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Parameters influencing asymmetric synthesis of (*R*)-mandelonitrile by a novel (*R*)-hydroxynitrile lyase from *Eriobotrya japonica*

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ABSTRACT

(*R*)-Mandelonitrile was successfully synthesized by an enzymatic transcyanation reaction of benzaldehyde and acetone cyanohydrin catalyzed by a hydroxynitrile lyase from *Eriobotrya japonica* (*Ej*HNL) in an aqueous-organic biphasic system. The effects of pH, temperature, organic solvent, substrate concentration and enzyme concentration on the initial activity and enantioselectivity of the enzyme were studied. Both pH and temperature had a large effect on the initial velocity and enantiomeric excess (*e.e.*) of the product, (*R*)-mandelonitrile. High enantiomeric purity of the product was observed at low pH and temperature because the non-enzymatic reaction producing racemates of mandelonitrile was almost suppressed. The optimum pH and temperature to obtain high *e.e.* were pH 4.0 and 10 °C, respectively. Surprisingly, the organic solvents had a significant influence on the initial velocity of the reaction but less influence on the enantiomeric purity of product. The *Ej*HNL was very stable in ethyl acetate, diethyl ether, methyl-*t*-butyl ether, diisopropyl ether, dibutyl ether and hexane for 12 h. The best solvent for the highest initial velocity and *e.e.* was diethyl ether with an optimum aqueous phase content of 50% (v/v). The initial reaction rate increase as the aqueous phase content rose, but when the content was more than 50%, a reduction of *e.e.* was observed. Increasing the concentration of the substrates accelerated the initial velocity, but caused a slight decrease in the *e.e.* of the product. Under the optimized conditions, the conversion and *e.e.* of (*R*)-mandelonitrile for 3 h were 40 and 99%, respectively. The aqueous phase containing the enzyme also showed considerably efficient reusability for 4 batch reactions.

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1. Introduction

Recently, the interest of researchers in the synthesis and application of chiral cyanohydrins has markedly increased. The enantiomeric purity of chiral cyanohydrins has become an important criterion in the synthesis of valuable structural moieties including α-hydroxy-aldehyde, vicinal diols, β-amino alcohols and β-hydroxy-α-amino acids which are building blocks for industrial products such as pharmaceuticals, veterinary products, crop-protecting agents, vitamins, food additives, etc. [1].

The asymmetric synthesis of chiral cyanohydrins has successfully employed hydroxynitrile lyases (HNLs) as the key enzyme. HNLs (EC 4.1.2.10; EC 4.1.2.11; EC 4.1.2.37; EC 4.1.2.39) are classified based on their enantioselectivity into two groups. (*R*)-HNLs catalyze the nucleophilic addition of HCN to aldehydes or ketones yielding (*R*)-cyanohydrins, while (*S*)-HNLs catalyze the formation of (*S*)-cyanohydrins [2,3]. Nevertheless, asymmetric synthesis of chiral cyanohydrins employing HNLs is influenced by many factors. To achieve the high enantiomeric purity of chiral cyanohydrins, several strategies have been used to suppress the undesired simultaneous non-enzymatic formation of racemic cyanohydrins. The reaction carried out in the aqueous process has been optimized based on the pH and temperature of the reaction [4]. Although methods employing water-miscible solvents to produce chiral cyanohydrins have been of much interest, but the non-enzymatic reaction is still a problem [5]. Therefore, biphasic systems with buffer and water-immiscible organic solvents have been developed to minimize the non-enzymatic reaction as described elsewhere [6].

Among the HNLs discovered up to date, only few of HNL was purified and characterized [7]. Moreover, some characteristics of HNLs in the asymmetric synthesis of cyanohydrins in biphasic systems have been studied with a few HNLs from *Manihot esculenta* (*Me*HNL), *Hevea brasiliensis* (*Hb*HNL) and *Prunus amygdalus* (*Pa*HNL), and differences in some characteristics in each enzyme were observed [8–12]. The activity and enantioselectivity of the

Abbreviation: *Ej*HNL, hydroxynitrile lyase from *Eriobotrya japonica*.

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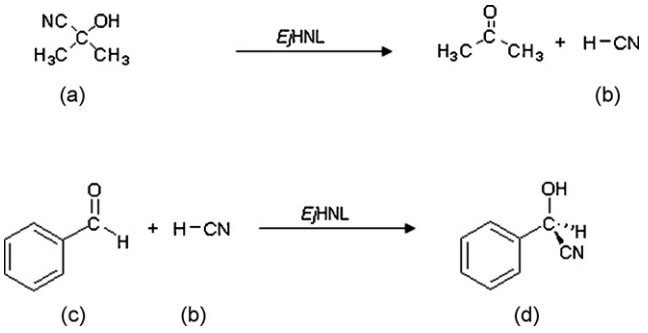

Scheme 1. The synthesis of (*R*)-mandelonitrile by transcyanation of acetone cyanohydrin and benzaldehyde using *Ej*HNL. Acetone cyanohydrin (a) was cleaved and released hydrocyanic acid (b) by the enzymatic reaction of *Ej*HNL. Then, the *Ej*HNL catalyze the asymmetric addition of hydrocyanic acid to benzaldehyde (c) yielding the (*R*)-mandelonitrile (d).

enzymes in biphasic systems were influenced by many parameters such as pH, temperature, organic solvent, aqueous phase content, source of enzyme, etc. [13–15]. However, only few paper describing on the biological characteristics of HNL, the unit of HNL used in cyanohydrin syntheses, and the actual initial reaction velocity have appeared in the literature leading to the lack of understanding of the enzyme characteristics and difficult to repeat the experiment. Therefore, the biological characteristics of the HNLs should be characterized to fully utilize the enzymes in their applications and the actual unit of enzyme should be described in the paper.

