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RESEARCH ARTICLE

Enantioselective resolution of (±)-1-phenylethyl acetate using the immobilized extracellular proteases from deep-sea *Bacillus* sp. DL-1

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ABSTRACT

Bacillus sp. DL-1 was isolated from the deep sea of the Western Pacific Ocean and behaved very good resistance to NaCl. The extracellular proteases of *Bacillus* sp. DL-1 were found to exhibit excellent enantioselectivity for the kinetic resolution of (±)-1-phenylethyl acetate. To improve the stability of the enzyme, the immobilized extracellular proteases were prepared by using 60 g kieselguhr and 1-L crude fermentation broth containing extracellular proteases of *Bacillus* sp. DL-1, shaking at 25 °C, 200 r/min for 10 h. Every gram of kieselguhr adsorbed 25.7 mg extracellular protease and the enzymatic activity recovery was 79.86%. The immobilized proteases preserved about 35.8% of its activity after 6 repeated uses, which were also used as biocatalyst to asymmetrically hydrolyse (±)-1-phenylethyl acetate for the preparation of (*R*)-1-phenylethanol and (*S*)-1-phenylethyl acetate with high optical purities. The effects of pH, temperature, enzyme concentration, substrate concentration, reaction time and additives (metal ions/surfactants) on the resolution were investigated by single factor experiments. Under the optimal reaction conditions (10 mM (±)-1-phenylethyl acetate, 40 mg/mL immobilized extracellular proteases, pH 7.5 (Tris-HCl), 5% (*v*/*v*) methanol and 45 °C for 2 h), (*R*)-1-phenylethanol was generated with the $e.e._p$ being > 97%, and the yield being 53%, respectively. Analogously, under the optimal reaction conditions (10-mM (±)-1-phenylethyl acetate, 360 mg/mL immobilized extracellular proteases, pH 6.0 (PB), 5% (*v*/*v*) DMSO and 35 °C for 1.5 h), (*S*)-1-phenylethyl acetate was generated with the $e.e._s$ being over 99% and the yield being 79%, respectively. Compared with the extracellular proteases from *Bacillus* sp. DL-2, the immobilized extracellular proteases from *Bacillus* sp. DL-1 exhibited higher hydrolytic activity and could asymmetrically hydrolyse (±)-1-phenylethyl acetate by using higher substrate concentrations, shorter reaction times to obtain higher yields. Notably, the extracellular proteases of *Bacillus* sp. DL-1 were demonstrated to behave the same enantiopreference as those of most other reported esterases/lipases. Proteases from deep-sea *Bacillus* sp. DL-1 are promising biocatalysts for the synthesis of valuable chiral chemicals.

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Introduction

Currently, the great demands of enzymes as biocatalysts in diverse industries are increasing rapidly, due to some attracting advantages such as mild working conditions, outstanding selectivity, high efficiency and benign to the environment (Hudlicky and Reed 2009). Protease, one important industrial hydrolase, has received special attention in some industries such as pharmacy, chemistry and food, and has accounted for nearly 60% of the total enzyme sale globally (Jemli et al. 2016). Proteases are robust and stable enzymes, active at mild conditions, with optimal pH in the range of 6 to 8 that do not require stoichiometric cofactors, being highly stereo- and regioselective (Bordusa 2002). These properties of proteases are important to their use as catalysts in organic synthesis, which may be better alternatives for chemical catalysts. Protease derived from microorganisms may be located intracellular, periplasmic, or extracellular (Ray et al. 2012). Considering availability and costs, extracellular proteases have advantages over intracellular one. A multitude of microorganism produce extracellular proteases, and most of the protease producing candidates belongs to *Bacillus* species (Banerjee and

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Supplemental data for this article can be accessed here.

Ray 2017). At present, using enzymes as promising biocatalysts to synthesize optically pure compounds is an important method. The conventional methods for purification of enzyme are ultrafiltration, precipitation and affinity chromatography. However, some of these methods are complicated, laborious, time-consuming and expensive. As a consequence, the crude enzymes with high stereoselectivity behave a better application prospect in industry.

With the development of the research on enantiomers, it has been recognized that due to the stereoselective interaction between drugs and their receptors, different enantiomers of chiral compounds have different or even opposite pharmacological and toxicological activities. Therefore, the synthesis of enantiomers with optical purities is particularly important. Biocatalytic reactions have become an integral part of modern technological processes and many chiral drug intermediates have been synthesized by biocatalysis (Zhao et al. 2006; Ghosh et al. 2015; Pinheiro et al. 2018, 2019). For instance, protease 6SD was covalently immobilized on multi-walled carbon nanotubes (MWCNT), and the as-prepared immobilizate P-6SD@NH_2-MWCNT was applied in the enantioselective resolution of (*R,S*)-2-chlorophenylglycine methyl ester to yield (*S*)-2-chlorophenylglycine methyl ester, a key chiral building block of clopidogrel (antiaggregatory and antithrombotic drug), with the *e.e.* being over 99%, and the conversion being 71% (Weng et al. 2021). (*S*)-pindolol, a non-selective β-blocker, acting effectively as antianginal, antihypertensive, and antiarrhythmic, 200 times more active than (*R*)-Pindolol, was synthesized by enzymatic kinetic resolution of rac-2-acetoxy-1-(1*H*-indol-4-yloxy)-3-chloropropane with lipase from *Pseudomonas fluorescens* to obtain (2*R*)-2-acetoxy-1-(1*H*-indol-4-yloxy)-3-chloropropane (97% ee) which was subjected to a hydrolysis reaction catalysed by *Candida rugosa* leading to (2*R*)-1-(1*H*-indol-4-yloxy)-3-chloro-2-propanol (97% ee), followed by a reaction with isopropylamine, with the *e.e.* being 97% (Lima et al. 2017). Lipase from *Pseudomonas fluorescens* was immobilized on the superparamagnetic nanoparticles coupled with APTES and then activated with glutaraldehyde for the kinetic resolution of secondary alcohols as rac-indanol, rac-1-phenylethanol, leading to (*S*)-indanol with high selectivity (*e.e.* $>$ 99%, $E > 200$) in 1.75 h at 50 °C, (*R*)-1-phenylethyl acetate with *e.e.* $>$ 99% and to the remaining (*S*)-phenylethanol with *e.e.* of 94%, conversion of 49% and $E > 200$, after 48 h of reaction at 40 °C (Galvão et al. 2018). Chiral secondary alcohols, represented by 1-phenylethanol, are important intermediates for the synthesis of many chiral compounds in pharmaceutical, fine chemical, and agrochemical industries (Tao and Xu 2009). (*R*)-1-phenylethanol is needed as a key precursor for the generation of pharmaceuticals and is also used as ophthalmic preservative, cholesterol intestinal adsorption inhibitor, and solvatochromic dye (Chua and Sarmidi 2004; Patel 2008). Chiral 1-phenylethyl acetate, 1-phenylethanol ester derivative, is widely used as additive in cosmetics, foods, and pharmaceuticals, owing to its mild floral odour (Gassenmeier et al. 2017; Boratyński et al. 2018).

Immobilization is one of the most useful strategies to improve various enzyme performance such as activity, selectivity, specificity, resistance to inhibitors, etc. (Garcia-Galan et al. 2011). There are several approaches of enzymatic immobilization, such as covalent attachment, multipoint covalent attachment, multipoint covalent attachment of an enzyme to a functionalized support, immobilization of enzymes by cross-linking, matrix entrapment, encapsulation, immobilization by adsorption, magnetic nanoparticles hybrids formation, enzyme immobilization via formation mechanism of hybrid nanoflowers (Qian et al. 2019; Reis et al. 2019; Xu et al. 2019; Lin et al. 2020; Zhu et al. 2020). The extracellular proteases contains many other enzymes in microbial fermentation broth, sometimes with activities against the same substrate, or with opposite (or just different) properties, such as different stereoselectivity, which may reduce the apparent performance, including catalytic activity and stereoselectivity, of the prepared biocatalyst. However, even in a best-case scenario, the contaminant proteins will reduce the volumetric activity of the biocatalyst by competitive inhibition. In some cases, a fine control of the support and immobilization conditions may realize the one-step immobilization and purification. The choice of solid activated support is significantly important in the immobilization of enzymes. The carriers need to have the following properties: enough mechanical strength, resistance of chemical attack and microbial decomposition. In addition, the surface of carriers should have abundant reactive groups and hydrophilic chain. A proper design of a support may permit to shift equilibrium of very weak protein complexes permitting their accumulation on the support, or improve the stability of an enzyme by several-thousand-fold, or its activity, or their selectivity (dos Santos et al. 2015a). Pinheiro and his colleagues (Pinheiro et al. 2019) used chitosan activated by divinyl sulphone as a novel support to immobilize *Candida antarctica* Lipase B (CALB). The result show that the thermal stability of immobilized enzyme was better

