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Enantioselective lipase-catalyzed kinetic resolution of *N*-(2-ethyl-6-methylphenyl)alanine

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Abstract

The enzyme (BSL2), a highly active lipase expressed from newly constructed strain of *Bacillus subtilis* BSL2, is used in the kinetic resolution of *N*-(2-ethyl-6-methylphenyl)alanine from the corresponding racemic methyl ester. Reaction conditions are optimized to enhance the enantioselectivity. The effects of various racemic alkyl esters, substrate concentration, operating temperature, pH of the aqueous medium and organic solvents on activity and enantioselectivity of BSL2 for kinetic resolution are also studied. A high enantiomeric ratio (E = 60.7) is reached in diisopropyl ether/water (10%, v/v) and the enantioselectivity is about 22-fold higher than that in pure buffered aqueous solution. The results show that the reaction medium greatly influences BSL2 reaction and its enantioselectivity in the hydrolysis of racemic methyl ester.

Keywords: *Bacillus subtilis*; BSL2; Kinetic resolution; Hydrolysis; (*S*)-(–)-*N*-(2-ethyl-6-methylphenyl)alanine

1. Introduction

Enantiopure *N*-substituted amino acid derivatives are important building blocks for the synthesis of many pharmaceuticals [1–3], liquid crystal materials [4] and pesticides [5], in which substances of very high enantiomeric excess are often needed. In particular, (*S*)-(–)-*N*-(2-ethyl-6-methylphenyl)alanine ((*S*)-(–)-NEMPA) is very useful in organic synthesis and is used for the synthesis of most widely used herbicides such as (*S*)-Metolachlor [6]. The most commonly used methodology for the preparation of enantiopure *N*-substituted amino acid derivatives is chemical synthesis [1,4,5]. Nevertheless, the chemical method has several disadvantages especially for highly functionalized chiral molecules. The required drastic reaction conditions may cause racemization, decomposition or side reactions. Especially for industrial process, long reaction time at high temperature is unfavorable due to high energy consumption [7].

An alternative processes will be to use a highly active biocatalyst in a very efficient manner. Requirements on such a biocatalyst will be ready availability for the organic chemist, for example, simple and reliable fermentation procedures, high activity for short reaction time and a broad substrate spectrum to ensure general applicability. The use of enzymes as chiral catalysts to prepare optically active compounds from either racemic or prochiral substrates has been widely studied [8,9]. Among a variety of enzymes, there has been a growing interest in the use of lipase (triacylglycerol ester hydrolase, EC 3.1.1.3) for kinetic resolution of racemic compounds through esterification [10–14], hydrolysis [15,16] and transesterification [17,18] reactions because of its wide substrate specificity and ability to recognize chirality. However, the use of lipase as chiral catalyst to prepare enantiopure *N*-substituted amino acid derivatives from the corresponding racemic esters has not been widely studied. Using the kinetic

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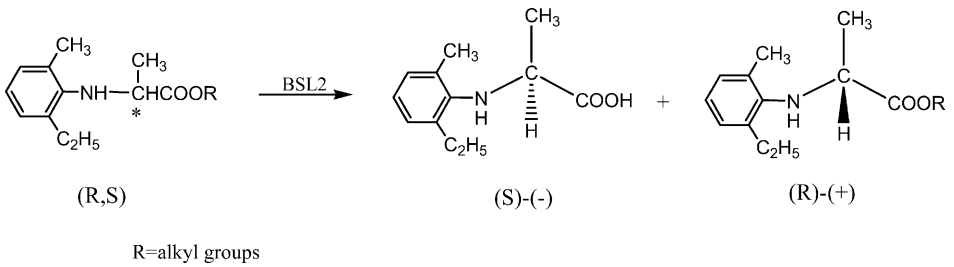

Scheme 1. Enantioselective hydrolysis of racemic alkyl esters of NEMPA using BSL2.

resolution of (*R*,*S*)-NEMPA as a model reaction, we herein have attempted to develop a stereoselectively hydrolytic process for studying the feasibility of resolution of the racemic *N*-substituted amino acid derivatives via enzyme.

The lipase from *Bacillus subtilis* is one of the most versatile and widely used enzymes due to its great activity and ability to accept a large number and variety of substrates [19,20]. Recently, we have isolated an intracellular lipase (BSL2, 24 kDa), which is produced by a newly constructed *B. subtilis* strain BSL2 (unpublished results) in our laboratory. In this paper, we first report a new method to prepare (*S*)-(–)-NEMPA (Scheme 1) using BSL2 as catalyst. The conditions of the effective kinetic resolution, such as various racemic alkyl esters, substrate concentration, reaction temperature, pH of aqueous medium and organic solvents are investigated.

