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Enantiopure Synthesis of (*R*)-Mandelonitrile Using Hydroxynitrile Lyase of Wild Apricot (*Prunus armeniaca* L.) [*Pars*HNL] in Aqueous/Organic Biphasic System

Mohammad Asif[1] · **Tek Chand Bhalla**[1]

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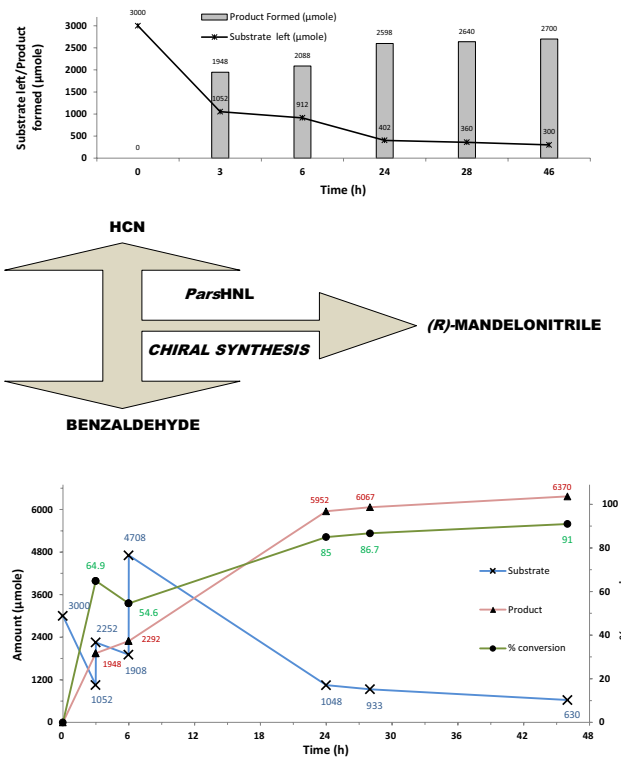
Abstract Hydroxynitrile lyases (HNLs) are increasingly finding application in the synthesis of enantiomerically pure cyanohydrins which are important intermediates in the production of pharmaceuticals and agrochemicals. Synthesis of enantiopure mandelonitrile was carried out using HNL of wild apricot (*Prunus armeniaca* L.) [*Pars*HNL] in aqueous/organic biphasic system. The optimum pH and temperature of the reaction were 4.0 and 15 °C respectively, which are important parameters to suppress the non-enzymatic catalysis. *Pars*HNL catalyses synthesis of (*R*)-mandelonitrile in methyl-*t*butyl ether (MTBE)/citrate buffer biphasic system with >99% ee. Synthesis of mandelonitrile was carried out in batch reaction at 40 ml scale and finally 2.7 mmoles of (*R*)-mandelonitrile was recovered which corresponded to 90% molar conversion in 46 h reaction. In fed batch reaction 6.37 mmoles of (*R*)-mandelonitrile could be produced which corresponds to 91% molar conversion in 46 h. In both reactions, enzyme produces (*R*)-mandelonitrile with >99% ee which showed enhanced selectivity as compared to aqueous reaction (96% ee) by *Pars*HNL. The results showed potential of *Pars*HNL to synthesize (*R*)-mandelonitrile in both, batch reaction and fed-batch reaction and can be effectively used in the synthesis of (*R*)-mandelonitrile.

Graphical Abstract



Keywords *Prunus armeniaca* L. · Wild apricot · (*R*)-mandelonitrile · Biphasic reaction · Hydroxynitrile lyase · *Pars*HNL · Cyanohydrin · Batch-fed batch reaction

1 Introduction

Hydroxynitrile lyases (HNL, EC 4.1.2.x) are the enzymes which mediate the release of hydrogen cyanide (HCN) and

✉ Tek Chand Bhalla
bhallatc@rediffmail.com

[1] Department of Biotechnology, Himachal Pradesh University, Summer Hill, Shimla, Himachal Pradesh 171005, India

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aldehyde or ketone from their corresponding cyanohydrins [1] and catalyses enantioselective synthesis of cyanohydrins through reverse reaction [2]. The cyanohydrins and their derivatives are finding wide applications in industries for production of pharmaceuticals, agrochemicals and cosmetics, as both functional groups of cyanohydrins, hydroxyl and cyanide are attached to same carbon atom, which can be readily converted into other synthetically relevant building blocks like α-hydroxy ketones, α-hydroxy carboxylic acids and β-amino acids [3].