Recently, a novel (*R*)-hydroxynitrile lyase from *Eriobotrya japonica* (*Ej*HNL) (EC 4.1.2.10) was discovered, purified and characterized by our group. *Ej*HNL showed promising ability in the synthesis of several cyanohydrins in an aqueous system [16,17]. Although the synthesis of cyanohydrins by HNL from loquat (*Eriobotrya* L.) was performed in a biphasic system and under micro-aqueous conditions by Lin et al., but there has no report regarding the actual unit of *Ej*HNL used in cyanohydrin synthesis [18] and the characteristics of the enzyme in biphasic systems have not been studied and described elsewhere. Therefore, the vigilant characterization of *Ej*HNL on the synthesis of cyanohydrins in biphasic systems was of much interest for further application of the enzyme.

In this paper, the asymmetric synthesis of (*R*)-mandelonitrile by *Ej*HNL in biphasic system was investigated and successfully realized for the first time. Transcyanation of benzaldehyde and acetone cyanohydrin was employed in this study (Scheme 1). Several important parameters influencing the biological characteristic of the *Ej*HNL including pH, temperature, organic solvents, aqueous phase content, substrate concentration and the enzyme concentration were studied and described. Moreover, all of these parameters were optimized to achieve high initial reaction velocity and enantiomeric purity of the products. The aqueous phase containing the enzyme was found to be reusable under the optimized conditions.

2. Experimental

2.1. Materials and chemicals

Seeds of *E. japonica* were purchased from the National Federation of Agricultural Co-operative Associations (Nagasaki, Japan) and stored at 4 °C. All chemicals utilized in the experiments were purchased from commercial sources and used without further purification. Benzaldehyde (redistilled, 99.5+%; Sigma–Aldrich Inc., USA) and acetone cyanohydrin (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) were also used. The chiral HPLC analysis was performed using a CHIRALCEL OJ-H column (Diacel Chemical Industries Ltd., Osaka, Japan) with a SPD-10A VP UV–vis detector (Shimadzu, Kyoto, Japan) at 254 nm. The eluting solvent was the mixture of *n*-hexane (85%) and isopropanol (15%).

2.2. Crude enzyme preparation

Seeds of *E. japonica* were sterilized by 0.1% (v/v) sodium hypochlorite and rinsed with de-ionized water. Sterilized seeds were homogenized with 3% polyvinylpyrolidone in 10 mM potassium phosphate buffer, pH 6.0 (100 ml/100 g fresh seed) in a SMT Process Homogenizer PH91 (SMT Company, Tokyo, Japan) at 4 °C. The homogenate was filtered through four layers of cheesecloth and centrifuged at 28,000 × *g* for 30 min. The supernatant was precipitated by 30–80% saturation of $(NH_4)_2SO_4$ and the precipitated enzyme was collected by centrifugation at 28,000 × *g* for 30 min at 4 °C. The precipitate was dissolved and dialyzed against the same buffer for 12 h. The enzyme solution was lyophilized. The lyophilized powder (2270 units/g powder) was used as a crude enzyme.

2.3. Activity assay

A reaction mixture in a total volume of 1.0 ml was prepared in a micro-tube. Bezaldehyde (1.0 M in DMSO, 40 μl) was added to sodium citrate buffer (400 mM, pH 4.0), followed by the enzyme solution and a KCN solution (1.0 M, 100 μl). The initial velocity of the reaction was monitored by taking a small aliquot of the reaction mixture (100 μl) and the reaction was stopped by extracting with 700 μl of organic solvent (85% *n*-hexane, 15% isopropanol by volume). The mandelonitrile formed was extracted into the organic layer, and the supernatant obtained by centrifugation at 15,000 × *g* for 1 min at 4 °C was analyzed by HPLC with a CHIRALCEL OJ-H column at 254 nm using a mobile phase of solvent (85% *n*-hexane, 15% isopropanol, v/v) at a flow rate of 1.0 ml/min. The retention times of benzaldehyde and (*R*)- and (*S*)-mandelonitrile were about 4.9, 10.2 and 12.7 min, respectively. The reaction progressed linearly in the first 5 min was used for calculating activity.

One unit of HNL activity was defined as the amount of enzyme that produced 1 μmol of optically active mandelonitrile from benzaldehyde per min under standard assay conditions.

2.4. Transcyanation reaction in biphasic system

The reaction (total volume of 1.5 ml) was performed in a 2.0 ml micro-tube. Organic solvent containing benzaldehyde was mixed with citrate-phosphate buffer (400 mM) containing the crude enzyme powder. The reaction was initiated by the addition of acetone cyanohydrin, and the mixture was shaken at 1500 rpm in an incubator shaker (BioShaker M-BR-022UP, Taitec Corporation, Tokyo, Japan). Aliquots of sample (50 μl) were withdrawn from the organic phase at different intervals time, and mixed with the HPLC solvent (100 μl; *n*-hexane:2-propanol, 85:15, v/v). The initial velocity, conversion and enantiomeric excess (*e.e.*) of (*R*)-mandelonitrile formed were analyzed by chiral HPLC. The initial velocity and conversion were calculated using a standard curve of (*R*)-mandelonitrile, while *e.e.* was determined by calculation of the peak areas of the two enantiomers using the following equation:

$$\%\text{enantiomeric excess} = \frac{R-S}{R+S}100 \quad (1)$$

where *R* and *S* represent the concentrations of the (*R*)-mandelonitrile and (*S*)-mandelonitrile, respectively.

