding and stability.

The influence of a biphasic system on the specific activity of *Bm*HNL 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, as compared to 43.2 U/mg without any organic solvent. Bauer et al. reported the stability of *Hb*HNL in different biphasic systems [24]. Their study showed the highest half-life of *Hb*HNL in the mixture of MTBE and hexane (40:60). However, this solvent system has shown ~55% of relative activity. The highest activity of *Hb*HNL was seen in hexane while rapid inactivation of the enzyme was observed in it.

Wehtje et al. have reported the effect of water content with DIPE toward the synthesis of chiral mandelonitrile using immobilized mandelonitrile lyase and concluded that insufficient water content in reaction medium leads to denaturation of the enzyme [20]. Costes et al. studied the stability of immobilized *Hb*HNL (Celite-*Hb*HNL) in a variety of organic solvents. The enzyme showed the highest half-life of 2500 h in the presence of dry toluene toward the synthesis of (*S*)-mandelonitrile while 260 h in buffer saturated hexane. The enzyme's stability was decreased when incubated in buffer saturated solvents with HCN [21]. Paravidino et al. have studied the effect of water concentration in organic medium on activity and enantioselectivity of CLEA-*Me*HNL [22]. They have selected MTBE, toluene, and octane as organic solvents in the synthesis of (*S*)-mandelonitrile. The immobilized enzyme showed excellent enantioselectivity with increase amount of water in organic medium while higher enzyme activity was observed with lower water concentration although complete drying of enzyme resulted in deactivation of the enzyme.

3.2.5. Effect of stabilizers

Addition of stabilizers such as polyols and sugars increases the stability of proteins at extreme pH [11,18,19,43–47]. There exist several shreds of evidence supporting the role of stabilizers in improved enzymatic activity [48,49], enzyme stability [50,51] as well as in both [52]. To investigate the same, we have selected a condition where *Bm*HNL shows poor stability. The enzyme was taken in 50 mM citrate-phosphate buffer pH 3.5. Addition of sucrose in high concentration (400 mg/mL) has improved *Bm*HNL's half-life at pH 3.5, more than five folds as compared to without stabilizer (Fig. 2). The stability increased 3, 3.6, and 4.8 fold with 50, 100, and 200 mg/mL of sucrose respectively. Improved enzymatic stability was observed with all the four tested polyols. Addition of 50 and 100 mg/mL of lactose increased *Bm*HNL's stability 2.75 (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 respectively compared to without addition. 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 [18,43,53,54]. 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 the 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 stability of *Bm*HNL. Stability of *Hb*HNL in the presence of different additives, e.g., saccharose, sorbitol, and different concentrations of *Hevea* extract have been reported [19]. Addition of these additives into *Hb*HNL in 20 mM potassium phosphate buffer pH 3.75 has increased its half-life by 3.84 to 58 fold as compared to without additives. *Hb*HNL's stability has also been tested by addition of *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 from its 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