2. Materials and methods

2.1. Materials

B. subtilis lipase (BSL2) was homely produced from a newly constructed *Bacillus subtilis* strain BSL2. *Pseudomonas* sp. Lipase (PSL) was purchased from Amano Pharmaceutical Co., Ltd. (Japan). Bacterial strain *B. subtilis* A.S.1.1655 and Bacterial strain *B. subtilis* A.S.1.1700 were provided by Institute of Microbiology, Chinese Academy of Sciences (China). Bacterial strain *B. subtilis* IFFI10210 was provided by Institute of Foodstuff and Fermentation (China). The authenticity of racemic esters prepared during the study was confirmed by spectroscopic analysis including NMR (300 MHz) and MS. Reactions were routinely monitored on silica gel plates (Qingdao Haiyang Chemical Co., Ltd., China) using UV light for detection of the spots. All the organic solvents were reagent grade and used without further purification. Other reagents were all analytical grade or better.

2.2. Determination of conversion and enantiomeric excess

The analysis of the reaction mixtures and the determination of enantiomeric excess of (*S*)-NEMPA were performed by capillary zone electrophoresis (P/ACE MDQ, Beckman, USA) with a 59 cm (49 cm to detector) × 50 μm i.d. eCAP^{TM} neutral capillary (Beckman, USA). The conversion of reaction was determined by using 100 mmol/L triethylamine/acetic acid buffer (TEAA, pH 5.5) as background electrolyte. The enantiomeric excess of (*S*)-NEMPA was successfully analyzed in 100 mmol/L TEAA, pH 5.5, by using 40 mmol/L of 2,6-di-*O*-methyl-β-cyclodextrin (DM-β-CD, Beckman, USA) as a buffer additive. The analysis was performed with applied voltage at −20 kV, and the absorbance was recorded at 200 nm [21].

2.3. Preparation of racemic alkyl esters

In general procedure, under the protection of nitrogen, the mixture, 8.4 ml (60 mmol) of 2-ethyl-6-methylaniline, 5.5 g (65 mmol) of $NaHCO_3$ and alkyl 2-bromopropionate (180 mmol), was stirred and slowly heated to 120–125 °C in one hour. The dark reaction mixture was kept at the temperature for 18 h with stirring. After it was cooled, the reaction mixture was pooled into 30 mL of ice-water and extracted with ethyl acetate. The ethyl acetate fractions were dried over sodium sulfate and concentrated in a rotary evaporator at 40 °C. The resulting ester after normal work up was purified by column chromatography on silica gel using ethyl acetate and petroleum ether (1:5) as the eluant to furnish the corresponding ester. *N*-(2-ethyl-6-methylphenyl)alanine methyl ester: ^{1}H NMR ($CDCl_3$): δ 7.02–6.96 (m, 2H, Ph), 6.88–6.83 (t, 1H, Ph, J=7.2 Hz), 3.96–3.94 (q, 1H, CH, J = 6.9 Hz), 3.81 (s, 1H, NH), 3.66 (s, 3H, CH_3), 2.69–2.66 (m, 2H, CH_2), 2.30 (s, 3H, CH_3), 1.38–1.35 (d, 3H, CH_3, J = 6.9 Hz), 1.26–1.21 (t, 3H, CH_3, J = 7.5 Hz); ^{13}C NMR ($CDCl_3$): δ 176.2, 143.6, 135.3, 129.6, 129.1, 126.9, 122.4, 55.8, 52.2, 24.6, 19.9, 19.2, 14.7; MS(EI) *m*/*z*: 221(25, M^+), 162 (100, M^+–(C=O)OCH_3), 133 (30, M^+–(C=O)OCH_3–C_2H_5), 77 (11, Ph).

2.4. Construction of Bacillus subtilis engineering strain BSL2

Bacterial strain *B. subtilis* A.S.1.1655 was used as an expression host for lipase (BSL2) and recombinant plasmid pBSR2. The recombinant plasmid pBSR2 originated from the plasmid pBD64 (from *B. subtilis* A.S.1.1700), in which the strong promoter of *B. subtilis* was designed by studying σ^{43} subunit-recognized sites, and the ribosome bind site and terminator sequence were also designed according to the information of NCBI Gene Bank. Genomic DNA from *B.*

subtilis IFFI10210 was isolated using a standard protocol. The PCR product was digested by *Hin*dIII and *Xho*I, and the resulting fragments were ligated into the same sites of the recombinant plasmid pBSR2. The ligation mixture was transformed into *B. subtilis* A.S.1.1655 and the resulting construct was named *B. subtilis* strain BSL2.