Details of the pH, temperature, benzaldehyde and acetone cyanohydrin concentrations, organic solvent, volume of the aqueous buffer and enzyme concentration were specific for each case and described in the figure legends.

2.5. Influence of organic solvents on enzyme stability

The organic solvents were mixed with 400 mM citrate-phosphate buffer, pH 4.0 (ratio 1:1, v/v) and equilibrated with shaking at 1500 rpm, 10 °C for 60 min in the incubator shaker (BioShaker M-BR-022UP, Taitec Corporation, Tokyo, Japan). The solution of *Ej*HNL (50 μl, 5 units) was injected into the aqueous phase and mixed gently so as not to disturb the interface. Then, the enzyme activity at zero time was determined with the saturated solvent in the aqueous phase. After that, the mixtures of enzyme solution and organic solvent were shaken to obtain the emulsion between the two phases at 1500 rpm, 10 °C for 12 h. The mixture was centrifuged at 15,000 × *g* for 1 min at 4 °C to separate the aqueous and organic phase, and the aqueous phase was then withdrawn to assay the remaining activity of the enzyme.

2.6. Determination of the partition coefficients for benzaldehyde and mandelonitrile

The partition coefficient for benzaldehyde between the buffer and various organic solvents was determined. Benzaldehyde (20 mM) was dissolved in the organic solvent (500 μl), then mixed with 400 mM citrate-phosphate buffer, pH 4.0 (500 μl). The mixture was equilibrated by shaking at 10 °C for 1 h. The benzalehyde concentration was determined by HPLC with a CHIRALCEL OJ-H column and the partition coefficient was calculated using the following equation:

$$\text{partition coefficient } (K) = \frac{C_{org}}{C_{aq}} \quad (2)$$

where C_{org} and C_{aq} represent the concentrations of benzaldehyde (g/l) in organic phase and aqueous phase, respectively.

2.7. Reusability

To test the stability of the enzyme in the aqueous phase in repeated use, a batch transcyanation reaction of benzaldehyde (200 mM) and acetone cyanohydrin (400 mM) in a biphasic system of 400 mM citrate-phosphate buffer, pH 4.0 (5 ml) and diethyl ether (5 ml) was conducted by addition of the *Ej*HNL powder (30 units) at 10 °C for 3 h. Then, the aqueous phase containing the enzyme was recovered and dialyzed against the 400 mM citrate-phosphate buffer, pH 4.0 and reused for the next batch reaction under the same conditions.

3. Results

3.1. pH and temperature

The effect of pH on the asymmetric synthesis of (*R*)-mandelonitrile from benzaldehyde and acetone cyanohydrin by *Ej*HNL in the biphasic system of diisopropylether (DIPE) and buffer was examined in the range of pH 3–7. The pH of the buffer influenced the reaction velocity and *e.e.* of the product significantly. The highest initial velocity of *Ej*HNL in the biphasic system was at pH 5 (Fig. 1a) and it lost activity at a pH lower and higher than this optimum. The highest *e.e.* (98%) was observed between pH 3.0 and 4.0, while an increase of pH from 4.5 to 7.0 caused a drastic loss in the *e.e.* of the product. At low pH, the spontaneous release of cyanide ion from acetone cyanohydrin was slow leading to the low initial velocity of the enzyme [19]. On the other hand, the low pH might suppress the non-enzymatic reaction leading to a high *e.e.* At higher pH, the acceleration of acetone cyanohydrin's decomposition occurred providing the low *e.e.* of the product [4].

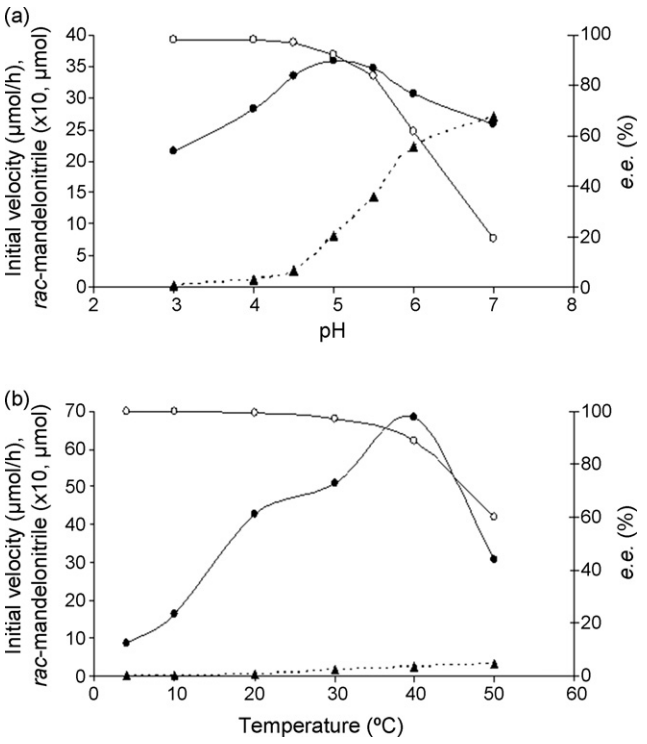

Fig. 1. Effect of pH (a) and temperature (b) on initial velocity (●), *e.e.* (○) and non-enzymatic reaction (▲) in the transcyanation of benzaldehyde (250 mM) and acetone cyanohydrin (500 mM) in biphasic system of 20% (v/v) of buffer and DIPE, containing 5 U of *Ej*HNL. The reaction was performed at 30 °C for pH effect and pH 4.0 for temperature effect.