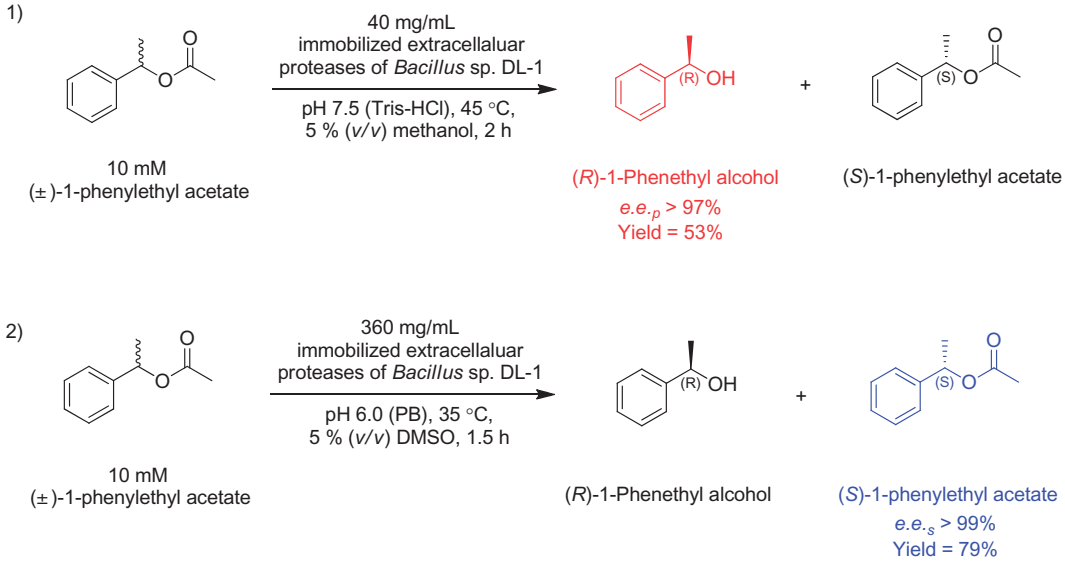
than that obtained when using glutaraldehyde as the support-activating reagent. Using a heterofunctional support, such as the heterofunctional glyoxyl-octyl agarose can overcome the problem that the enzyme easily released from the octyl-agarose support (physical absorption), which couples the numerous advantages of the octyl-agarose support to covalent immobilization and creates the possibility of using the biocatalyst under any experimental conditions without the risk of enzyme desorption and leaching (Rueda et al. 2016). Fernandez-Lopez improved immobilization and stabilization of lipase from *Rhizomucor miehei* (RML) on octyl-glyoxyl (OCGLX) agarose beads by using $CaCl_2$, and the final preparation is 2.7 fold more active than the free enzyme and a 50% more active than the standard OCGLX-RML and more stable than thestandard glyoxyl (Fernandez-Lopez et al. 2016). Adsorption using inorganic matrices such as kieselguhr, combined with enzyme by the force of hydrogen bonds, van der Waals forces and hydrophobic bonds, is an attractive option, due to its procedural simplicity, cost-effective, retention of the enzyme activities, high efficiency and ease of industrial application (Satar and Husain 2009; Contesini 2012; Lin et al. 2019). In the case of adsorption immobilization, excessive enzyme loading may cause intermolecular steric hindrance, resulting in a decline in enzyme activity. A long spacer arm will increase the possibilities of multi-interaction to absorb more proteins, but the selectivity of the adsorption will decrease (Armisén et al. 1999). In the case of covalent immobilization, a long spacer arm may reduce steric hindrances for the enzyme-support reaction, and involve more percentage of the protein surface in the immobilization, increasing the number of covalent attachment groups but reducing the rigidity conferred through multipoint covalent attachment (dos Santos et al. 2015a). If the arm is hydrophobic, this may have some negative effects on enzyme thermal stability. Kieselguhr (celite) is white to off-white natural sedimentary rock powder, similar to pumice powder and very light, and has high porosity, which is desirable/recommended for the immobilization of enzyme so that proper amounts of enzymes can be adsorbed without conformational changes (Verma et al. 2009). Enzymes immobilized on celite via adsorption have been used in many reactions, such as effluent treatment, ester synthesis, dye decolorization, and biodiesel production (Prieto et al. 2002; Sagiroglu 2008; Andrade et al. 2013; Saun et al. 2014; Kanmani et al. 2015; Sharma et al. 2015; Zhou et al. 2017).

Notably, most of the kinetic resolution of racemic mixtures by asymmetric hydrolysis were carried out by lipases/esterases (Musidlowska-Persson and Bornscheuer 2003; Cross et al. 2004; Wang et al. 2011; de los Ríos et al. 2012; Merabet-Khelassi et al. 2012; Jia et al. 2013; Xia et al. 2014; Zaïdi et al. 2015; Cao et al. 2016; Huang et al. 2016; Liang et al. 2016a; Deng et al. 2018; Gong et al. 2018; Wang et al. 2019), a few studies utilized proteases for kinetic resolution (Huang et al. 2018; Dong et al. 2019, 2020). In this work, one deep-sea microorganism *Bacillus* sp. DL-1 could produce extracellular proteases, which can resolve (±)-1-phenylethyl acetate to (*R*)-1-phenylethanol and (*S*)-1-phenylethyl acetate. To improve the stability and enantioselectivity, the extracellular proteases were immobilized by adsorption on kieselguhr. And the results indicated that extracellular proteases of *Bacillus* sp. DL-1 were effectively immobilized on the natural diatomite. But due to steric resistance, the amount of enzymes adsorbed by diatomite is limited. We will activate the surface of diatomite and then use it for the immobilization of the extracellular protease to further improve the activity, rigidity and stability of the immobilized enzyme. In addition, the prepared immobilized proteases, further utilized to asymmetrically hydrolyse (±)-1-phenylethyl acetate, showed good stability, resolution activity and stereoselectivity and generated (*R*)-1-phenylethanol and (*S*)-1-phenylethyl acetate with high optical purities (Scheme 1).

Materials and methods

Microorganisms and reagents

Bacillus sp. DL-1 was isolated from the deep-sea sediment samples of the Western Pacific Ocean by the Research Vessel "KEXUE". High protein level skimmed milk powder was purchased from Inner Mongolia Yili Industrial Group Co., Ltd. (Inner Mongolia, China). Yeast extract and tryptone were bought from Guangdong Huankai Microbial Science and Technology Co., Ltd. (Guangdong, China). PCR reagents were obtained from Shanghai Generay Biotech Co., Ltd. (Shanghai, China). Kieselguhr was purchased from Tianjin Damao Chemical Reagent Co., Ltd. (Tianjin, China). (±)-1-phenylethyl acetate and corresponding chiral enantiomers were gained from Adamas Reagent Co., Ltd. (Shanghai, China). Dodecane used as an internal standard was obtained from Aladdin Industrial Corporation (Shanghai, China). Other reagents were of analytical grade.



Scheme 1. Kinetic resolution of (±)-1-phenylethyl acetate using immobilized extracellular proteases from *Bacillus* sp. DL-1 for the generation of (*R*)-1-phenylethanol (1) and (*S*)-1-phenylethyl acetate (2).

PCR amplification and sequencing of 16S rDNA gene

The 16S rDNA of *Bacillus* sp. DL-1 was obtained by PCR with the following primers: 5′-AGAGTTTATC CTGGCTCAG-3′ and 5′-GGTTACCTTGTTACGACTT-3′. The PCR system included 1 μL of incubating medium as template directly, 2 μL of both 10 μM forward and reverse primers, 25 μL of 2×*Taq* Master Mix containing 4 mM $MgCl_2$ and 0.4 mM dNTP mix and 20 μL aseptic ddH_2O in 50 μL reaction volume. The PCR program comprised a pre-denaturation step at 94 °C for 10 min and 30 cycles of 30 s denaturation at 94 °C, 30 s annealing at 56 °C, and 150 s extension at 72 °C. A final extension of 10 min was given at 72 °C. The PCR products were purified by using 0.8% agarose gel electrophoresis.

16s rDNA gene sequence analysis for molecular identification of strains

The sequencing of the 16S rDNA of *Bacillus* sp. DL-1 was carried out by Shanghai Majorbio Bio-Pharm Technology Co., Ltd. The BLASTN program was used to blast against the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/blast/).

Biochemical characterization of bacillus sp. DL-1

In order to measure the growth curve of *Bacillus* sp. DL-1, Lysogeny broth (LB) liquid medium with 1% inoculation was cultured in a rocking incubator at 37 °C and 200 r/min and the optical densities of bacteria liquid at the wavelength of 600 nm (OD_{600}) were tested every 2 h during the culture process by using a Tecan NanoQuant Infinite M200 Pro plate reader. Furthermore, the growth curve of *Bacillus* sp. DL-1 under different concentrations of NaCl (0% – 15%) at four culture time (4 h, 14 h, 24 h, and 36 h) were also analysed by measuring OD_{600}. Subsequently, 1% bacteria liquid was inoculated to milk powder liquid medium (1% high protein level skimmed milk powder) for fermentation cultivation at 37 °C and 200 r/min. According to the SB/T 10317-1999 standard, the enzymic activities of extracellular proteases were measured at culture times of 14 h, 24 h, 36 h and 48 h. Meanwhile, the extracellular proteases at the same culture times were used to hydrolyse (±)-1-phenylethyl acetate and the reaction products were analysed by chiral gas chromatograph (GC) to evaluate the enantioselectivity of the kinetic resolution.