2.5. Cultivation of Bacillus subtilis strain BSL2

A culture medium (LB), comprising of 1.0% tryptone, 0.5% yeast extract and 1.0% sodium chloride (NaCl) (pH 7.0 before sterilization and pH 6.8 after sterilization) was prepared and dispensed in shake flasks (100 mL and 5 L, respectively). The preculture of *B. subtilis* strain BSL2 was made by shaking the cells in LB supplemented with 30 mg/L kanamycin and incubating the flask at 30 °C on a rotary shaker. The 28 h old preculture thus produced was inoculated (1%, v/v) into 1 L of LB with 30 mg/L kanamycin, and fermentation was carried out at 500 rpm keeping at a constant temperature of 30 °C for 28–30 h. The culture was thereafter centrifuged to collect the cells. The cell pellet was washed twice with 30 mmol/L phosphate buffer (pH 7.0) and the wet cell mass (5.8 g) was thus obtained.

2.6. Extraction and partial purification of enzyme (BSL2)

The wet cell mass prepared as described above was sonicated (at 40%, w/v solid suspension) for 5 min at 0–4 °C, following centrifugation at 8000 rpm for 20 min at 0–4 °C, then the cell free extract was obtained. The crude enzyme was partially purified by ammonium sulfate fractionation procedure. The optimum ammonium sulfate precipitation procedure for concentrating BSL2 is that we firstly prepare the fraction of 30% saturation of ammonium sulfate to remove the hetero-protein, then measure the volume of the supernatant and slowly add enough solid ammonium sulfate to bring the solution to 60% saturation. The 60% ammonium sulfate precipitation was dissolved in 30 mmol/L phosphate buffer (pH 7.0) and dialyzed to remove this excess salt. The dialyzate was lyophilized with the resulting solid (0.5 g) showing hydrolysis activity of 2.1 U/mg solid, using tributyrin as assay substrate (one unit of enzyme corresponds to liberation of 1 μmol of organic acid from tributyrin in 1 min). The total protein amount was 0.3 g, according to Lowry's method using bovine serum albumin (BSA) as the reference protein [22].

2.7. Biocatalytic hydrolysis of racemic methyl ester of NEMPA

BSL2 (105 units, with tributyrin assay) was added to a suspension of racemic methyl ester of NEMPA ((*R,S*)-NEMPAME, 0.5 mmol, 110 mg) of phosphate buffer (100 mmol/L, pH 8.0, 5.0 mL). The resulting mixture was stirred at 40 °C and the pH was maintained by a pH stat using 0.1 mol/L NaOH solution. Periodically aliquots were drawn and analyzed on capillary electrophoresis. The reaction was terminated after 40 h (conversion 48.7%). The mixture was first extracted with ether (3 mL × 20 mL) at the initial pH to remove the unchanged ester. The aqueous mixture was then acidified to pH 5.5 with 0.1 mol/L HCl and was again extracted with ether (3 × 20 mL) to remove the acid product. The acid extracts was dried over $MgSO_4$ and evaporated to give (*S*)-(–)-NEMPA.

Enantiomeric ratio (*E*) of hydrolysis of (*R,S*)-NEMPAME was calculated from the conversion (*c*) and enantiomeric excess (e.e.$_p$) of (*S*)-NEMPA, using the equation [23]: $E = \ln[1 - c\ (1 + \text{e.e.}_p)]/\ln[1 - c\ (1 - \text{e.e.}_p)]$, where $\text{e.e.}_p = (c_S - c_R)/(c_S + c_R)$, while c_S and c_R are concentrations of the (*S*)- and (*R*)-NEMPA, respectively. The absolute configuration of the enantiomer was established by comparison of the measured optical rotation with the literature data [5].