The optimum conditions to obtain a high *e.e.* of the product were reported to be pH 5.0–5.5 and a temperature of between −5 and 4 °C [20]. The effects of pH and temperature on *e.e.* were studied. The higher *e.e.* was obtained at pH 4.0 than 5.5 at the same temperature because the non-enzymatic reaction was almost suppressed (data not shown). Therefore, the effect of temperature was studied at pH 4.0 to minimize the non-enzymatic reaction.

The effect of temperature in the range of 4–50 °C was investigated in the biphasic system of DIPE and citrate-phosphate buffer (pH 4.0). As demonstrated in Fig. 1b, the enantioselectivity of *Ej*HNL was certainly influenced by reaction temperature. The initial velocity increased as the temperature increased from 4 to 40 °C, followed by a decrease at higher temperature. When the reaction temperature increased, the chance of a collision between the enzyme and both substrates also increased. This might explain why the formation of enzyme–substrate complexes was enhanced and the reaction rate was improved. Although the highest initial velocity of the reaction was observed at 40 °C, the highest *e.e.* (>99%) was obtained at low temperature, 4–10 °C. The *e.e.* value decreased with an increase of temperature due to an acceleration of the non-enzymatic reaction at high temperature. High temperature might increase the possibility of non-enzymatic collision between molecules of benzaldehyde and HCN released from acetone cyanohydrin and cause the racemization of the product.

3.2. Organic solvent

Organic solvent is essential to activity and enantioselectivity of enzymes. It has a large influence on enzyme enantioselectivity, but in some cases it was found to have no effect [21,22]. Ethyl acetate (EA; log *P* 0.67), diethyl ether (DEE; log *P* 0.85), methyl-*t*-butyl ether (MTBE; log *P* 1.4), diisopropyl ether (DIPE; log *P* 1.9), dibutyl ether

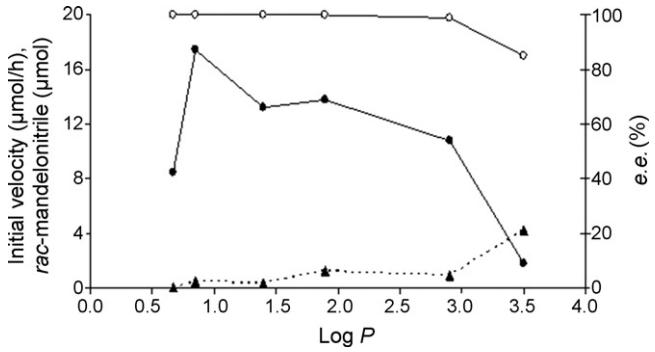

Fig. 2. Effect of organic solvent on initial velocity (●), *e.e.* (○) and non-enzymatic reaction (▲) in the transcyanation of benzaldehyde (250 mM) and acetone cyanohydrin (500 mM) in biphasic system of 20% (v/v) of buffer, pH 4.0, 10 °C, containing 5 U of *Ej*HNL with various organic solvents.

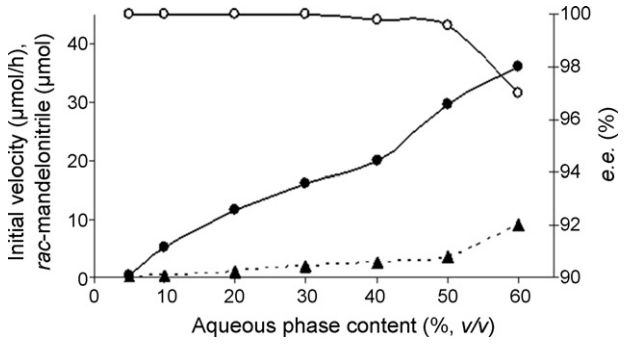

Fig. 4. Effect of aqueous phase content on initial velocity (●), *e.e.* (○) and non-enzymatic reaction (▲) in biphasic system of DEE (1 ml) and buffer (pH 4.0, vary volume of aqueous phase content) at 10 °C containing benzaldehyde (250 mM), acetone cyanohydrin (500 mM) and 5 U of *Ej*HNL.