Effect of pH on the activity and stability of the extracellular protease

In order to determine the optimal pH, the crude fermentation broth cultivated for 14 h was freeze-dried by freeze drier to obtain extracellular proteases powder which were dissolved by various pH values buffer: pH 5.0–5.5 (sodium citrate buffer), pH 6.0–8.0 (sodium phosphate buffer), and pH 8.0–9.0 (Tris/HCl buffer). Subsequently, the activities of extracellular proteases with different pH were measured according to the SB/T 10317-1999 standard. The extracellular proteases in the different pH buffers were incubated for 2 h at

room temperature, and then the residual activities were analysed at 45 °C to confirm the pH stability of extracellular proteases.

Effect of temperature on the activity and stability of the extracellular protease

The effect of temperature on the activities of extracellular proteases were evaluated by incubating the extracellular proteases at temperatures ranging from 25 to 70 °C by using 2% casein as the substrate. The thermal stability of the extracellular proteases was determined by incubating the enzymes at different temperatures ranging from 25 to 70 °C for 2 h, and then analysing the residual activities at 45 °C.

Immobilization of extracellular proteases and measurement of enzyme activities

Bacillus sp. DL-1 was cultured in milk powder liquid medium for 14 h. Subsequently, the cultured filtrates, crude extracellular proteases of *Bacillus* sp. DL-1, were gained by centrifugation and filtration. Sixty-gram kieselguhr was put into 1-L crude fermentation broth containing extracellular proteases. The adsorption reaction was performed in shaking table at pH 8.0, 25 °C, 200 r/min for 10 h. Finally, the kieselguhr in the reaction mixture was obtained by vacuum suction filtration, which was dried at 38 °C so as to gain the immobilized extracellular proteases, whose activity was measured according to the SB/T 10317-1999 standard. Some parameters of immobilization process can fully understand the effect of immobilization on enzyme activity (Boudrant et al. 2020). But the activities of mixed proteases did not represent the activities of resolution reactions, so we did not optimize the immobilization process, but only optimized the resolution reaction conditions.

Kinetic resolution of (±)-1-phenylethyl acetate for the preparation of (R)-1-phenylethanol using immobilized extracellular proteases

Standard 500-μL pH 7.5 (50 mM Tris-HCl) reaction system containing 40 mg/mL immobilized extracellular proteases and 10 mM (±)-1-phenylethyl acetate was implemented at 45 °C for 2 h. After the enzyme-catalysed reaction was completed, 500-μL ethyl acetate was used to extract substrates and reaction products and the ethyl acetate phase was tested by chiral GC to evaluate the enzymatic resolution of (±)-1-phenylethyl acetate.

A series of pH (5.0 ∼ 10.0) were carried out in standard enzymatic reaction system to optimize the pH for the kinetic resolution of (±)-1-phenylethyl acetate. The optimal reaction temperature for the kinetic resolution was determined by incubating standard enzymatic reactions at temperatures ranging from 20 °C to 50 °C at pH 7.5 (Tris-HCl). In order to confirm the optimal immobilized extracellular proteases concentrations, 20 mg/mL to 160 mg/mL immobilized enzymes were loaded in standard reaction systems reacted at 45 °C for 2 h. Various concentrations of (±)-1-phenylethyl acetate ranging from 5 mM to 50 mM were employed to confirm the effect of substrate concentration on the kinetic resolution of (±)-1-phenylethyl acetate. The effect of reaction time was studied by investigating the reaction time ranging from 1 h to 12 h. The effects of metallic ions and surfactants on the kinetic resolution of (±)-1-phenylethyl acetate were researched by adding different metallic ions and surfactants into standard reaction systems at final concentrations of 2 mM and 0.05% (*w*/*v*), respectively. Similarly, a variety of organic solvents at final concentrations of 5% (*v*/*v*) were also put into standard enzymatic reactions to measure the effect of organic solvents on the kinetic resolution of (±)-1-phenylethyl acetate.

Kinetic resolution of (±)-1-phenylethyl acetate for the preparation of (S)-1-phenylethyl acetate by immobilized extracellular proteases

Standard reactions were executed by adding 360 mg/mL immobilized extracellular proteases to a 500 μL reaction mixture containing 50 mM phosphate buffer (pH = 6.0) and 10 mM (±)-1-phenylethyl acetate followed by incubation at 35 °C, 200 r/min for 1.5 h. An equal volume of ethyl acetate was used to terminate the reaction and extract both substrates and products. Afterwards, the ethyl acetate phase was analysed by a chiral GC to determine the efficiency of kinetic resolution reaction.

The effect of pH on the kinetic resolution of (±)-1-phenylethyl acetate was ascertained by assaying the enantiomeric excess and conversion rate of the substrate at different pH in the range of 5.0–6.5. The optimal temperature was determined from 20 °C to 50 °C at pH 6.0 (50 mM phosphate buffer). 120 mg/mL to 400 mg/mL immobilized extracellular proteases were added to the standard reaction to investigate the effect of enzyme concentration on the kinetic resolution of (±)-1-phenylethyl acetate. The optimal substrate concentration for the kinetic resolution of (±)-1-phenylethyl acetate was tested by adding different concentrations of (±)-1-phenylethyl acetate ranging from 5 mM to 60 mM.

Subsequently, the hydrolytic reaction system containing 360 mg/mL immobilized enzyme and 10 mM (±)-1-phenylethyl acetate were carried out at 35 °C and pH 6.0 for different times (from 0.5 to 12 h) to investigate the optimal reaction time. Under the optimal reaction conditions for the preparation of (*S*)-1-phenylethyl acetate, 2 mM various metallic ions, 0.05% (*w*/*v*) surfactants, and 5% (*v*/*v*) organic solvents were added to the kinetic resolution reactions catalysed by immobilized extracellular proteases of *Bacillus* sp. DL-1, to test the effect of additives on the kinetic resolution of (±)-1-phenylethyl acetate by the biocatalyst.

Operational stability of immobilized extracellular proteases

The operational stability of immobilized extracellular proteases was studied by repeating the hydrolysis process, and the residual enzyme activities were measured after each hydrolysis process. The enzyme activities of each reaction were calculated as relative activity by taking the activity of the first cycle as 100%. Meanwhile, the celite-bound extracellular proteases were repetitively used to perform asymmetric hydrolysis of (±)-1-phenylethyl acetate for five times under the optimized conditions.

Analytical methods

The reaction products were analysed by gas chromatograph (FULI GC-9700II) equipped with H_2 flame ionization detector and 1112-6632 CYCLOSIL-B chiral capillary column (30 m × 0.25 mm × 0.25 μm). Nitrogen served as the carrier gas at a flow rate of 1.20 mL/min. The column temperature was ranging from 80 to 210 °C, and the injector and detector temperature were 210 °C. The enantiomeric excess (*e.e.*), conversion rate (*C*), and yield (Y) were calculated by the equation as follows (Chen et al. 1982).

$$C = \frac{S_0 + R_0 - (S + R)}{S_0 + R_0}$$

$$Y_R = A/A_0; Y_S = B/B_0$$

C stands for the conversion ratio of the substrate, and $e.e._p$ and $e.e._s$ represent the enantiomeric excess of the product and substrate, respectively. S_0 and R_0, respectively, stand for the concentrations of the (*S*)- and (*R*)-enantiomers of 1-phenylethyl acetate before reaction, while *S* and *R* represent the concentrations of the (*S*)-enantiomer and (*R*)-enantiomer of 1-phenylethyl acetate after reaction, respectively. Y_R and Y_S stand for the yields of (*R*)-1-phenylethanol and (*S*)-1-phenylethyl acetate, respectively. A represents the concentration of (*R*)-1-phenylethanol after the reaction. A_0 represent the concentration of (*R*)-1-phenylethanol when (*R*)-1-phenylethyl acetate completely transform into (*R*)-1-phenylethanol. B and B_0 represent the concentration of (*S*)-1-phenylethyl acetate after and before the reaction, respectively.