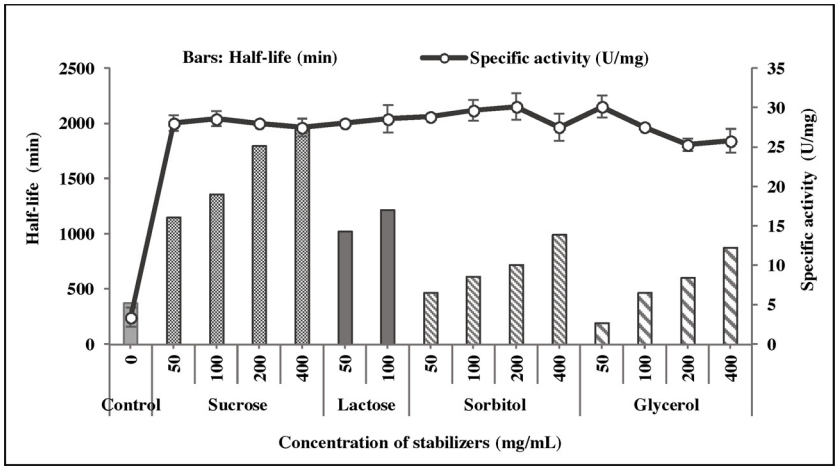


Fig. 2. Half-life and specific activity of *Baliospermum montanum* hydroxynitrile lyase (*Bm*HNL) in presence of different stabilizers. (Bars): Half-life of enzyme; (o): specific activity of enzyme. The substrate was racemic mandelonitrile. Control experiment was carried out without addition of stabilizers.

pH 3.5 at 30 °C [18]. Among all the tested stabilizers, sorbitol (400 mg/mL) has shown six-fold improved stability of *Hb*HNL while all others could show a maximum of 3 fold increased stability.

Guterl et al. studied the stability of *Me*HNL and *At*HNL at low pH in the presence of sorbitol and saccharose as stabilizers. Among the tested concentrations, *At*HNL has shown > 36 fold increased stability in case of 400 mg/mL of saccharose and also with 200 mg/mL of sorbitol. *Me*HNL with the same additives and concentration has shown only 2.75 to 6.5 fold increased stability. They also tested the stability of both the enzymes in the presence of cell lysate and observed that stability of *Me*HNL increased from 2 h to > 48 h while the stability of *At*HNL was unaffected [25].

We have studied the effect of stabilizers on *Bm*HNL's specific activity. All four stabilizers tested in various concentrations have increased the HNL activity. 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. The addition of sorbitol or glycerol has increased ~ 9 fold of specific activity.

3.2.6. 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 (Fig. 3). 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 reason for lower stability is due to inhibition of *Bm*HNL with an increase in benzaldehyde concentration [2]. A similar study was also reported by Bauer et al. with *Hb*HNL [24]. They determined the stability of *Hb*HNL in the presence of varying concentrations of benzaldehyde in 20 mM CPB pH 5.0 at 20 °C. They observed *Hb*HNL's half-life of 450 min without the addition of benzaldehyde, but after addition of benzaldehyde, it decreased drastically. In the presence of 2 mM benzaldehyde, *Hb*HNL's 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, the half-life of *Hb*HNL was less than < 20 min.

The effect of benzaldehyde concentrations over the activity was also studied. Similar to stability, a decrease in activity with increasing benzaldehyde concentration was observed. The highest activity of 41 U/mg was found without the addition of benzaldehyde. The reason for decreasing the specific activity of *Bm*HNL is due to its inhibition by benzaldehyde [2].

3.2.7. Effect of chemical additives/inhibitors

The effect of various inhibitors over stability and activity of *Bm*HNL was studied. Addition of chemical additives or inhibitors decreased both the stability and activity of *Bm*HNL compared to control having no inhibitor (Fig. 4). *Bm*HNL in the absence of any inhibitor was stable up to 67 days and showed 42.8 U/mg of specific activity. Addition of 2-