(DBE; log *P* 2.9) and hexane (HEX; log *P* 3.5) were used as the organic phase in biphasic system. The initial velocity of the reaction was significantly affected by the organic solvents. The best solvent giving the highest initial velocity was DEE, while a decrease in the initial velocity of the reaction was observed with solvents having a log *P* value higher and lower than 0.85 (Fig. 2). *Ej*HNL showed weak activity in the EA and HEX biphasic systems. Good stability of *Ej*HNL in all biphasic systems was observed and the stability was not correlated with the log *P* of the organic solvent as shown in Fig. 3a. The stability of *Ej*HNL after incubation with various organic solvents was determined and compared to that of initial activity in aqueous phase saturated with the organic solvent. The remaining activity of the enzyme was more than 80% in EA, DBE, DEE, DIPE, MTBE and HEX after incubation for 12 h. Log *P* values of between 0.67 and 2.9 had no significant effect on the *e.e.* value, while a decrease in *e.e.* was observed at a log *P* of 3.5 for HEX. The partition coefficient for

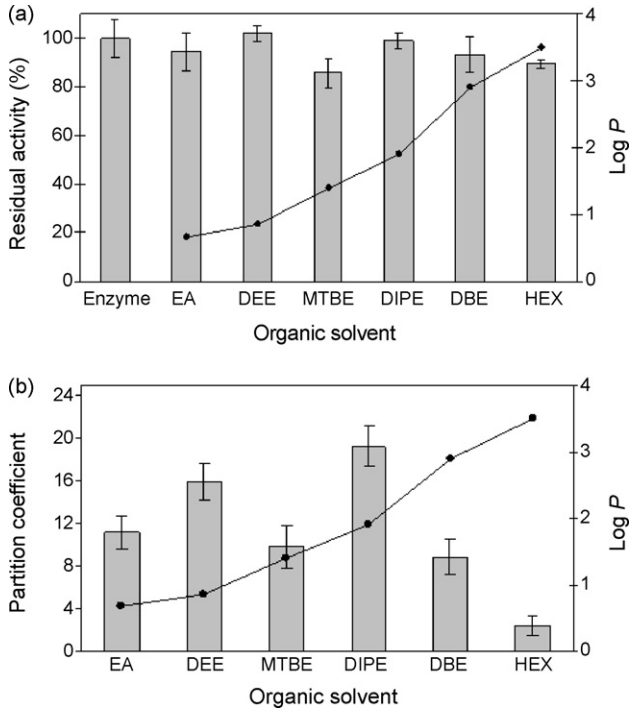

Fig. 3. Effect of organic solvents on stability of *Ej*HNL (a) and partition coefficient of mandelonitrile (b). (□) Residual activity of *Ej*HNL after incubated for 12 h or partition coefficient of mandelonitrile, (●) log *P* of organic solvent.

benzaldehyde in biphasic systems was determined (Fig. 3b). The partition coefficient for benzaldehyde in the organic phase should be high to minimize the inhibition of enzyme benzaldehyde in the aqueous phase and reduce the non-enzymatic reaction rate. DIPE and DEE were suitable solvents for the partitioning of benzaldehyde to the organic phase, while EA, MTBE and DBE showed moderate values for the partition coefficient. In the case of HEX, the partition of benzaldehyde was very low, therefore benzaldehyde almost dissolved in the aqueous phase with acetone cyanohydrin. This might incite the non-enzymatic reaction and cause the lower *e.e.* of the product.

3.3. Aqueous phase content

The aqueous phase content is an important factor in the use of an enzyme in an organic solvent. The effect of aqueous phase content on the enzyme reaction was studied at 5–60% (v/v) with the volume of organic phase fixed at 1 ml. A content of less than 5% (v/v) caused aggregation of the enzyme.

The aqueous phase content affected the reaction rate more clearly than the *e.e.* (Fig. 4). Increasing the aqueous phase content in biphasic system caused the initial reaction rate to rise linearly. The high water content might provide more flexibility to the enzyme molecules [23], increase the mass transfer of the enzyme and substrates [24] and reduce the chance of contact with the interface of the biphasic reaction which might inactivate the enzyme [25]. Furthermore, the high aqueous phase content stimulated the decomposition of acetone cyanohydrins to HCN [19], yielding the corresponding high reaction rate.

In our study, there was no straightforward correlation between *e.e.* and the initial velocity of the reaction. A high *e.e.* (≥99%) was obtained at aqueous phase content of 5–50% (v/v). On the other hand, an excess of aqueous phase (more than 50%) accelerated the spontaneous non-enzymatic reaction and led to a fall in the *e.e.* of the product.

3.4. Substrate concentration

The effect of benzaldehyde and acetone cyanohydrin on the initial reaction velocity and enantiomeric purity of (*R*)-mandelonitrile was studied. The benzaldehyde concentration was varied in the range of 10–300 mM, while keeping the acetone cyanohydrin concentration at 500 mM. The initial reaction velocity of *Ej*HNL increased linearly when the benzaldehyde concentration increased up to 200 mM and a constant initial velocity was observed at 200–300 mM (Fig. 5a). The effect of the acetone cyanohydrin concentration was investigated while the benzaldehyde concentration