Results and discussion

Isolation and characterization of protease-producing strain which can resolve (±)-1-phenylethyl acetate

One bacterium strain isolated and purified from deep-sea sediments was found to produce extra-cellular proteases which could asymmetric hydrolyse (±)-1-phenylethyl acetate. The 16S rRNA nucleotide sequence of this deep-sea bacterium was used to blast against the NCBI nucleotide sequence database, which revealed 99% identity with those of some *Bacillus* species, such as *Bacillus cereus* strain RTR, *Bacillus* sp. F221, *Bacillus cereus* strain L1.3, *Bacillus anthracis* strain ES-9, and *Bacillus subtilis* strain 263AY4. Consequently, the deep-sea bacterium was named as *Bacillus* sp. DL-1. The growth curve of *Bacillus* sp. DL-1 was determined by measuring the OD_{600} during the time of culture, which showed that the logarithmic phase was 1–10 h, the stationary phase was 10–20 h, and the decline phase was after 20 h (Supplementary Figure S1). The growth curve of *Bacillus* sp. DL-1 under different concentrations of NaCl revealed that *Bacillus* sp.

$$e.e._p = \frac{[(R)-1-\text{phenyletha nol}]-[(S)-1-\text{phenyletha nol}]}{[(R)-1-\text{phenyletha nol}] + [(S)-1-\text{phenyletha nol}]}$$

$$e.e._s = \frac{[(S)-1-\text{phenylethyl acetate}]-[(R)-1-\text{phenylethyl acetate}]}{[(S)-1-\text{phenylethyl acetate}] + [(R)-1-\text{phenylethyl acetate}]}$$

DL-1 exhibited very good resistance to NaCl at concentrations of 0%∼6% and *Bacillus* sp. DL-1 could grow quite well even in the presence of 5% NaCl (Supplementary Figure S2). Thus, *Bacillus* sp. DL-1, one bacterium isolated from the deep sea of the Western Pacific Ocean was characterized to be a strain with very good resistance to NaCl.

Comparation of the protease activity and resolution ability of bacillus sp. DL-1

The highest enzyme activity (208.8 U/mL) of the extracellular proteases from *Bacillus* sp. DL-1 was observed when cultured for 36 h, while the extra-cellular proteases from 14-h cultures were proved to be most appropriate to resolve (±)-1-phenylethyl acetate, with the $e.e._p$ being 91%, but the enzyme activity was only 99.9 U/mL (Figure 1). The reason why the highest enzymatic activity of the extracellular proteases was inconsistent with the best resolution ability is possibly due to the fact that *Bacillus* sp. DL-1 could produce multiple extracellular proteases and the kinetic resolution of (±)-1-phenylethyl acetate might be carried out by one of them.

Effect of pH on the activity and stability of the extracellular protease

The activity of extracellular proteases under pH 5.0–9.0 (Supplementary Table S1) were measured to evaluate the effect of pH on the enzyme activity. As shown in Supplementary Figure S3, the hydrolytic activities of extracellular proteases increased first and then decreased with the increase of pH, showing that the highest hydrolytic activity of the extracellular proteases was in Tris–HCl buffer of pH 8.0. The pH stability of the proteases was determined after incubating the enzymes in different buffers (pH 5.0–9.0) for 2 h. The extracellular proteases were relatively stable when pH was over 6.0, and exhibited the highest activity and stability in Tris–HCl buffer of pH 8.5 (Supplementary Figure S4).

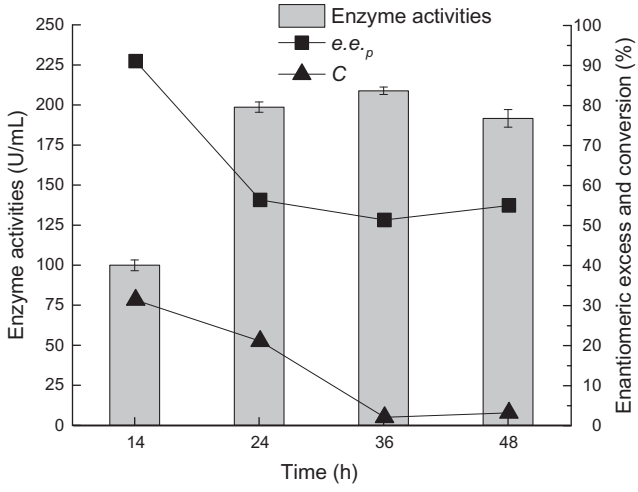


Figure 1. The hydrolytic activities and the kinetic resolutions of (±)-1-phenylethyl acetate by the extracellaluar proteases of *Bacillus* sp. DL-1 at different culture times.

Effect of temperature on the activity and stability of the extracellular protease

The hydrolytic activities of the extracellular proteases at different temperatures (25 °C–70 °C) were shown in Supplementary Figure S5, which demonstrated that the hydrolytic activity of the extracellular proteases was the highest at 45 °C. When the temperature was over 55 °C, the activities of the proteases decreased sharply. After incubating the extracellular proteases at different temperatures (25 °C–70 °C) for 2 h, the residual hydrolytic activities of the enzyme were measured to evaluate the thermal stability of the proteases. As shown in Supplementary Figure S6, the extracellular protease was relatively stable below 45 °C, but when the temperature was over 50 °C the activities of the proteases decreased distinctly, and when the temperature was over 60 °C the hydrolytic activities of the free enzyme were almost zero, indicating that high temperature had great influence on free extracellular protease of *Bacillus* sp. DL-1.

The activities of extracellular proteases

Before immobilization, the enzymatic activity of the free extracellular proteases cultivated for 14 h in the fermentation medium was 129.9 U/mL. After immobilization, every gram of kieselguhr adsorbed 25.7 mg extracellular protease and the hydrolytic activity of immobilized protease was 1729.0 U/g. The enzymatic activity recovery was 79.86%.

Process optimization for the preparation of (R)-1-phenylethanol through the kinetic resolution of (±)-1-phenylethyl acetate using immobilized extracellular proteases

Effect of pH on the kinetic resolution of (±)-1-phenylethyl acetate

The influence of pH on the stereo-selectivity and enzymatic activity of biocatalysts is one major concern in enzymatic reactions. Both the *C* and the $e.e._p$ during the enzymatic kinetic resolution of (±)-1-phenylethyl acetate were investigated at different pH ranging from 5.0 to 10.0 (Supplementary Table S1) to determine the

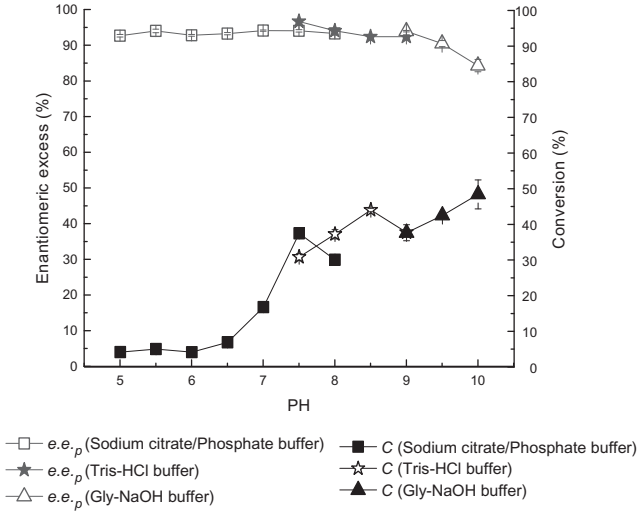


Figure 2. Effect of pH on the kinetic resolution of (±)-1-phenylethyl acetate catalysed by the immobilized extracellaluar proteases of *Bacillus* sp. DL-1 for the generation of (*R*)-1-phenylethanol. The reaction condition: 40 mg/mL immobilized extracellular proteases, 10 mM (±)-1-phenylethyl acetate, total 500-μL, reaction at 45 °C for 2 h.

effect of pH on kinetic resolution. As shown in Figure 2, the highest $e.e._p$ (96.6%) was observed at pH 7.5 (Tris-HCl), and significant decrease of $e.e._p$ was observed at pH above 9. Thus, the optimal pH for the asymmetric hydrolysis of (±)-1-phenylethyl acetate using immobilized proteases was determined to be 7.5 (Tris-HCl), with the *C* being 30.8%.

Effect of temperature on the kinetic resolution of (±)-1-phenylethyl acetate

The effect of temperature on the enzyme-catalysed reaction was investigated at various temperatures ranging from 20 to 50 °C at pH 7.5 (Tris-HCl). The $e.e._p$ during the kinetic resolution of (±)-1-phenylethyl acetate increased as the temperature increased from 25 °C to 45 °C and decreased when the temperature was over 45 °C. Therefore, 45 °C was determined to be the optimal temperature for the enzymatic kinetic resolution of (±)-1-phenylethyl acetate for the preparation of (*R*)-1-phenylethanol, with the $e.e._p$ being 95.7% and the conversion being 35.7%, respectively (Figure 3).