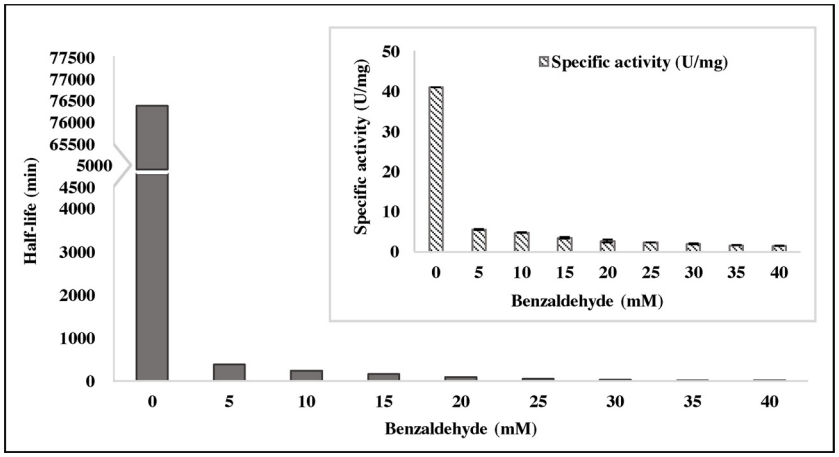


Fig. 3. Half-life and specific activity of *Baliospermum montanum* hydroxynitrile lyase (*Bm*HNL) with different benzaldehyde concentrations. Half-life (solid bars) and specific activity (pattern bars) of enzyme was measured using substrate racemic mandelonitrile with addition of 5–40 mM benzaldehyde.

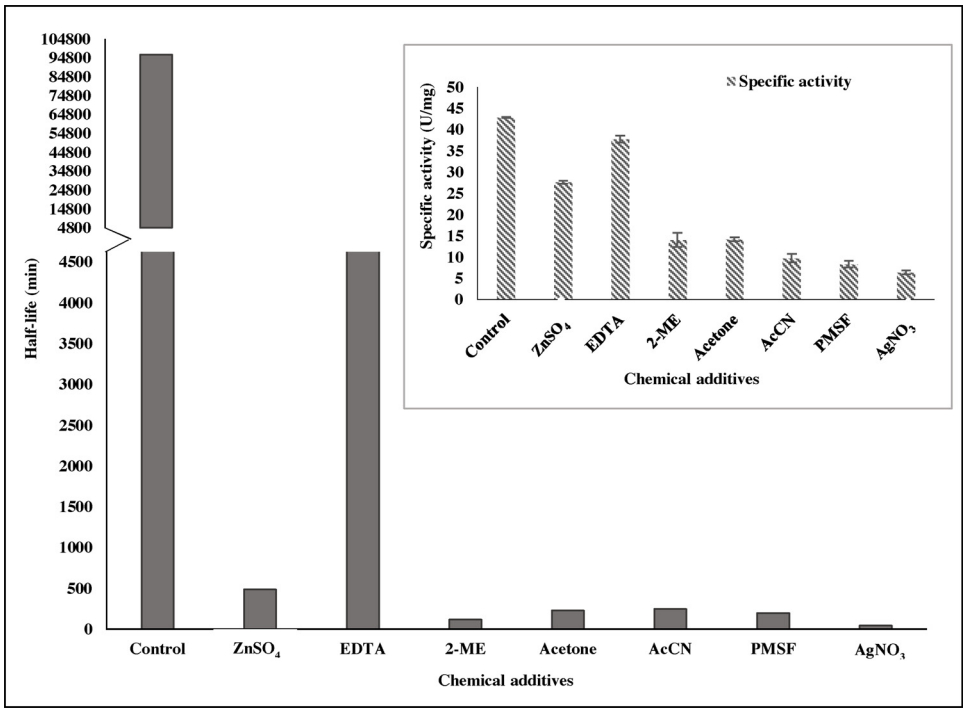


Fig. 4. Half-life and specific activity of *Baliospermum montanum* hydroxynitrile lyase (*Bm*HNL) in the presence of different chemical additives. Half-life (solid bars) represents the stability of enzyme with addition of chemical additives and specific activity (pattern bar) refers initial activity of enzyme in the presence of additives. The substrate was racemic mandelonitrile.

mercaptoethanol has decreased enzymatic activity to 14 U/mg, possibly because of the disruption of the homo-dimer of the enzyme complex. It also reduced the half-life of the enzyme to 112 min. EDTA addition showed little or no change in enzyme's activity as well as stability. The half-life of the enzyme was 4651 min, and specific activity was 37.71 U/mg in the presence of EDTA. *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. In the presence of acetone, the half-life and residual activity were 226 min and 14.17 U/mg respectively whereas in case of AcCN they were 245 min and 9.68 U/mg respectively. Similar observations were earlier made by Dadashipour et al [2]. In the case of PMSF addition, 27% loss of enzyme activity and low stability, i.e. 196 min half-life was observed. This is probably due to the binding of active site serine (Ser80) of *Bm*HNL with PMSF. Use of $AgNO_3$ has completely inhibited the activity and stability both; they were found to be 6.35 U/mg and 40 min respectively. In the presence of $ZnSO_4$ the enzyme showed an activity of 27.59 U/mg and half-life of 487 min. 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 [2].