The enantiomeric purity of the synthesized caynohydrins is highly dependent on the spontaneous non-enzymatic catalysis which is major drawback of aqueous monophasic reaction. To overcome this problem and to maintain the substrate and product concentration to avoid catalytic inhibition, immiscible biphasic reactions are introduced. Biphasic reactions are also preferred due to easy recovery of product formed from the organic phase. HNL mediated synthesis of cyanohydrins involves two substrates and therefore it is a complex reaction that involves several parameters [1, 4]. The role of organic solvent has been well studied on *Pa*HNL activity and stability [5]. Biphasic reaction is known for its suppression of non-enzymatic catalysis and hence enhancing the enantiopurity of product formed. Previous studies on the syntheses of chiral cyanohydrins in biphasic systems were carried out on the HNLs from *Manihot esculenta* (*Me*HNL), *Hevea brasiliensis* (*Hb*HNL), *Sorghum bicolour* (*Sb*HNL), *Prunus amygdalus* (*Pa*HNL), *Passiflora edulis* (*Pe*HNL) and *Prunus dulcis* (*Pd*HNL) [6–15], but no further information on cyanohydrins synthesis is available for recently characterized HNLs.

Recently purification and characterization of HNL from wild apricot (*Prunus armeniaca* L.) has been reported [16]. The HNL from *P. armeniaca* (*Pars*HNL) catalyzed the synthesis of (*R*)-mandelonitrile from benzaldehyde in a citrate buffer system employing potassium cyanide as a cyanide source [16], but the yield and enantiomeric excess of the (*R*)-mandelonitrile was low in this aqueous medium.

Therefore, in the present studies, an attempt has been made to synthesize (*R*)-mandelonitrile using *Pars*HNL in aqueous/organic biphasic system and study productivity in batch and fed batch conditions with aim to enhance both volumetric yield and enantiomeric excess of the product.

2 Materials and Methods

2.1 Plant Material

Seeds of wild apricot (*P. armeniaca*) used as source of HNL were obtained from the Department of Food Science and Technology, Dr. Y S Parmar University of Horticulture and Forestry, Solan, India.

2.2 Preparation of Crude Enzyme Extract

Crude hydroxynitrile lyase was extracted from the kernel of *P. armeniaca* seeds according to method outlined by Han et al. [17] with slight modification [16]. The crude enzyme obtained was concentrated using vacuumed-freeze drier. The protein concentration in the enzyme preparation was determined following the method of Bradford [18].

2.3 Preparation of HCN Solution

Preparation of cyanide solution was carried out following the method used by Alagöz et al. [15] with slight modification. In brief potassium cyanide (13.0 g) was added into 100 ml of citrate buffer (50 mM, pH 5.0) on ice and then 25 ml of methyl-*t*butyl ether (MTBE) was added onto cyanide solution. The mixture was continuously stirred for 25 min followed by addition of HCl (35%) until the final pH was dropped to 3.0. MTBE layer containing HCN was separated and further MTBE was added repeatedly onto the citrate buffer so that HCN formed was extracted further. The combined MTBE layers were collected together and stored over citrate buffer (50 mM, pH 4.0) in a dark bottle at 4 °C. Concentration of HCN was determined as described in the literature [19].

2.4 Effect of pH

The effect of pH on the synthesis of mandelonitrile was studied. For this purpose reaction mixture (20 ml) was prepared with aqueous/organic phase (MTBE) [1:3 ratio (v/v)] containing 1500 µmole of benzaldehyde dissolve in DMSO (0.5 ml), and HCN was used in excess amount with citrate buffer of molarity 400 mM was used and pH was varied from 4.0 to 6.0. The reaction was carried out at 15 °C at shaking condition and the percentage conversion and % ee was recorded.

2.5 Effect of Temperature

Temperature plays an important role being a reversible catalytic property of HNLs. Effect of temperature was studied and the synthesis of mandelonitrile was carried out at different temperatures (10–20 °C). For this purpose reaction mixture (20 ml) was prepared with aqueous/organic phase (MTBE) [1:3 ratio (v/v)] containing 1500 µmole of benzaldehyde dissolve in DMSO (0.5 ml), and HCN was used in excess amount with citrate buffer of molarity 400 mM at pH 4.0 was used.