3. Results and discussion

3.1. Effect of various racemic alkyl esters on the reactivity and enantioselectivity of BSL2

To date, little has been reported on the effectiveness of BSL2 with respect to various racemic alkyl esters of NEMPA, so, several esters of NEMPA are prepared to investigate its behavior. Table 1 summarizes the results of the hydrolysis of various racemic alkyl esters using BSL2. In all the experiments, the biocatalytic system shows the same stereopreference for racemic ester giving the (–)-(*S*) acid and the unreacted (+)-(*R*) ester as hydrolysis products. The enantioselectivity of BSL2 is almost independent from the short-chain alkyl esters (*E* is about 2.5 for all esters from methyl ester to n-propyl ester). The rate of hydrolysis is influenced by the size of alkyl group of the ester moiety. For the methyl ester, the enzyme displayed a fairly good conversion (48.7%) and a higher enantioselectivity (*E* = 2.7) to produce (*S*)-NEMPA. The lowest enantiomeric excess (e.e.$_p$ ∼ 0) was observed when n-octyl ester was used as a substrate. The

Table 1
Hydrolysis of racemic esters of NEMPA using BSL2[a]

Entry (*R*)	Reaction time (h)	Conversion (%)	e.e.$_p$ (%)	*E*-value
CH_3	40	48.7	33.6	2.7
C_2H_5	40	46.3	32.2	2.5
n-C_3H_7	96	40.5	30.0	2.2
i-C_3H_7	96	24.7	27.5	1.9
n-C_4H_9	96	15.9	13.5	1.3
i-C_4H_9	96	10.8	6.2	1.1
t-C_4H_9	96	23.8	14.3	1.4
n-C_6H_{13}	96	9.2	1.2	1.0
n-C_8H_{17}	96	1.8	0.5	1.0
$CH_2C_6H_5$	96	17.4	29.6	2.0

[a] Reaction conditions: substrate: 0.5 mmol, BSL2: 105 U, phosphate buffer (pH 8.0, 100 mmol/L, 5.0 ml), temperature 40 °C.

n-octyl group ester proved to be the least accepted substrate. Since BSL2 showed a higher conversion rate for the methyl ester of racemic NEMPA, NEMPAME was selected as the substrate for further optimization studies such as the influence of substrate concentration, reaction temperature, pH as well as the effect of organic solvents, to improve enantioselectivity.

3.2. Influence of substrate concentration on reaction kinetics

In a process of enzymatic resolution, substrate concentration is an important parameter worthy of careful investigation, since it may influence sufficient expression of enzyme activity, or even result in the substrate inhibition. A series studies of influence of substrate concentration were performed in which keeping all other reaction parameters as constant and the substrate concentration was varied from 5.0 to 90 g/L. Fig. 1 shows that initial rate increased with the increasing of the substrate concentration, indicating that substrate inhibition did not exist at the substrate concentrations tested. After the substrate concentration reached the saturation point (45 g/L), the initial rate of reaction became a constant. The reaction rate could be increased if more enzymes were added. However, the use of higher enzymatic concentration turned out to be impractical because of the viscosity of the resulting mixture. According to the kinetic curve, BSL2 did not show the phenomnon of interfacial activation under these assay conditions either. The observed lack of such an interfacial activation of BSL2 suggests that it can be in direct relationship with the absence or partial deletion of the lid domain, according to the structural analysis of other *B. subtilis* lipase [24,25].

3.3. Effect of temperature on the activity and enantioselectivity of BSL2

The BSL2-catalyzed hydrolysis of (*R*,*S*)-NEMPAME at pH 8.0 in a temperature range from 20 to 60 °C was investigated. The results plotted in Fig. 2 show the activity and

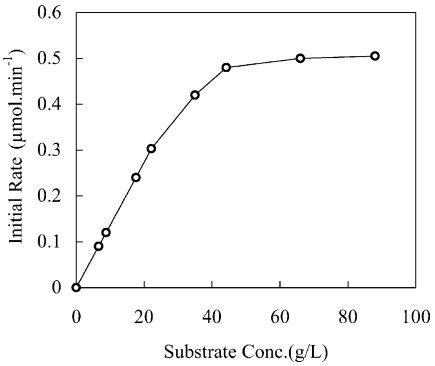

Fig. 1. Initial reaction rates for the BSL2-catalyzed hydrolysis of NEMPAME at varying substrate concentrations. Reaction conditions are the same as described in Table 1.