Effect of enzyme concentration on the kinetic resolution of (±)-1-phenylethyl acetate

The influence of biocatalyst loading on the kinetic resolution of (±)-1-phenylethyl acetate was investigated. As shown in Figure 4, with the increase of enzyme concentration, the *C* increased, but the $e.e._p$ increased first and then decreased. When the biocatalyst loading was 40 mg/mL, the $e.e._p$ was the highest (96.5%), with the conversion being 21.4%. Thus,

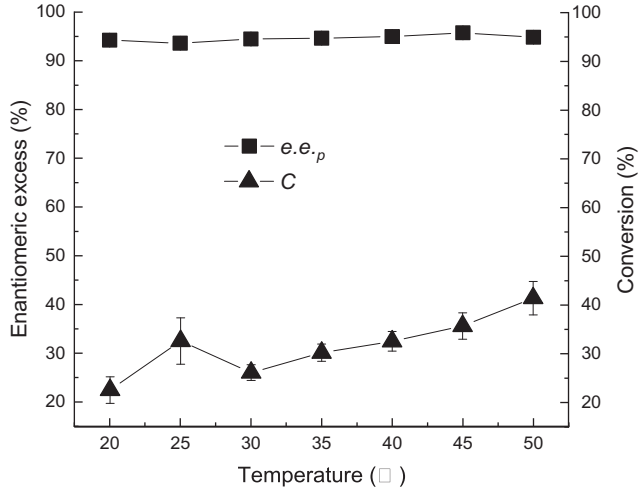


Figure 3. Effect of temperature on the kinetic resolution of (±)-1-phenylethyl acetate catalysed by the immobilized extracellaluar proteases of *Bacillus* sp. DL-1 for the generation of (*R*)-1-phenylethanol. The reaction condition: 50 mM pH 7.5 Tris-HCl, 40 mg/mL immobilized extracellular proteases, 10 mM (±)-1-phenylethyl acetate, total 500-μL, reaction for 2 h.

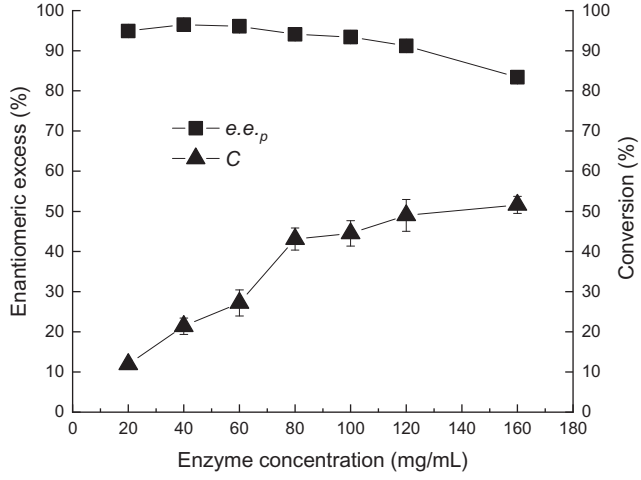


Figure 4. Effect of enzyme concentration on the kinetic resolution of (±)-1-phenylethyl acetate catalysed by the immobilized extracellaluar proteases of *Bacillus* sp. DL-1 for the generation of (*R*)-1-phenylethanol. The reaction condition: 50 mM pH 7.5 Tris-HCl, 10 mM (±)-1-phenylethyl acetate, total 500-μL, reaction at 45 °C for 2 h.

40 mg/mL immobilized extracellular proteases were selected to carry out the kinetic resolution for the preparation of (*R*)-1-phenylethanol.

Effect of substrate concentration on the kinetic resolution of (±)-1-phenylethyl acetate

Substrate concentration was also a key factor which can greatly affect enzymatic reactions. The kinetic resolution of (±)-1-phenylethyl acetate using the immobilized extracellular proteases of *Bacillus* sp. DL-1 with different substrate concentrations were

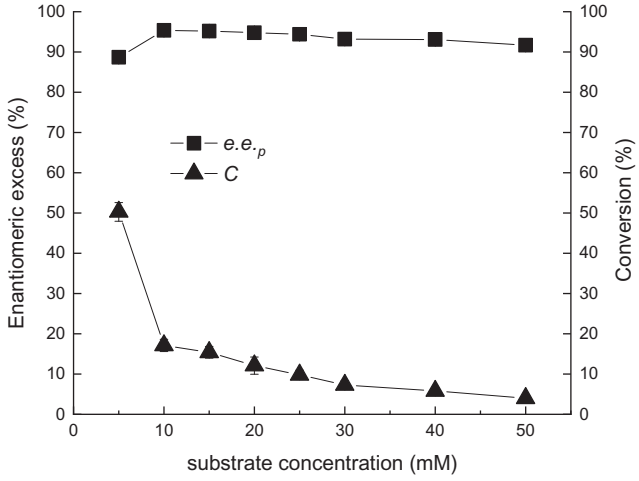


Figure 5. Effect of substrate concentration on the kinetic resolution of (±)-1-phenylethyl acetate catalysed by the immobilized extracellaluar proteases of *Bacillus* sp. DL-1 for the generation of (*R*)-1-phenylethanol. The reaction condition: 50 mM pH 7.5 Tris-HCl, 40 mg/mL immobilized extracellular proteases, total 500-μL, reaction at 45 °C for 2 h.

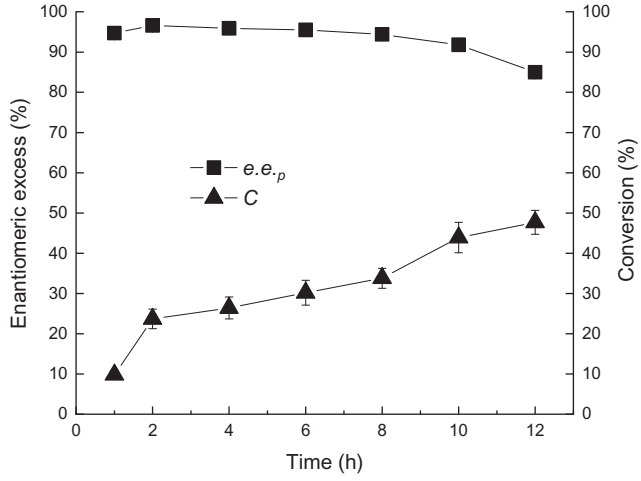


Figure 6. Effect of reaction time on the kinetic resolution of (±)-1-phenylethyl acetate catalysed by the immobilized extracellaluar proteases of *Bacillus* sp. DL-1 for the generation of (*R*)-1-phenylethanol. The reaction condition: 50 mM pH 7.5 Tris-HCl, 40 mg/mL immobilized extracellular proteases, 10 mM (±)-1-phenylethyl acetate, total 500-μL, reaction at 45 °C.

investigated. As shown in Figure 5, the $e.e._p$ reached its maximum 95.4% with the *C* of 17.1% at a substrate concentration of 10 mM. With the further increase of substrate concentration, the *C* of (±)-1-phenylethyl acetate decreased significantly. Thus, the optimal resolution results were gained when the substrate concentration was set at 10 mM.

Effect of reaction time on the kinetic resolution of (±)-1-phenylethyl acetate

The effect of reaction time (1 h − 12 h) on both the *C* and the enantio-selectivity of chiral products was studied. When the reaction time was 2 h, the $e.e._p$ reached 96.6%, with the conversion being 23.7%, and when the reaction time was longer than 2 h, the $e.e._p$ gradually decreased (Figure 6). Thus, 2 h was chosen as the optimal reaction time to asymmetrically hydrolyse (±)-1-phenylethyl acetate for the preparation of (*R*)-1-phenylethanol.

Effect of metallic ions and surfactants on the kinetic resolution of (±)-1-phenylethyl acetate

Metallic ions and surfactants could possibly greatly affect both the hydrolytic activity and the resolution ability of enzyme biocatalysts. In this study, thirteen different metallic ions and eight surfactants were added to investigate the effect of metallic ions and surfactants on the enzymatic kinetic resolution of (±)-1-phenylethyl acetate (Table 1). Of all the tested metallic ions and surfactants, only Na^+, Ba^{2+}, Co^{2+} and CMC-Na increased the enantio-selectivity of the immobilized proteases, compared with control experiments without the addition of additives. Notably, the presence of Co^{2+} increased the $e.e._p$ to 97%. While surfactant SDBS (Sodium dodecylbenzene sulfonate) showed obvious inhibitory activity on the kinetic resolution of (±)-1-phenylethyl acetate.

Effect of organic co-solvents on the kinetic resolution of (±)-1-phenylethyl acetate

Different organic co-solvents at final concentrations of 5% (*v*/*v*) were added into standard reaction systems. As shown in Table 2, the $e.e._p$ was enhanced in the presence of *n*-hexane and methanol, with the $e.e._p$ in the existence of methanol being the highest (97.1%). Most of the rest organic solvents used in this reaction systems exhibited negative effects on both the $e.e._p$ and the *C*.

To summarize, after careful process optimization for the kinetic resolution of (±)-1-phenylethyl acetate using immobilized extracellular proteases of *Bacillus* sp. DL-1, the optimal resolution reaction condition was carried out at 45 °C for 2 h in pH 7.5 Tris-HCl buffer with 10 mM (±)-1-phenylethyl acetate, 40 mg/mL immobilized proteases, 5% (*v*/*v*) methanol, which led to the $e.e._p > 97\%$, the *C* and the yield being 27% and 53%, respectively (Figure 7).