2.6 Effect of Time

Effect of time on % molar conversion and %ee was studied. The reaction was continued for 46 h and % molar conversion and %ee was determined. For this purpose reaction mixture (40 ml) was prepared with aqueous/organic phase (MTBE) [1:3 ratio (v/v)] containing 3000 µmole of benzaldehyde dissolve in DMSO (1 ml), and HCN was used in excess amount with citrate buffer of molarity 400 mM at pH 4.0 was used. The reaction was carried out at 15 °C at shaking condition, samples were withdrawn (3, 6, 24, 28 and 46 h) and the percentage conversion and % ee was recorded.

2.7 Synthesis of Chiral Mandelonitrile in Batch Reaction

Batch reaction was performed using 3000 µmole of benzaldehyde dissolve in DMSO (1 ml), and HCN was used in excess amount to ensure the availability throughout the carboligation reaction. Reaction mixture (40 ml) was prepared with aqueous/organic phase (MTBE) [1:3 ratios (v/v)] containing 75 mg crude *Pars*HNL, citrate buffer of pH 4.0, molarity 400 mM. The reaction was carried out at 15 °C in shaking condition at 160 rpm.

2.8 Fed Batch Reaction

A fed batch reaction was carried out starting with 3000 µmole of benzaldehyde dissolved in DMSO (1 ml). Reaction mixture (40 ml) was prepared with aqueous/organic phase (MTBE) [1:3 ratios (v/v)] containing 75 mg crude *Pars*HNL, citrate buffer of pH 4.0, molarity 400 mM. The reaction was carried out at 15 °C in shaking condition at 160 rpm. Feeding of substrates were done at 3 h (1200 µmole) and 6 h (2800 µmole), hence total of 7000 µmole of substrate was used. Samples were withdrawn and amount of product formed, substrate left and %ee was determined at regular interval of time.

2.9 Analysis of Mandelonitrile Produced

The mandelonitrile synthesized in reactions was quantified by HPLC using 65% acetonitrile in water as mobile phase using Shimadzu Prominence System with auto sampler equipped with C18 5 µm (4.6×250 mm) column and dual wavelength detector with flow rate of 60 ml/h. Absorbance was measured at 210 nm wavelength and retention time (RT) of 4.0 and 5.0 min were observed for mandelonitrile and benzaldehyde respectively.

2.10 Determination of Enantioselectivity

To determine the enantiomeric excess (%ee), chiral chromatography was performed using Chiralcel-OJH 5 µm (4.6×250 mm) column (Daicel) and SPD-20A vp UV detector, with mobile phase of *n*-hexane:isopropanol in ratio of 80:20 respectively with flow rate of 60 ml/h. Sample prepared in isopropanol was injected using auto sampler and the absorbance was recorded at 210 nm and data analyzed using Shimadzo LC solution software. The %ee was calculated as $\frac{R-S}{R+S} \times 100$, where *R* and *S* are the amount of mandelonitrile formed as (*R*)-mandelonitrile and (*S*)-mandelonitrile respectively. A blank reaction was also performed without enzyme and amount of mandelonitrile formed in blank was subtracted from enzyme catalyzed reaction.

3 Results and Discussion

3.1 Effect of pH

The effect of pH on asymmetric synthesis of (*R*)-mandelonitrile in the biphasic system (citrate buffer-MTBE) is shown in Fig. 1. It was observed that at pH 4.0, 65% molar conversion of substrate and ee (>99%) were obtained at the end of 3 h reaction time. On increasing the pH above 4.0, upto 6.0 caused increase in the molar conversion (85%) but

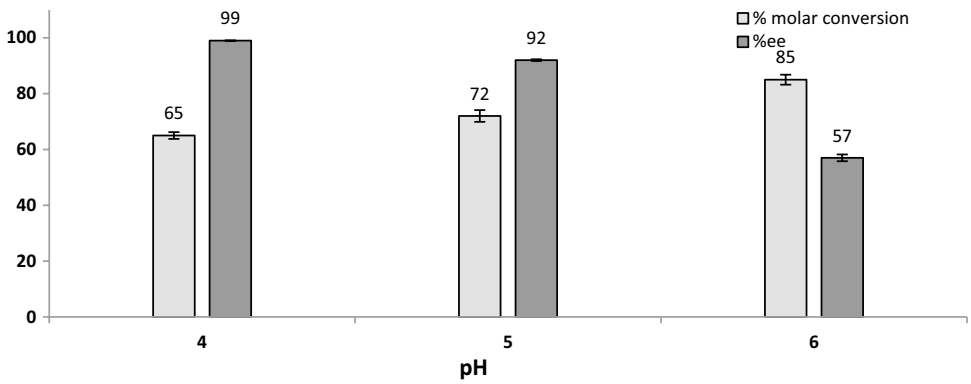

Fig. 1 The effect of pH on molar conversion (in percent) and %ee value of (*R*)-mandelonitrile synthesized by *Pars*HNL in biphasic condition

a drastic loss in the %ee of (*R*)-mandelonitrile (57%) was observed.