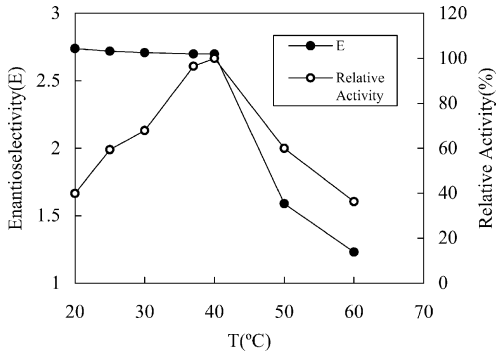

Fig. 2. Effect of temperature on the activity and enantioselectivity of BSL2 in the kinetic resolution of NEMPA. Reaction conditions are the same as described in Table 1.

enantioselectivity exhibited by BSL2 under different sets of operating temperatures. It could be concluded that higher selectivity (E = 2.7) could be achieved by operating at lower temperatures (20–40 °C) and little enantioselective changes occurred in this range. In contrast, at higher temperatures (50–60 °C), the reaction occurred with low enantiopreference. The reduction in enantioselectivity at higher temperatures had also been reported by Z. Ujang et al. [14] in the resolution of 2-(4-chlorophenoxy)propionic acid using *Candida rugosa* lipase. The increases of temperature generally led to increasing the reaction rates. However, there was a limit to the increase because the enzyme became deactivated at a higher temperature, resulting in loss of activity as well as selectivity. BSL2 shows maximum catalytic activity at 40 °C.

3.4. Effect of pH on the activity and enantioselectivity of BSL2

Fig. 3 illustrates the effect of pH on the activity and enantioselectivity of BSL2 in the hydrolysis of (*R*,*S*) -NEMPAME at 40 °C in the pH range of 6.0–11.0. The *E*-value increased in the pH range of 6.0–8.0, and the higher enantioselectivity was stabilized at pH 7.5–8.0 (E = 2.7), then decreased at pH 8.0–11.0. The pH values below 7.5 were unfavorable for the

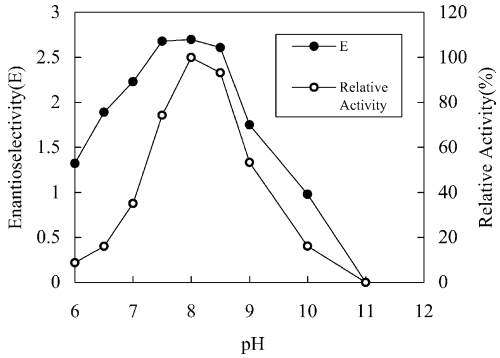

Fig. 3. Effect of pH on the activity and enantioselectivity of BSL2 in the kinetic resolution of NEMPA. Experiments were performed in 100 mmol/L sodium phosphate (pH 6–8), Gly–NaOH buffer (pH 8–9), 1–100 mmol/L NaOH (pH 9–11), using 0.5 mmol of substrate as described in Table 1.

hydrolysis. An increase beyond pH 8.0 marginally increased the rate of reaction but at the cost of activity and selectivity of BSL2. We checked if the spontaneous hydrolysis of (*R*,*S*)-NEMPAME occurred at alkaline pH in the absence of BSL2 and found that hydrolysis did occur in which 20% hydrolysis product with 0% $e.e._p$ was obtained at pH 10.0. The *E*-value was corrected for this effect. At pH 8.0, an optimal reaction rate and selectivity were recorded. The results may be explained as that the pH can influence enzyme activity by altering the three dimensional structure of the enzyme by breaking weak bonds such as ionic, and hydrogen bonds [26].

3.5. Effect of different organic solvents on the activity and enantioselectivity of BSL2

The effect of organic solvents on the reaction is also investigated. The organic solvents are chosen on the basis of their log *P* [27,28] even though no reliable relationship has been reported between the various proposed parameters that describe the solvent properties and biocatalytic effects [29]. The results of the hydrolysis of (*R*,*S*)-NEMPAME by BSL2 at 40 °C in different aqueous-organic media are summarized in Table 2. Under the given reaction conditions, the biocatalytic system shows the same stereopreference for (*R*,*S*)-NEMPAME giving the (*S*)-acid. The conversions are lower in aqueous-organic media than that in aqueous medium, but higher enantiomeric excesses are obtained. The highest enantiomeric ratio (*E* = 60.7) is achieved with diisopropyl ether (DIPE)/water medium, which is about 22-fold more enantioselective than that in pure buffered aqueous solutions (*E* = 2.7).

To establish optimum reaction conditions, the hydrolysis of (*R*,*S*)-NEMPAME in the presence of BSL2 in DIPE/water at different solvent ratios (%, v/v) is studied. The effect of the DIPE content on the enantioselectivity of BSL2 is shown in Fig. 4. The *E*-value increases with the increasing of the content of DIPE in phosphate buffer. At 10% (v/v) DIPE, the maximum value for the enantioselectivity of BSL2 is obtained.