Table 1. Effect of metallic ions and surfactants on the kinetic resolution of (±)-1-phenylethyl acetate catalysed by immobilized extracellular proteases of *Bacillus* sp. DL-1 for the preparation of (*R*)-1-phenylethanol.

Metallic ions (2mM)	$e.e._p$ (%)	*C* (%)	Metallic ions (2mM)	$e.e._p$ (%)	*C* (%)
Control	96.0 ± 0.3	33.3 ± 1.5	Cu^{2+}	95.3 ± 0.3	23.1 ± 0.4
Li^{+}	95.7 ± 0.6	33.3 ± 1.5	Mg^{2+}	95.9 ± 0.1	23.8 ± 2.2
Na^{+}	96.9 ± 0.1	34.1 ± 0.4	Mn^{2+}	95.6 ± 0.4	14.2 ± 2.1
K^{+}	95.8 ± 0.5	32.8 ± 1.6	Ni^{2+}	93.9 ± 0.3	42.7 ± 1.9
Ba^{2+}	96.8 ± 0.1	23.7 ± 0.4	Zn^{2+}	96.0 ± 0.3	17.2 ± 1.8
Ca^{2+}	96.1 ± 0.6	30.3 ± 1.0	Al^{3+}	92.6 ± 1.5	32.4 ± 1.7
Co^{2+}	97.0 ± 0.1	30.1 ± 1.5	Fe^{3+}	96.0 ± 1.6	28.4 ± 1.4
Surfactants 0.05 % (w/v)	$e.e._p$ (%)	C (%)	Surfactants 0.05 %(w/v)	$e.e._p$ (%)	C (%)
Control	95.4 ± 0.3	40.3 ± 0.9	STPP	94.4 ± 1.2	38.6 ± 2.1
Tween-20	94.6 ± 0.3	38.5 ± 2.4	CTAB	90.4 ± 1.4	11.1 ± 1.7
Tween-80	95.6 ± 0.4	38.6 ± 1.9	SDBS	60.4 ± 2.5	0.9 ± 0.4
TritonX-100	94.6 ± 0.9	36.1 ± 2.0	CMC-Na	96.2 ± 0.7	34.7 ± 1.1
Span 65	94.9 ± 1.0	40.9 ± 1.8			

Note: The reaction condition: 50 mM pH 7.5 Tris-HCl, 40 mg/mL immobilized extracellular proteases, 10 mM (±)-1-phenylethyl acetate, total 500-μL, reaction at 45 °C for 2 h.

Table 2. Effect of organic solvents on the kinetic resolution of (±)-1-phenylethyl acetate catalysed by immobilized extracellular proteases of *Bacillus* sp. DL-1 for the preparation of (*R*)-1-phenylethanol.

Organic solvents 5 % (v/v)	$e.e._p$ (%)		Organic solvents 5 % (v/v)	$e.e._p$ (%)	*C* (%)
Control	95.9 ± 0.8	25.9 ± 0.6	1,4-Dioxane	95.5 ± 0.5	29.7 ± 2.1
Acetone	95.6 ± 1.4	34.8 ± 1.7	DMF	96.1 ± 0.8	34.8 ± 2.0
Acetonitrile	96.0 ± 0.5	28.0 ± 1.8	DMSO	95.2 ± 1.5	33.9 ± 2.3
Dichloromethane	91.7 ± 1.5	38.3 ± 1.8	Tetrahydrofuran	95.1 ± 0.6	36.2 ± 2.4
n-Hexane	96.6 ± 0.5	19.4 ± 0.5	*n*-Propanol	90.1 ± 0.7	6.4 ± 1.0
Cyclohexanone	94.0 ± 0.2	17 ± 1.4	*tert*-Butanol	94.1 ± 0.9	7.3 ± 1.8
Cyclohexane	96.3 ± 0.5	12.2 ± 0.4	*sec*-Butanol	89.7 ± 1.0	4.6 ± 1.2
Chloroform	95.5 ± 0.3	12.4 ± 0.5	*n*-Butanol	89.3 ± 1.1	7.5 ± 1.8
Ethanol	96.1 ± 0.2	14.1 ± 1.6	Isopropanol	93.2 ± 0.3	7.2 ± 1.6
Methanol	97.1 ± 0.1	27.4 ± 0.6	Isobutanol	84.1 ± 1.5	2.3 ± 0.7

Note: reaction conditions are the same as in Table 1.

Process optimization for the preparation of (S)-1-phenylethyl acetate through the kinetic resolution of (±)-1-phenylethyl acetate using immobilized extracellular proteases

The immobilized extracellular proteases of *Bacillus* sp. DL-1 preferentially hydrolysed (*R*)-1-phenylethyl acetate firstly to generate (*R*)-1-phenylethanol and thus (*S*)-1-phenylethyl acetate was generated during the process of asymmetric hydrolysis of (±)-1-phenylethyl acetate. Suitable increasing enzyme loading can enhance the conversion rate in enzymatic kinetic resolution. With the increase of conversion rate, (*R*)-1-phenylethyl acetate was consumed completely, (*S*)-1-phenylethyl acetate began to be transformed into (*S*)-1-phenylethanol. Therefore, in order to generate (*S*)-1-phenylethyl acetate with high optical purity, enhancing the concentration of immobilized proteases in hydrolytic reaction system might be useful. (*S*)-1-phenylethyl acetate was gained with high $e.e._s$ through optimizing the asymmetric hydrolysis conditions as follows.

Effect of pH on the kinetic resolution of (±)-1-phenylethyl acetate

pH may exhibit great effect on the enantio-selectivity and conversion rate in enzymatic kinetic resolution. Under standard hydrolytic reaction system of producing (*S*)-1-phenylethyl acetate, when pH was equal to or greater than 7.0, (±)-1-phenylethyl acetate was completely hydrolysed. Thus, for the generation of (*S*)-1-phenylethyl acetate, the kinetic resolution of (±)-1-phenylethyl acetate catalysed by immobilized extracellular proteases was analysed after incubating enzyme reactions at pH ranging from 5.0 to 6.5. As shown in Supplementary Figure S7, the highest $e.e._s$ of (*S*)-1-phenylethyl acetate was found to be 98.1% at pH 6.0 (PB), with the *C* being 64.8%.

Effect of temperature on the kinetic resolution of (±)-1-phenylethyl acetate

To evaluate the effects of different temperatures on enzyme reaction, the temperature range was varied from 20 °C to 50 °C under standard working