3.3. Secondary structure study by CD analysis

3.3.1. CD analysis of BmHNL at different temperatures

The effect of temperature on the secondary structure of *Bm*HNL was investigated by circular dichroism spectrophotometer. The enzyme was incubated at different temperatures like 10 to 70 °C in the CD spectrophotometer, and the spectra were recorded over a range of wavelength from 195 to 255 nm (Fig. S3). The spectra showed a change in the content of both α-helix and β-sheets, but a negligible change in the content of random coil of the enzyme with an increase in temperature (Table 1). The percentage of the 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 from 12.5 to 15.4%. 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.

Table 1
Secondary structure elements of *Baliospermum montanum* hydroxynitrile lyase (*Bm*HNL) at different temperatures. 0.1 mg/mL of purified enzyme was used in the study.

Temp (°C)	% α-Helix	% β-sheets	% Random coils
10	38.3	12.8	48.9
20	38.2	13.0	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	18.0	49.8
60	31.0	19.7	49.3

3.3.2. CD analysis of BmHNL in different organic solvents

The influence of organic solvents on the structure of *Bm*HNL was also investigated by CD spectrophotometer over a range of wavelength from 195 to 255 nm. The spectra were recorded in two different organic solvents, i.e., *n*-BA and DIPE (Table 2, Fig. S4). The enzyme was most stable in DIPE whereas the activity of the enzyme was highest in *n*-BA as mentioned in section 3.2.4. After incubating the enzyme in organic solvents, the spectra showed a change in the content of both α-helix and β-sheets, but minimum change in the content of random coil of the enzyme. The percentage of α-helix has 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 has decreased from 38.3 to 32.8, whereas the percentage of β-sheets increased from 15.4 to 17.2. Both of

Table 2
Secondary structure elements of *Baliospermum montanum* hydroxynitrile lyase (*Bm*HNL) in the presence of different organic solvents. 0.1 mg/mL of purified enzyme with 10% organic solvents was used in the study.

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

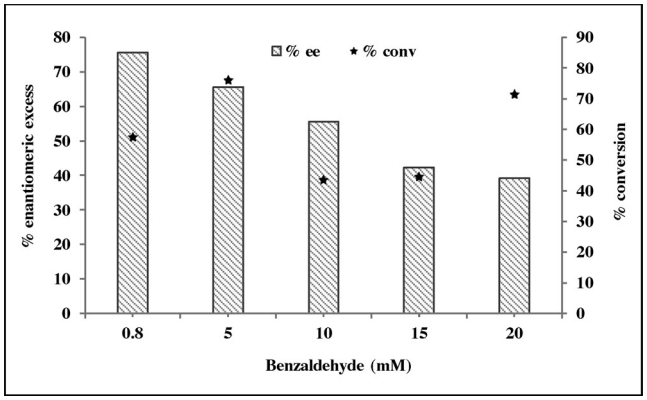


Fig. 5. Effect of benzaldehyde concentration in the synthesis of (*S*)-mandelonitrile. (Bars): % enantiomeric excess (ee) and (★): % conversion (conv). The standard reaction conditions (300 mM citrate buffer pH 4.2, 100 mM KCN solution, 4U of purified *Bm*HNL) were used with 0.8–20 mM benzaldehyde.

the above solvents did not destabilize the enzyme's secondary structure to that extent.