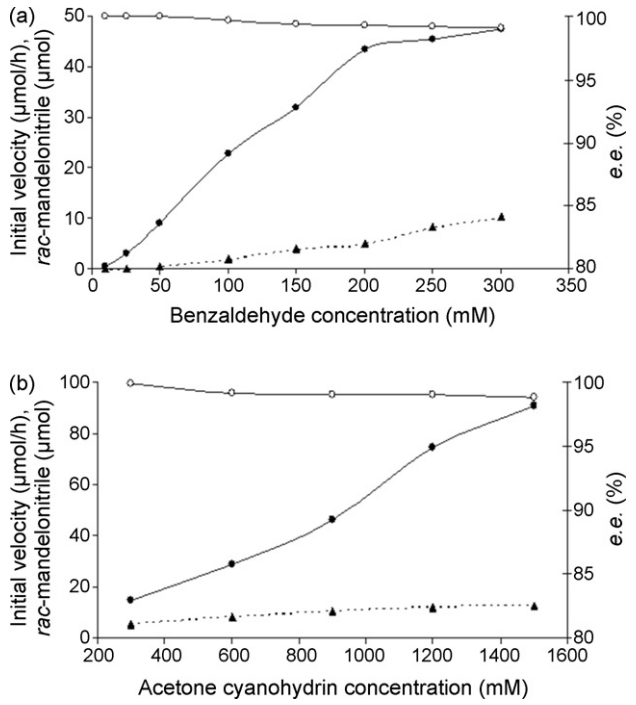

Fig. 5. Effect of benzaldehyde (a) and acetone cyanohydrin (b) concentration on initial velocity (●), *e.e.* (○) and non-enzymatic reaction (▲) in biphasic system of buffer (pH 4.0; 50%, v/v) and DEE at 10 °C. To determine effect of benzaldehyde, concentration of acetone cyanohydrin was kept constantly at 500 mM, while concentration of benzaldehyde was kept at 200 mM when studied on effect of acetone cyanohydrin. The 5 Units of *Ej*HNL was used in all cases.

was fixed at 200 mM. The increase in the initial reaction velocity of *Ej*HNL was linearly correlated with the acetone cyanohydrin concentration (Fig. 5b). The result indicated that the enzyme was found not to be inactivated by a high concentration of acetone cyanohydrin. In contrast, increasing the benzaldehyde or acetone cyanohydrin concentration caused a slight decrease in the *e.e.* of the product due to an enhanced spontaneous chemical reaction between substrates, however an *e.e.* of more than 98% was observed in all cases. KCN was not suitable as a source of cyanide because the non-enzymatic reaction was occurred rapidly yielding mandelonitrile with a significantly low *e.e.* (data not shown).

3.5. Enzyme concentration

A linear increase in the initial velocity with an increase in enzyme concentration up to the optimum of 13.3 units/ml reaction mixture was observed (Fig. 6). The increase of enzyme concentration in the biphasic reaction might cause the mass transfer rate to rise [24] and might be involved in the raising of initial velocity. The excess amount of enzyme (more than 13.3 units/ml reaction) caused a decrease in initial velocity that might be due to the aggregation of the enzyme powder. In this case, the viscosity and turbidity of the enzyme solution were observed when the enzyme was added at a concentration beyond the optimum. The aggregation might limit the mass transfer between the enzyme and substrates. A slight drop in the *e.e.* of the product was found when the amount of the enzyme was increased.

3.6. Synthesis of (R)-mandelonitrile and reusability of EjHNL

The synthesis of (*R*)-mandelonitrile by *Ej*HNL was demonstrated for the first time. The time-course of (*R*)-mandelonitrile synthesis by the transcyanation reaction is shown in Fig. 7a. Linear

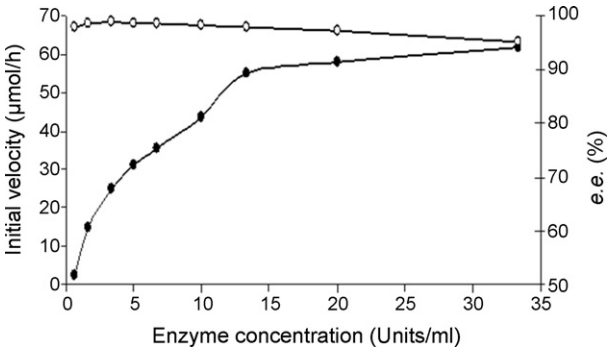

Fig. 6. Effect of enzyme concentration on initial velocity (●) and *e.e.* (○). The reaction was performed in biphasic system of buffer (pH 4.0; 50%, v/v) and DEE, at 10 °C containing benzaldehyde (200 mM), acetone cyanohydrin (400 mM) and various concentration of *Ej*HNL.

progress in the reaction was observed in the first hour with the conversion and *e.e.* of (*R*)-mandelonitrile being 40 and 99%, respectively. A constant *e.e.* value was observed from 3 to 24 h which indicated the total suppression of the non-enzymatic reaction. The transcyanation of (*R*)-mandelonitrile was compared between *Ej*HNL and HNL from *P. amygdalus* as shown in Fig. 7b. The time course was not significantly different in *e.e.* (99%) or conversion (35–38%).

The reusability of *Ej*HNL in asymmetric (*R*)-mandelonitrile synthesis employing a biphasic system was studied. The enzyme in aqueous phase was recovered and successfully used for the next reaction. As the results show in Table 1, a residual activity of 90% of