This result indicated that the rate of non-enzymatic carboligation of cyanide molecule to benzaldehyde increased at pH 5.0 and 6.0 and this decreased the %ee value of (*R*)-mandelonitrile in the reaction. pH 4.0 has been earlier reported to be the optimum pH for synthesis of (*R*)-mandelonitrile catalysed by HNL from *P. amygdalus turcomanica* [20], *P. dulcis* [15] and *Passiflora edulis* [14].

3.2 Effect of Temperature

The effect of the temperature on the enantioselectivity of *Pars*HNL was examined at 10–20 °C and the molar conversion and %ee change of synthesized (*R*)-mandelonitrile depending on temperature were shown in Fig. 2.

*Pars*HNL catalysed the reaction effectively in the temperature range varying from 10 to 20 °C. Increasing the temperature above 20 °C, favours the reverse reaction i.e. conversion of mandelonitrile to benzaldehyde and hence avoided. The molar conversion and %ee were obtained as 60% and >99 respectively at 10 °C, pH 4.0 at the end of 3 h. On increasing the temperature, the % molar conversion and %ee values of (*R*)-mandelonitrile were 65 and >99% at 15 °C. When the temperature was increased to 20 °C, 64% molar conversion was recorded whereas the %ee observed for (*R*)-mandelonitrile was 95%. This observation can be explained that the increase in temperature slightly accelerates the non-enzymatic carboligation, thus leading to decrease in %ee of (*R*)-mandelonitrile and similar trends have been reported earlier [14, 21].

3.3 Time Course Reaction

As demonstrated in Fig. 3, the % molar conversion increases with respect to reaction time while the %ee of (*R*)-mandelonitrile was constant. The % molar conversion of (*R*)-mandelonitrile increased from 64.9 to 90% when the reaction time was increased from 3 to 46 h at pH 4.0

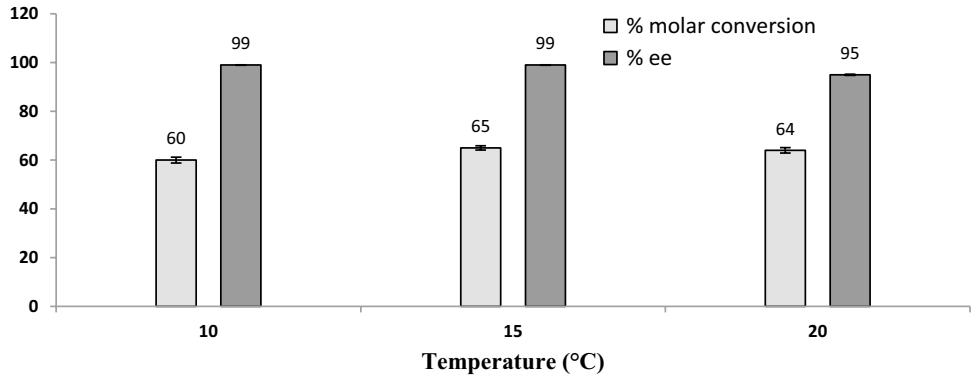

Fig. 2 The effect of temperature on molar conversion (in percent) and % ee value of (*R*)-mandelonitrile synthesized by *Pars*HNL in biphasic condition