Ulf Hanefeld et al. studied the inhibitory effect of acetic acid and hydrochloric acid on the secondary structure of *Hb*HNL by CD spectroscopy. They observed 41.5 and 39.1% α-helix when the enzyme was incubated in 20 mM KPB pH 6.5 and water respectively. The percentage of α-helix decreased with an increasing amount of acetic acid in enzyme solution as well as lowering of pH. In 0.5% acetic acid, 30.1% of α-helix was observed while it decreased up to 15.5% in the presence of 2% acetic acid [55].

3.4. 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 (Fig. 5) by performing the biotransformation for 5 min as per 2.5. Benzaldehyde concentration was varied from 0.8 to 20 mM in different biocatalysis. In the 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 55.5% ee and 44% conversion of the product was found with 10 mM benzaldehyde.

The decrease in % ee with increasing benzaldehyde concentration could be due to the inhibition of *Bm*HNL [2]. Hanefeld et al. reported a similar trend with *Hb*HNL catalyzed cyanohydrin synthesis [56]. With increasing concentration of benzaldehyde (5–16 mM), *Hb*HNL inactivation and decrease in % ee were observed [56].

3.5. 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 the case of the sorbitol addition, the biocatalysis has produced 97.6% ee and 56.2% conversion of (*S*)-mandelonitrile in 10 min while in the presence of glycerol it showed the highest, i.e. 99.2% ee and 47.7% conversion in 15 min (Fig. 6, Figs. S5–S8). A control experiment with purified enzyme having no polyol showed ~75% ee and ~33% conversion in 5 min. This indicates that polyol addition has increased the % ee of product. 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 was observed in the case of sorbitol, and glycerol added biocatalysis. In sorbitol added biocatalysis, the % ee of product decreased to 93% in 15 min and 36.2% in 20 min, however, highest conversion, i.e. 65.6% was found in 20 min.

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 were carried out as described in section 2.6. In the *Bm*HNL catalyzed biocatalysis with glycerol, benzaldehyde concentration was varied from 0.8 to 20 mM (Fig. 7, Figs. S9–S10). 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 found without glycerol in 5 min. Even in the 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. With further increase in benzaldehyde concentration, % ee decreased both in the presence and absence of glycerol which could be due to the inhibitory effect of benzaldehyde on *Bm*HNL [2].

As we observed 95–96% ee, in the biocatalysis with 5 and 10 mM benzaldehyde in the 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 (Fig. 8). With 5 mM benzaldehyde, enzyme without polyol showed 71% ee and 51% conversion; in the presence of sorbitol 71% ee and 50% conversion while glycerol addition has improved the % ee to 99.3% in 5 min. 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 the case of 10 mM benzaldehyde, enzyme without polyol gave 65.2% ee and 55.2% conversion; in the 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 min. In this case also, decreased % ee was observed with increased reaction time. Sorbitol addition did not improve the % enantiomeric excess of the purified enzyme in this study.

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 a HNL has improved the % ee of product. Purified *Bm*HNL has been reported to synthesize (*S*)-mandelonitrile in 54% ee [2] while the double mutant *Bm*HNL-H103C-N156 G improved the % ee to 93% [30]. We observed 99.8% ee of (*S*)-mandelonitrile with immobilized *Bm*HNL [29]. 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 is an intermediate in the synthesis of pyrethroids. We studied *Bm*HNL catalyzed synthesis of (*S*)-2-hydroxy-2-(3-phenoxyphenyl) acetonitrile in the presence of glycerol. The biotransformation was carried out by varying substrate concentration from 0.8 to 10 mM (Fig. 9). *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 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 the enzyme showed ~44% ee and 13.5% conversion without glycerol. Thus there is a decrease in both the % ee and conversion of product with increase in substrate concentration. Jangir et al. reported the synthesis