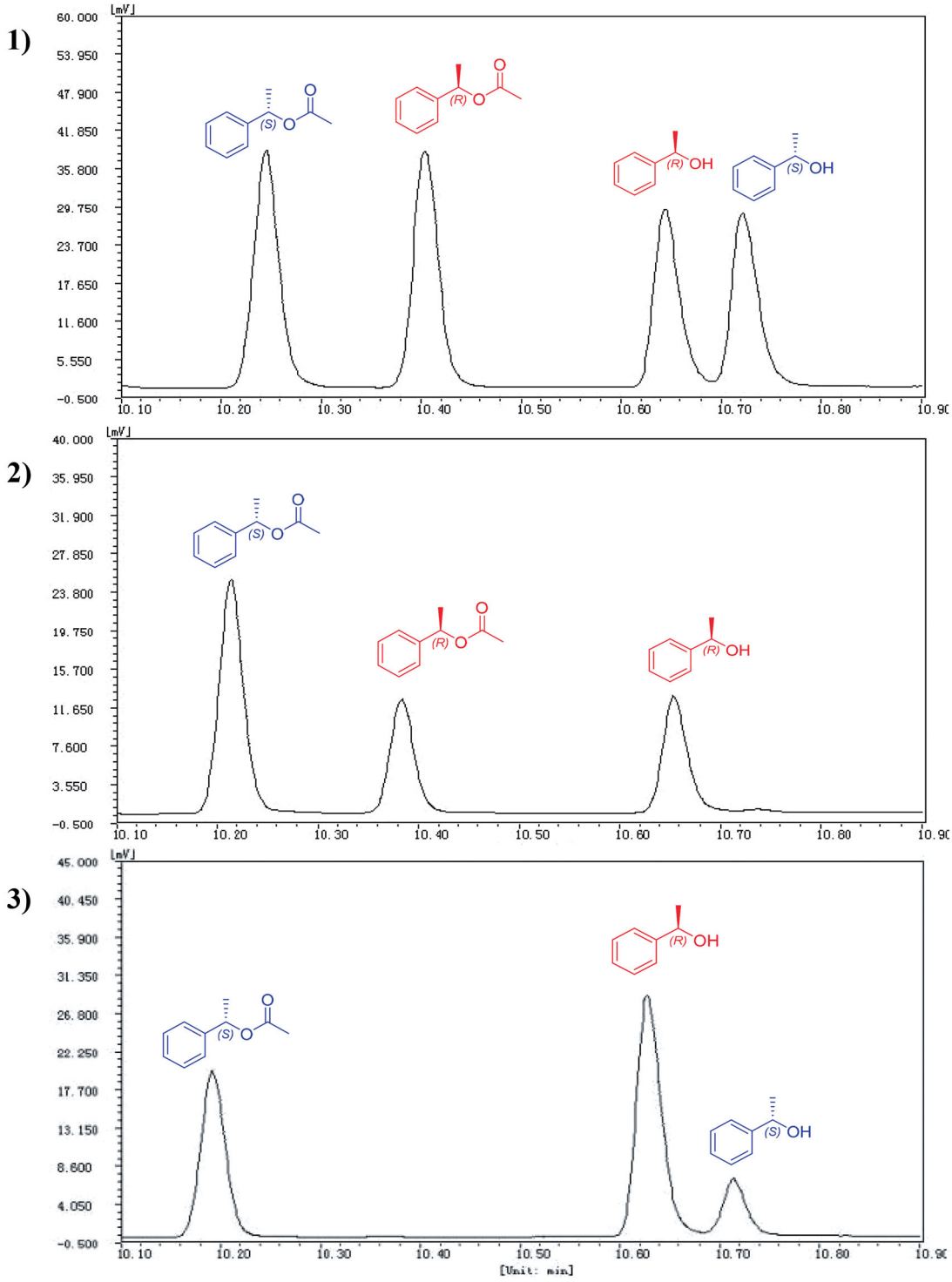


Figure 7. (*R*)-1-phenylethanol and (*S*)-1-phenylethyl acetate prepared through asymmetric hydrolysis of (±)-1-phenylethyl acetate by immobilized extracellular proteases after process optimization (1: standards of (±)-1-phenylethyl acetate and (±)-1-phenylethanol; 2: (*R*)-1-phenylethanol prepared after enzymatic kinetic resolution; 3: (*S*)-1-phenylethyl acetate prepared after enzymatic kinetic resolution).

conditions. With the increase of temperature, $e.e._s$ increased, and the maximal $e.e._s$ was recorded at 35 °C, with the $e.e._s$ and the *C* being 98.0% and 63.1%, respectively (Supplementary Figure S8). When the reaction temperature was further increased, the $e.e._s$ decreased. Thus, the optimal working temperature was characterized as 35 °C.

Table 3. Effect of metallic ions and surfactants on the kinetic resolution of (±)-1-phenylethyl acetate catalysed by immobilized extracellular proteases of *Bacillus* sp. DL-1 for the preparation of (*S*)-1-phenylethyl acetate.

Metallic ions (2mM)	$e.e._s$ (%)	C (%)	Metallic ions (2mM)	$e.e._s$ (%)	C (%)
control	95.6 ± 1.1	52.9 ± 0.7	Cu^{2+}	90.5 ± 0.6	49.8 ± 0.1
Li^{+}	93.7 ± 1.3	48.5 ± 1.4	Mg^{2+}	96.9 ± 0.3	54.7 ± 0.4
Na^{+}	97.3 ± 0.2	55.1 ± 0.5	Mn^{2+}	60.0 ± 1.4	38.7 ± 2.4
K^{+}	96.8 ± 0.6	54.7 ± 0.6	Ni^{2+}	49.2 ± 1.3	34.0 ± 1.4
Ba^{2+}	86.7 ± 0.4	54.2 ± 0.5	Zn^{2+}	84.4 ± 0.9	47.7 ± 1.5
Ca^{2+}	92.5 ± 0.5	50.7 ± 1.7	Al^{3+}	47.4 ± 1.7	33.1 ± 1.9
Co^{2+}	85.1 ± 2.2	47.8 ± 0.7	Fe^{3+}	72.0 ± 1.2	43.2 ± 1.3
Surfactants 0.05 % (w/v)	$e.e._s$ (%)	C (%)	Surfactants 0.05 % (w/v)	$e.e._s$ (%)	C (%)
control	95.8 ± 0.5	53.5 ± 0.4	STPP	96.8 ± 0.4	55.0 ± 2.0
Tween-20	97.3 ± 0.2	71.4 ± 1.2	CTAB	93.7 ± 1.3	48.4 ± 1.1
Tween-80	97.0 ± 0.6	56.5 ± 2.5	SDBS	1.8 ± 0.9	2.1 ± 1.3
TritonX-100	97.5 ± 0.2	68.5 ± 1.8	CMC-Na	96.2 ± 0.4	54.3 ± 0.6
Span 65	97.2 ± 0.5	58.0 ± 1.8			

Note: The reaction condition: 50 mM pH 6.0 PB, 360 mg/mL immobilized extracellular proteases, 10 mM (±)-1-phenylethyl acetate, total 500-μL, reaction at 35 °C for 1.5 h.

Effect of enzyme concentration on the kinetic resolution of (±)-1-phenylethyl acetate

Under standard hydrolytic reaction system for the preparation of (*S*)-1-phenylethyl acetate, different concentrations (120 mg/mL ∼ 400 mg/mL) of immobilized extracellular proteases were added to the enzymatic reactions. As shown in Supplementary Figure S9, both the $e.e._s$ and the *C* slowly increased when the enzyme concentration was less than 280 mg/mL, and a sharp increase of both the $e.e._s$ and the *C* was observed from 280 mg/mL to 360 mg/mL. When the concentrations of proteases were higher, both the $e.e._s$ and the *C* decreased. Thus, the optimal enzyme concentration was found to be 360 mg/mL, with the highest $e.e._s$ and the *C* being 96.5% and 59.2%, respectively.

Effect of substrate concentration on the kinetic resolution of (±)-1-phenylethyl acetate

High substrate loading in biocatalysis is of great importance for its practical use. In order to enhance the volumetric productivity of kinetic resolution of (±)-1-phenylethyl acetate, the substrate concentration was optimized. As illustrated in Supplementary Figure S10, (*S*)-1-phenylethyl acetate was produced with the highest $e.e._s$ (96.8%) and a 46.5% conversion rate, when the (±)-1-phenylethyl acetate concentration was 10 mM. With the further increasing of substrate concentration, the $e.e._s$ and the conversion of substrate gradually decreased. Therefore, 10 mM was set as the optimal substrate concentration for generating (*S*)-1-phenylethyl acetate.

Effect of reaction time on the kinetic resolution of (±)-1-phenylethyl acetate

To investigate the influence of reaction time on the resolution of (±)-1-phenylethyl acetate by the immobilized extracellular proteases, the reaction time was varied within the range of 0.5 h to 12 h. The results in Supplementary Figure S11 indicated a marked tendency for the $e.e._s$ to increase as the reaction time prolonged to 1.5 h. Then, the $e.e._s$ decreased with subsequent increases in reaction time. Thus, the highest $e.e._s$ (97.9%) was observed when the reaction time was set as 1.5 h, with the conversion rate being 58.5% (Supplementary Figure S11).

Effect of metallic ions and surfactants on the kinetic resolution of (±)-1-phenylethyl acetate

Under optimal reaction conditions, various metallic ions (2 mM) and surfactants (0.05%) were added to the enzyme-catalysed reaction systems to determine the effect of metallic ions and surfactants on the kinetic resolution of (±)-1-phenylethyl acetate. As shown in Table 3, the presence of Na^{+}, K^{+}, Mg^{2+} and surfactants Tween-20, Tween-80, TritonX-100, Span 65, STPP promoted the stereoselectivity to some extent, compared with control experiments. While most of the tested additives especially Mn^{2+}, Ni^{2+}, Al^{3+}, Fe^{3+}, and SDBS inhibited the activity of the biocatalyst.

Effect of organic co-solvents on the kinetic resolution of (±)-1-phenylethyl acetate

We also tested the effects of different organic co-solvents at final concentrations of 5% (*v/v*) on the biocatalytic process. From the results we found that the presence of acetonitrile, ethanol, methanol, DMF, DMSO, and THF significantly improved the

Table 4. Effect of organic solvents on the kinetic resolution of (±)-1-phenylethyl acetate catalysed by immobilized extracellular proteases of *Bacillus* sp. DL-1 for the preparation of (*S*)-1-phenylethyl acetate.