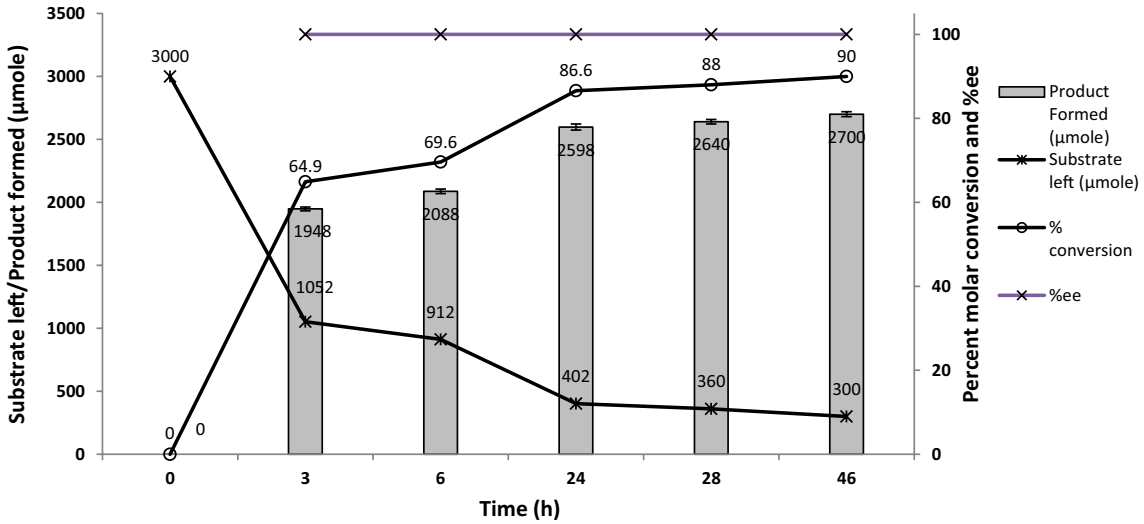

Fig. 3 The effect of time course on molar conversion (in percent) and % ee value of (*R*)-mandelonitrile, product formation and substrate left in batch reaction synthesized by *Pars*HNL in biphasic condition

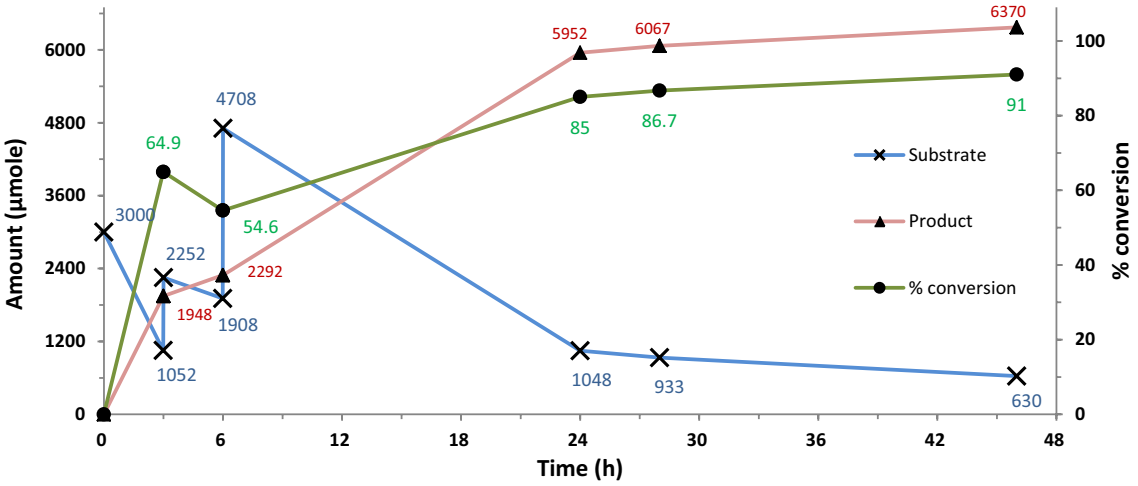

Fig. 4 Effect of time on product formation (*filled triangle*), substrate left (*multiply symbol*) and percentage conversion (*filled circle*) in fed-batch reaction synthesized by *Pars*HNL in biphasic condition

and 15 °C and no increament in % molar conversion was observed on increasing the reaction time further.

The % molar conversion of (*R*)-mandelonitrile was 90% whereas its %ee value was still >99%. These results showed that *Pars*HNL had a unique enantio-specificity and the non-emzymatic spontaneous addition of HCN and benzaldehyde has completely suppressed at pH 4.0 and 15 °C during long reaction time.