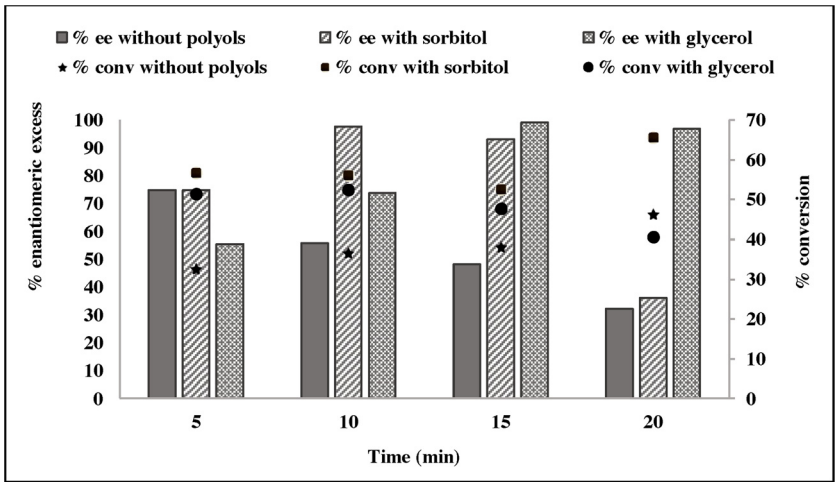


Fig. 6. Effect of polyols in the *Baliospermum montanum* hydroxynitrile lyase (*Bm*HNL) (stored in pH 3.5 buffer) catalyzed synthesis of (*S*)-mandelonitrile. (Bars) represent % enantiomeric excess (ee) while (●), (★), (■) represent % conversion (conv). The standard reaction conditions (300 mM citrate buffer pH 4.2, 0.8 mM benzaldehyde, 100 mM KCN solution) were used with 4U of enzyme and 50 or 200 mg/mL glycerol or sorbitol.

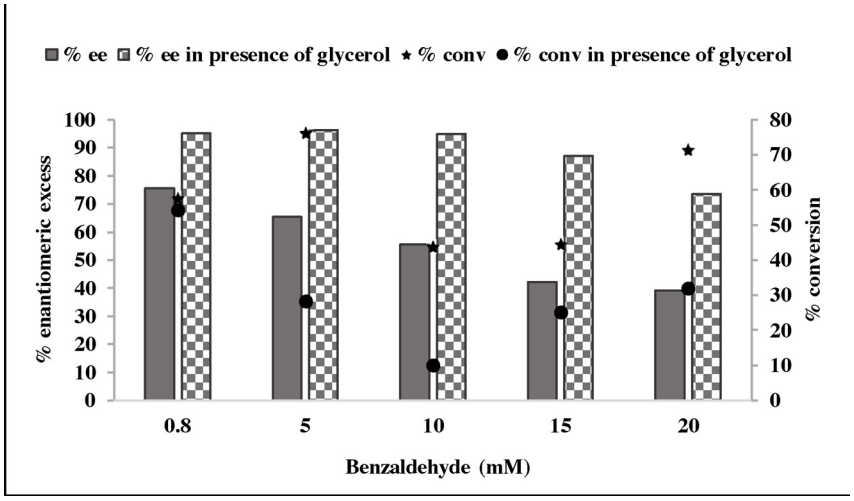


Fig. 7. Effect of benzaldehyde concentration in *Baliospermum montanum* hydroxynitrile lyase (*Bm*HNL) (stored in pH 7.0) catalyzed synthesis of (*S*)-mandelonitrile in the presence of glycerol. (Bars) represent % enantiomeric excess (ee) while (●) and (★) represent % conversion (conv). The standard reaction conditions (300 mM citrate buffer pH 4.2, 100 mM KCN solution, 4U of enzyme) were used with 0.8–20 mM benzaldehyde and 50 mg/mL glycerol.

of (*S*)-2-hydroxy-2-(3-phenoxyphenyl) acetonitrile by crude enzyme with 0.8 mM substrate concentration in a biphasic system that produced only 7.4% ee of the product [23]. CLEA-*Bm*HNL catalyzed synthesis of this (*S*)-cyanohydrin has also been reported that produced 97.6% ee of the product [29].