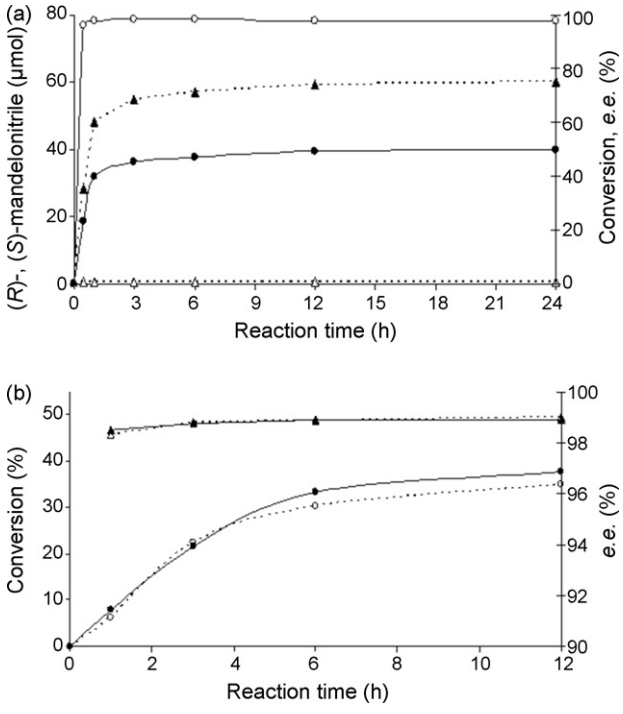

Fig. 7. (a) Time course for transcyanation of (*R*)-mandelonitrile synthesis by *Ej*HNL. The *e.e.* (○), conversion (●), (*R*)-mandelonitrile (▲) and (*S*)-mandelonitrile (△) was monitored for 24 h. (b) A comparison of transcyanation of (*R*)-mandelonitrile synthesis by *Ej*HNL and *Pa*HNL. The conversion of *Ej*HNL (●) and *Pa*HNL (○), and the *e.e.* of *Ej*HNL (▲) and *Pa*HNL (△) was monitored for 12 h. The (*R*)-mandelonitrile synthesis reaction was performed in biphasic system of buffer (pH 4.0; 50%, v/v) and DEE (50%, v/v) at 10 °C containing benzaldehyde (200 mM), acetone cyanohydrin (400 mM), and enzyme (13.3 units/ml).

Table 1
(*R*)-Mandelonitrile synthesis in repeated batch process by *Ej*HNL

Batch	Initial velocity (μmol/h)	*e.e.* (%)	Conversion (%)
1	63.7	99.7	40.4
2	62.1	99.4	39.3
3	59.8	98.5	38.1
4	57.3	98.2	36.8

the reused *Ej*HNL in aqueous phase remained after four cycles and *e.e.* did not change markedly.

4. Discussion

Among the HNLs discovered up to date, a few of these enzymes were purified and characterized. Many reports have dealt with the synthesis of cyanohydrins to obtain the high yield and enantiomeric purity of product. The lack of information on the biological properties of HNLs has limited not only the understanding of the enzyme, but also their applications.

A novel (*R*)-hydroxynitrile lyase from *E. japonica* (EC 4.1.2.10) was isolated, purified and characterized for the first time by our group. *Ej*HNL performed considerably well in the synthesis of various asymmetric cyanohydrins in a buffer system, but the non-enzymatic reaction was still a problem, leading to poor enantiomeric purity of the desired chiral products [16,17]. A biphasic system was an interesting way to minimize the amount of undesired product. So far the synthesis of cyanohydrins employing a HNL from loquat (*Eriobotrya* L.) in biphasic systems and single phase organic solvents was reported [18], however, there is no literature of the biological characteristics of the *Ej*HNL in the system containing organic solvent. Most of the cyanohydrin synthesis catalyzing by HNLs always employ EA or DIPE as organic phase [11,18] since it might be commonly believed that these organic solvents are suitable for the enzymes. Moreover, an excess amount of crude enzyme was added to achieve the high *e.e.* of products without concern about enzyme activity. Therefore, the objective of this study is to fill the lack information of biological characteristics of *Ej*HNL and optimized the reaction condition to achieve high initial reaction velocity and enantiomeric purity of the products.

We successfully demonstrated the asymmetric synthesis of (*R*)-mandelonitrile by *Ej*HNL in a biphasic system and achieved a higher *e.e.* (99%) of the product for the first time, as compared with the *e.e.* of 81% in the biphasic system reported in a previous work [18]. Furthermore, several parameters influencing the initial velocity and enantiomeric purity of the product were investigated and optimized. Especially, diethyl ether was found to be the most suitable organic solvent in biphasic system for *Ej*HNL which differ from previous reports of other HNLs [6,8,9,12,18].

In our study, both pH and temperature absolutely affected the *e.e.* of the product. The pH and temperature (pH 5 and 40 °C, respectively) giving the highest initial velocity of the reaction were not suitable for asymmetric synthesis of the (*R*)-mandelonitrile. A low *e.e.* of the desired product was obtained when using the pH and temperature giving the highest initial velocity. Under these conditions, the non-enzymatic reaction was incited and produced the racemate of mandelonitrile leading to the fall in *e.e.* As demonstrated in Fig. 1, a low pH and temperature should be maintained during the reaction to suppress the non-enzymatic reaction and achieve the highest *e.e.* of the product. These results agreed with previous reports on HNLs from *H. brasiliensis*, *M. esculenta*, *Sorghum bicolor* and *P. amygdalus* [9,12]. A similar effect of temperature on the enantioselectivity of enzymes was found for lipase and hydrolase which are enantioselective biocatalysts [26–28]. Therefore, pH and temperature might be important parameters to control the enantioselectivity of enzymes to achieve a high *e.e.* of their products.