3.4 Synthesis of (*R*)-Mandelonitrile in Batch Reaction

A batch reaction was carried out using 3000 µmole of benzaldehyde dissolved in DMSO, at 15 °C, pH 4.0 and it was observed that a total of 90% molar conversion of substrate added was achieved in 46 h reaction and no further increase in conversion was observed on extending the reaction time. The %ee was >99% throughout the reaction. A total of 2700 µmole of (*R*)-mandelonitrile was produced which corresponds to 90% molar conversion of substrate used (Fig. 3). In earlier studies Alagöz et al. have observed 91% yield with 93%ee for (*R*)-mandelonitrile after 3 days of reaction with *Pat*HNL [20]. Cabirol et al. have reported 98% yield with 97%ee for (*R*)-mandelonitrile with crude *Pa*HNL extract after 4 h of incubation in batch scale [22] while Nanda et al. obtained 65% yield with 95% ee for (*R*)-mandelonitrile using crude *P. mume* HNL after 24 h of incubation in 50 ml batch reaction [23].

3.5 Fed Batch Reaction

A fed batch reaction was performed starting with 3000 µmole of benzaldehyde dissolved in DMSO, pH 4.0, at 15 °C. In 3 h 64.9% molar conversion was observed yielding 1948 µmole of product. After feeding of 1200 µmole substrate at this point, total 2292 µmole of product was synthesized after 6 h which corresponded to 54.6% molar conversion. At 6 h finally 2800 µmole of substrate was added and reaction was continued up to 46 h. At 24, 28 and 46 h, the % molar conversion observed was 85, 86.7 and 91 respectively. Hence a total of 91% molar conversion was achieved in 46 h reaction which corresponded to 6370 µmole of mandelonitrile produced (Fig. 4). The %ee was >99% though out the reaction. As compared with batch reaction, the fed batch reaction was observed to be more efficient in terms of % molar conversion and the amount of product formed.

The productivity of mandelonitrile produced (µmol/h) was calculated for both batch and fed batch reactions and it was observed that fed batch reaction seemed better than batch reaction (Fig. 5). At the end of 46 h the productivity for (*R*)-mandelonitrile (µmol/h) was 2.4 times for fed-batch reaction as compare with batch reaction. These results suggest that the fed batch mode of reaction can be employed for achieving higher volumetric yield and % enantiomeric excess of (*R*)-mandelonitrile in a biphasic reaction condition.

4 Conclusion

The potential of the *Pars*HNL for enantioselective synthesis of (*R*)-mandelonitrile has been explored. The results showed that the catalytic performance of *Pars*HNL was significantly influenced by reaction conditions like pH and temperature. The fed-batch reaction seemed more efficient as compared to batch reaction in the term of percentage conversion as well as amount of product synthesized. These results demonsitrate that *Pars*HNL is a powerful and

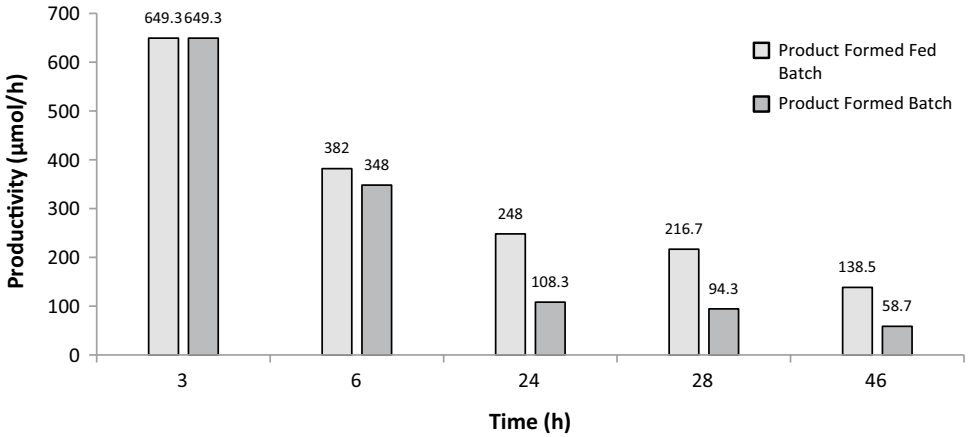

Fig. 5 Comparison of productivity of (*R*)-mandelonitrile synthesized in batch and fed-batch conditions with time catalysed by *Pars*HNL in biphasic condition

cheap biocatalyst for the synthesis of (*R*)-mandelonitrile in biphasic conditions. Moreover, the appropriate biphasic system for synthesis of (*R*)-mandelonitrile indicated that *Pars*HNL has high potential for enantiopure synthesis of (*R*)-mandelonitrile.

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