*Bm*HNL catalyzed synthesis of (*S*)-2-hydroxy-2-(3,5-dimethoxyphenyl) acetonitrile was carried out in the presence of glycerol (Fig. 10) while the study with addition of sorbitol was excluded due to negligible increase in % ee by it (Fig. 8). 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 the absence

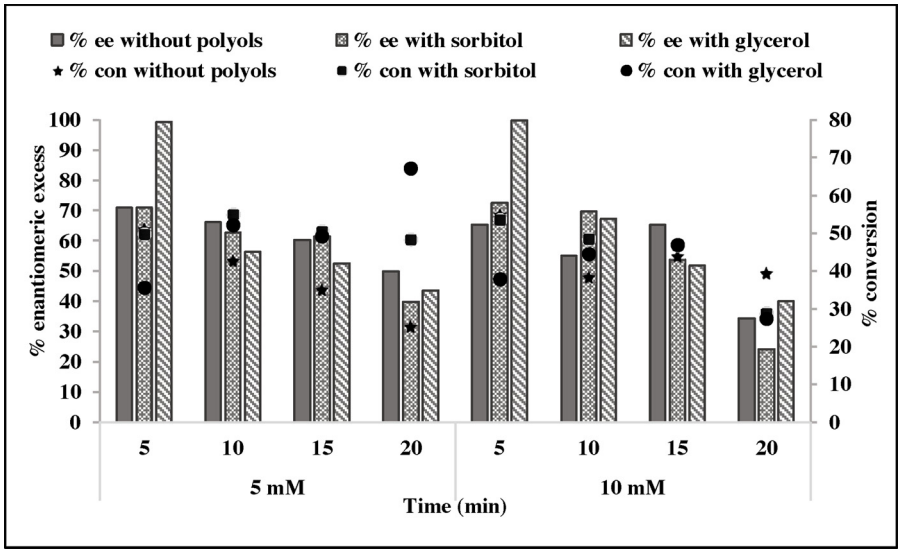


Fig. 8. Effect of polyols in the *Baliospermum montanum* hydroxynitrile lyase (*Bm*HNL) (stored in pH 7.0 buffer) catalyzed synthesis of (*S*)-mandelonitrile. (Bars) represent % enantiomeric excess (ee) while (●), (★), (■) represent % conversion (conv). The standard reaction conditions (300 mM citrate buffer pH 4.2, 100 mM KCN solution, 4U of enzyme) were used with 5–10 mM benzaldehyde and 50 or 200 mg/mL glycerol or sorbitol.