Surprisingly, the initial velocity of *Ej*HNL was significantly affected by the type of organic solvent. *Ej*HNL exhibited the best initial activity in DEE system which differed from previous work on other HNLs which almost performed the enzymatic reaction in biphasic system of DIPE or EA [11,18]. Therefore, it is necessary to study the characteristics of the enzymes in organic solvent before employing them in the system containing organic solvent, since the conformation of the enzyme might be affected by the change in dielectric properties of the reaction medium caused by introducing an organic solvent, leading to unfolding and a change in activity of the enzyme [29]. Organic solvents do not only influence the initial velocity of the enzyme, but affect enzyme stability [8,25]. *Ej*HNL was very stable in all biphasic systems used, while HNL from *P. amygdalus* showed no lose activity after incubation in DIPE, and MTBE, but unstable in heptane and DBE [11]. In our study, the change in log *P* of organic solvent was not correlated with the *e.e.* value of the product, but the partition of benzaldehyde and HCN in the organic and aqueous phases was important to control the enantiomeric purity of the product. For biphasic systems, solvents having no harmful effects on the activity and stability of the enzyme, and a high partition for the aldehyde substrate and cyanohydrin product but a low partition for cyanide, are required. In the case of the asymmetric synthesis of (*R*)-mandelonitrile by *Ej*HNL, DEE was the best solvent, while EA and HEX were not suitable for the enzyme. EA was also reported to be an unsuitable solvent for *Hb*HNL causing strong inhibition of its activity [8], while *Pa*HNL showed the lowest level of activity with MTBE and much higher levels found in a more non-polar solvent than MTBE such as BME (bultyl methyl ether), DIPE, DBE and heptane [11]. Enantioselectivity of *Hb*HNL catalyzing the addition of HCN to 3-phenylpropanal was not affected by log *P* of organic solvent [12], whereas that of *Me*HNL catalyzing the transcyanation of acetyltrimethylsilane and acetone cyanohydrin was, and high hydrophobicity of the organic solvent caused the low *e.e.* of the product [30].

A small amount of water is required for the catalytic activity of an enzyme. However, the quantity of water required varies [18,31–33]. Actually, the optimum amount of water depends on several parameters, including type of enzyme, polarity of the enzyme active site, type of organic solvent, substrate and reaction conditions. In the case of the transcyanation of (*R*)-mandelonitrile by *Ej*HNL in the biphasic system of DEE and buffer, the optimum amount of water was 50% (v/v) to obtain high initial velocity of enzyme and *e.e.* of the product. Several previous papers reported differently on the optimum aqueous content for initial velocity of HNLs in cyanohydrin synthesis. A similar result was observed for the transcyanation of acetyltrimethylsilane by *Me*HNL in which a raise in initial velocity was provided with the increase in aqueous phase content from 13 to 60% (v/v) [30]. An optimum water content of 1.0–1.5% (v/v) was reported for *Hb*HNL catalyzing the synthesis of 3-phenylpropionaldehyde cyanohydrin in DBE as the organic phase [9]. Furthermore, optimum water content of 0.54% was observed for *Hb*HNL catalyzing the synthesis of the same product in DIPE [12].

A difference in the correlation between the optimum water content and *e.e.* of product in cyanohydrin synthesis was reported previously. In the case of *Hb*HNL, the presence of enough water phase to form a biphasic system did not affect the enantioselectivity of the enzyme [12], while increasing the aqueous phase led to a decrease of *e.e.* in the transcyanation of acetyltrimethylsilane by *Me*HNL [30]. Micro-aqueous

conditions were also found to suppress the non-enzymatic reaction sufficiently [18,31,32]. Therefore, it is necessary to optimize the aqueous phase content for cyanohydrin synthesis.

Substrate and enzyme concentrations are parameters influencing the initial velocity of a reaction and enantiomeric purity of the product. In this study, benzaldehyde and acetone cyanohydrin were the substrates. An increase of substrate concentration cause an increase in initial velocity but a slight decrease in *e.e.* due to the increase in the non-enzymatic reaction. Stability of *Ej*HNL at high concentrations of benzaldehyde and acetone cyanohydrin was observed in this study. However, *Hb*HNL was stable with 3-phenylpropionaldehyde while the high concentration of HCN had a negative influence on the enzyme [9].

The previous studies on cyanohydrin synthesis used an excess amount of enzyme to achieve a high *e.e.* without measuring the initial activity and optimizing the amount of enzyme [18,19,34]. In this study, we did optimize the enzyme concentration, where the highest reaction velocity and enantiomeric purity were achieved. The excess amount of enzyme employed in the system caused the decrease in the initial activity. The optimum enzyme concentration should be used for the production of cyanohydrins to achieve the highest level of activity and the lowest cost.

In the present study, we successfully demonstrated the transcyanation of (*R*)-mandelonitrile by a novel *Ej*HNL for the first time. All parameters play an important role in the activity and enantioselectivity of the enzyme, therefore it is necessary to study and optimize all parameters to achieve the highest level of activity and enantiomeric purity of the product. Furthermore, the reusability of the enzyme and ease of product separation show the potential of *Ej*HNL in (*R*)-mandelonitrile synthesis especially on an industrial scale.

5. Conclusions

The study on biological characteristics of *Ej*HNL demonstrated that the initial activity and enantioselectivity of *Ej*HNL in the aqueous-organic biphasic system were influenced by several parameters. The organic solvent is a markedly important parameter influencing on the initial reaction velocity. For *Ej*HNL, the suitable organic solvent for (*R*)-mandelonitrile synthesis was found to be DEE that gave the highest initial velocity for *Ej*HNL. The synthesis of (*R*)-mandelonitrile in a biphasic system at low pH and temperature proved to be useful for improving the enantiomeric purity of the product. The highest initial velocity of the reaction and *e.e.* of the product were obtained after optimization of the organic solvent, aqueous phase content, substrate concentration, and enzyme concentration. Furthermore, the possibility of efficiently reusing of the enzyme in the aqueous phase makes the enzyme and system attractive for practical applications.

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