Table 2
Hydrolysis of (*R*,*S*)-NEMPAME by BSL2 in different aqueous-organic media[a]

Organic solvent	log *P*	Time (h)	Conversion (%)	$e.e._p$ (%)	*E*-value
None	–	40	48.2	34.0	2.7
Acetonitrile	−0.33	72	23.0	38.0	2.5
Acetone	−0.23	72	27.3	47.8	3.4
THF	0.49	72	26.6	54.7	4.1
Diethyl Ether	0.85	72	33.0	78.3	11.9
DIPE	1.90	72	47.0	92.0	60.7
Toluene	2.50	72	35.1	60.6	5.6
Cyclohexane	3.20	72	44.2	38.1	2.9
n-Hexane	3.50	72	37.9	60.7	5.8
n-Heptane	4.00	72	39.3	64.6	6.9

[a] Reaction conditions: substrate: 0.5 mmol, BSL2: 105 U, organic solvent/phosphate buffer (100 mmol/L, pH 8.0, 5.0 mL) 1:9 temperature 40 °C.

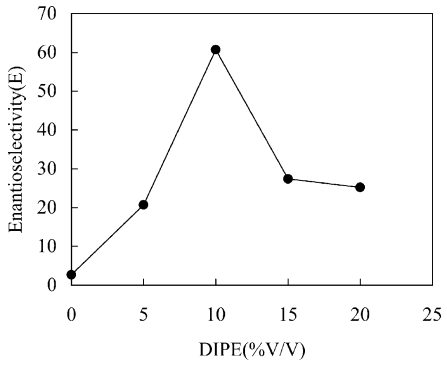

Fig. 4. Enantioselectivity of BSL2 at different volume fractions of DIPE in the hydrolysis of NEMPAME. Reaction conditions are the same as described in Table1.

Experimental data support the idea that the activity and enantioselectivity of BSL2 in the kinetic resolution of (*S*)-NEMPA by hydrolysis are influenced by the choice of organic solvents and the proportion between the water phase and organic phase. However, only a qualitative suggestion can be advanced to explain these results because no reliable relationship between the single parameters that describe the solvent properties and the biocatalytic effect has been proposed nor is the crystalline structure of the enzyme. Changes in the activity and selectivity of BSL2 induced by slight change in the reaction medium may be attributed to small conformational changes in the enzyme and/or substrate molecules. In additions, the lower dielectric constant of organic solvents as compared to an aqueous environment may decrease enzyme flexibility by increasing the strength of the intramolecular electrostatic interactions [30]. The higher enantioselectivity of BSL2 in ether/water may also be ascribed to the molecular structure of ether, which may change the stabilities of BSL2 by intermolecular interrelations, because it has an oxygen atom that can provide lone-paired electron. The improvement of the enantioselectivity of lipase in DIPE/water had also been reported by Y.H. Zhang et al.[31].

3.6. Comparing hydrolytic activity and enantioselectivity of BSL2 with PSL

The hydrolytic activity and enantioselectivity of BSL2 were compared with commercially available enzyme namely PSL under the optimum conditions, respectively. The conversion (47.0%) and enantiomeric excess ($e.e._p$ 92.0%) of BSL2 were achieved in DIPE/phosphate buffer (10%, v/v) at 40 °C and pH 8.0, While the conversion (45.5%) and enantiomeric excess ($e.e._p$ 94.5%) of PSL were obtained in phosphate buffer at 50 °C and pH 8.0. These data indicate that both BSL2 and PSL give excellent conversion and display comparable enantioselectivity, however, the former produces high stereospecificity towards the *S*-enantiomer of the acid while PSL possesses opposite stereopreference.

4. Conclusion

A kinetic resolution process for producing (*S*)-*N*-(2-ethyl-6-methylphenyl)alanine from racemic alkyl esters using BSL2-catalyzed hydrolysis was first investigated. The results show that the transition from aqueous medium to aqueous-organic media leads to a better enantiomeric excess of (*S*)-NEMPA, and the highest enantioselectivity ($E = 60.7$) of BSL2 is achieved in DIPE/water (10%, v/v), which is about 22-fold more enantioselective than that in pure buffered aqueous solutions ($E = 2.7$). Under the optimum conditions, BSL2 displays comparable enantioselectivity with respect to PSL. With this step, a practical process has been developed for the conversion of (*R*,*S*)-NEMPA ester to (*S*)-NEMPA using BSL2. The process can also be applied to stereoselective resolution of *N*-substituted amino acid derivatives; the research is currently in progress and will be reported in due course.

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