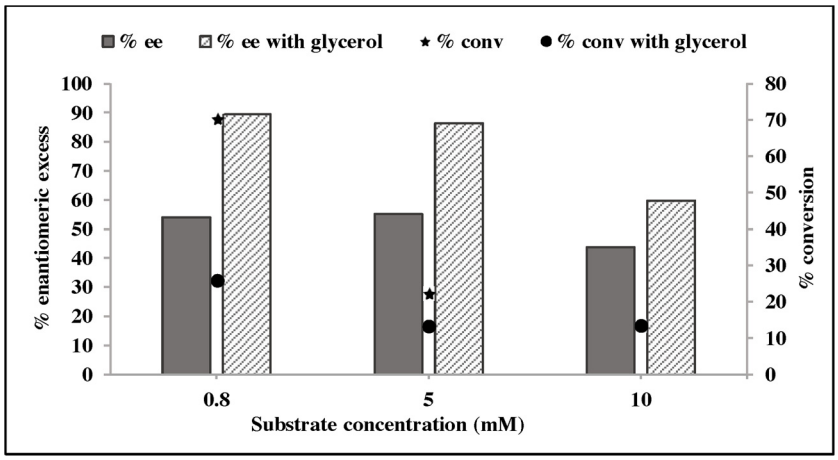


Fig. 9. Synthesis of (*S*)-2-hydroxy-2-(3-phenoxyphenyl) acetonitrile by purified *Baliospermum montanum* hydroxynitrile lyase (*Bm*HNL) (stored in pH 7.0) in presence of glycerol as stabilizer. (Bars) represent % enantiomeric excess (ee) while (●) and (★) represent % conversion (conv). The standard reaction conditions (300 mM citrate buffer pH 4.2, 100 mM KCN solution, 4U of enzyme) were used with 0.8–10 mM 3-phenoxybenzaldehyde and 50 mg/mL glycerol.

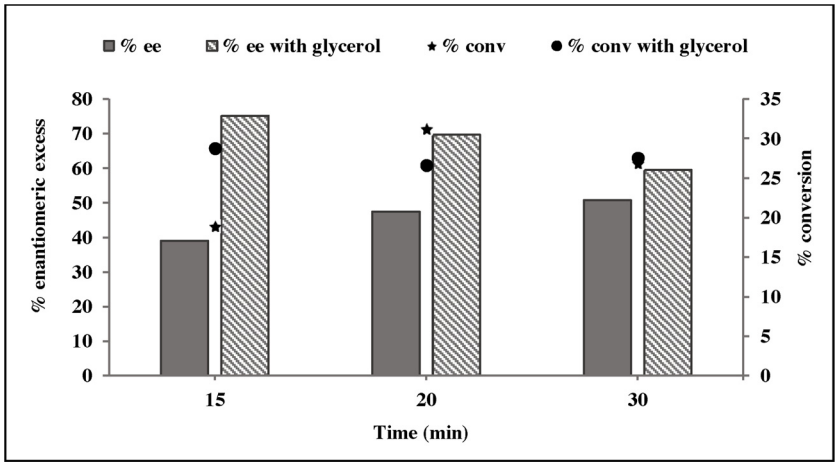


Fig. 10. Synthesis of (*S*)-2-hydroxy-2-(3,5-dimethoxyphenyl) acetonitrile by purified *Baliospermum montanum* hydroxynitrile lyase (*Bm*HNL) (stored in pH 7.0) in the presence of glycerol as stabilizer. (Bars) represent % enatiomeric excess (ee) while (●) and (★) represent % conversion (conv). The standard reaction conditions (300 mM citrate buffer pH 4.2, 100 mM KCN solution, 4U of enzyme) were used with 10 mM 3,5-dimethoxybenzaldehyde and 50 mg/mL glycerol. Reaction was carried out 15–30 min.

of glycerol in 15 min. While the % ee of product in the glycerol added biocatalysis decreased with time, % conversion of product was not affected much. Purified *Bm*HNL catalyzed synthesis of (*S*)-2-hydroxy-2-(3,5-dimethoxyphenyl) acetonitrile has been reported by Dadashipour et al. with 85% ee [2]. 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 [23]. Immobilized *Bm*HNL catalyzed synthesis of (*S*)-2-hydroxy-2-(3,5-dimethoxyphenyl) acetonitrile produced the product in 91.4% ee and 12.5% conversion in 100 min [29].

4. 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 °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-lives among all the currently known 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 increased its half-life by fivefold, and the addition of sorbitol or glycerol increased ~ 9 fold specific activity. Importantly, glycerol added *Bm*HNL biocatalysis has shown > 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 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.

Author contributions

SKP conceived/ and designed the experiments. NJ and Preeti performed and analyzed the stability and activity of *Bm*HNL experiments. NJ performed the stereoselective synthesis of cyanohydrin experiments. SKP, NJ, and Preeti validated the experiments/results of the study. NJ wrote the manuscript. SKP revised and reviewed the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.procbio.2019.10.014.

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