Organic solvents 5 % (v/v)	$e.e._s$ (%)	C (%)	Organic solvents 5 % (v/v)	$e.e._s$ (%)	C (%)
Control	97.2 ± 0.2	55.2 ± 0.4	1,4-Dioxane	97.1 ± 0.4	77.0 ± 0.2
Acetone	97.8 ± 0.1	81.6 ± 2.9	DMF	99.1 ± 0.1	80.9 ± 1.1
Acetonitrile	98.6 ± 0.2	70.4 ± 0.1	DMSO	99.2 ± 0.1	60.3 ± 2.2
Dichloromethane	69.8 ± 1.3	44.2 ± 2.1	Tetrahydrofuran	99.3 ± 0.1	77.4 ± 1.4
n-Hexane	90.9 ± 0.7	53.8 ± 0.5	*n*-Propanol	46.1 ± 1.2	32.9 ± 1.9
Cyclohexanone	88.9 ± 1.2	50.6 ± 0.4	*tert*-Butanol	59.0 ± 0.8	38.6 ± 1.6
Cyclohexane	97.4 ± 0.3	53.9 ± 0.3	*sec*-Butanol	25.4 ± 1.6	21.5 ± 0.8
Chloroform	59.7 ± 1.3	39.9 ± 1.5	*n*-Butanol	54.9 ± 1.3	38.7 ± 1.5
Ethanol	98.6 ± 0.3	70.3 ± 1.1	Isopropanol	90.3 ± 0.4	49.8 ± 1.7
Methanol	99.1 ± 0.1	67.6 ± 0.5	Isobutanol	14.5 ± 1.2	13.7 ± 2.7

Note: reaction conditions are the same as in Table 3.

stereoselectivity of the proteases compared with control experiments (Table 4). Particularly, DMSO, methanol, THF, and DMF could enhance the $e.e._s$ to 99% with the *C* being 60.3%, 67.6%, 77.4%, and 80.9%, respectively. As a consequence, considering the conversion rate, DMSO was characterized to be the optimal organic cosolvent for the kinetic resolution of (±)-1-phenylethyl acetate to generate (*S*)-1-phenylethyl acetate using immobilized extracellular proteases.

Finally, 10 mM (±)-1-phenylethyl acetate, 360 mg/mL immobilized extracellular proteases, pH 6.0 (PB), 5% (*v*/*v*) DMSO, and 35 °C for 1.5 h was determined as the optimal reaction conditions for the kinetic resolution of (±)-1-phenylethyl acetate to prepare (*S*)-1-phenylethyl acetate using immobilized extracellular proteases of *Bacillus* sp. DL-1. Under the optimal resolution conditions, the immobilized proteases could yield (*S*)-1-phenylethyl acetate with high $e.e._s$ (99%), conversion rate (60%) and yield (79%) (Figure 7).

Operational stability of immobilized proteases

As shown in Supplementary Figure S12, the immobilized extracellular proteases were reused for ten times to measure their enzyme activity. The bound enzyme preserved about 35.8% of its activity after 6 repeated uses. The operational stabilities of immobilized extracellular proteases were also assayed by reusing the immobilized enzyme five times for the kinetic resolution of (±)-1-phenylethyl acetate under the optimal conditions (Supplementary Figure S13). Both the conversion rate and the enantiomeric excess decreased after repeated usage in the asymmetric hydrolysis. This unsatisfactory reusability of the immobilized enzyme could be due to the weak physical adsorption between the proteases and kieselguhr. In other words, desorption or leakage of the enzyme (resulting from changes in temperature, pH, and ionic strength) may happen (Ertan et al. 2006; Nunes and Marty 2006; Silva et al. 2018). Different immobilization protocols may greatly alter enzyme features. Lipase B from *Candida antarctica* (CALB) was covalently immobilized on epoxy Immobead-350 (IB-350) for the circularly hydrolytic resolution of rac-indanyl acetate, which showed that the reusability of the CALB-IB-350 was efficient in five consecutive resolution cycles (Pinheiro et al. 2018). Lipase from *Pseudomonas fluorescens* was immobilized on the superparamagnetic nanoparticles coupled with APTES and then activated with glutaraldehyde (Fe_3O_4@APTES-GLU-PF) for the circularly kinetic resolution of rac-indanol, which showed that the enzyme system remained with high activity and values of selectivity unchanged during the first three cycles of reaction, and in the fourth and fifth cycles, only a slight decrease in selectivity and with conversion value close to 50% (Galvão et al. 2018). In consequence, covalent immobilization may be more advantageous in improving the reusability of the immobilized enzyme. The conditions of immobilization, especially, the pH, may have great influence on the performance of immobilization enzyme. Changing the immobilization pH can altere the orientation of the enzyme molecules on the support. For instance, using nucleophiles (e.g. ethylenediamine) to block the support permitted to eliminate the chemical reactivity of the support avoiding undesired enzyme-support interaction and being a useful reaction endpoint (dos Santos et al. 2015b). Near to the isoelectric point, the free enzyme may experience protein aggregation caused by undesired enzyme-support interactions where utilization of changing pH for inactivation that can stabilize incorrect enzyme structures (Sanchez et al. 2016). Therefore, pH of immobilization will be investigated carefully in our future research. In order to further improve the thermal stability, storage stability and reusability of immobilized enzyme, cross-linking after

Table 5. Comparation of extracellular proteases of *Bacillus* sp. DL-1 with other proteases/esterases/lipases in the asymmetric hydrolysis of (±)-1-phenylethyl acetate.

Origin of Enzymes	*Bacillus* sp. DL-1	*Bacillus* sp. DL-2	*Bacillus* sp. SCSIO 15121	*Bacillus* sp. SCSIO 15029	*Candida antarctica* Lipase B	*Candida rugosa*	*Candida antarctica*	*Pseudomonas cepacia*	*Candida cylindracea*
Enzyme species	Proteases	Proteases	Esterase BSE01281 (Liang	Esterase BSE00077 (Huang	(Novozym 435)	Lipase	Lipase B	Lipase	Lipase
Reference	This work	(Dong et al. 2019)	et al. 2016b)	et al. 2017)	(Fan et al. 2011)	(Ou et al. 2008)	(Melais et al. 2016)	(Melais et al. 2016)	(Melais et al. 2016)
1-Phenylethyl acetate									
Configuration	***S***	*S*	*S*	–	–	*S*	*S*	*S*	S
Substrate concentration (mM)	**10**	2.5	10	–	–	71.4	166.7	166.7	166.7
pH	**6.0**	7.5	8.0	–	–	7.2	7.0	7.0	7.0
Temperature (°C)	**35**	35	40	–	–	Room temperature	40	40	40
Time (h)	**1.5**	10	8	–	–	22	24	24	24
e.e. (%)	**> 99**	> 99	> 95	–	–	43	96	67	14
Conversion (%)	**60**	64	49	–	–	51	49	40	21
Yield (%)	**79**	71	–	–	–	–	36	30	78
1-Phenyl ethanol									
Configuration	***R***	*R*	*R*	*R*	*S*	*R*	*R*	*R*	*R*
Substrate concentration (mM)	**10**	5	10	50	62.6	71.4	166.7	166.7	166.7
pH	**7.5**	6.5	8.0	7.0	7.5	7.2	7.0	7.0	7.0
Temperature (°C)	**45**	20	40	30	40	Room temperature	40	40	40
Time (h)	**2**	2	8	2	1.5	31	24	24	24
e.e. (%)	**> 97**	97	> 99	> 99	> 99	44	99	99	54
Conversion (%)	**27**	17	49	> 30	41.2	46	49	40	21
Yield (%)	**53**	41	–	–	–	–	42	28	20

Note: "–" denotes no report.

adsorption of the extracellular proteases on the support, various conditions of immobilization, even some new activated supports and other immobilization methods, will be tried and optimized in our following work.

Comparation of the immobilized extracellular proteases from bacillus sp. DL-1 with other proteases/eaterases/lipases in the resolution of (±)-1-phenylethyl acetate through asymmetric hydrolysis

To the best of our knowledge, in the field of biocatalysis, most of the kinetic resolution of (±)-1-phenylethyl acetate through asymmetric hydrolysis were carried out by using esterases or lipases (Ou et al. 2008; Fan et al. 2011; Melais et al. 2016; Liang et al. 2016b; Huang et al. 2017). Protease, one of most important industrial enzymes, was rarely used in the resolution of racemic esters. The only example of asymmetrically hydrolysing (±)-1-phenylethyl acetate by using proteases was in our previous work (Dong et al. 2019). Generally, (*S*)-1-phenylethyl acetate and (*R*)-1-phenylethanol were generated in the asymmetric hydrolysis of (±)-1-phenylethyl acetate, which was catalysed by esterases/lipases/proteases. A commercial immobilized *Candida antarctica* lipase B (Novozym 435) was found to exhibited opposite stereoselectivity to generate (*S*)-1-phenylethanol with the *e.e.* being over 99%, and the conversion being 41.2% in the kinetic resolution of (±)-1-phenylethyl acetate. Compared with esterases/lipases, proteases showed its unique advantages of producing (*S*)-1-phenylethyl acetate with high optical pure in the asymmetric hydrolysis of (±)-1-phenylethyl acetate (Table 5).

Compared with the extracellular proteases from *Bacillus* sp. DL-2, the immobilized extracellular proteases from *Bacillus* sp. DL-1 exhibited higher hydrolytic activity and could asymmetrically hydrolyse (±)-1-phenylethyl acetate by using higher substrate concentration, shorter reaction time to obtain higher yield, which also generated (*S*)-1-phenylethyl acetate with the $e.e._s$ being over 99%, and (*R*)-1-phenylethanol with the $e.e._p$ being over 97% (Table 5).

Conclusion

The extracellular proteases of *Bacillus* sp. DL-1, one bacterium isolated from the deep sea of the Western Pacific Ocean, were immobilized by kieselguhr and then utilized in the kinetic resolution of (±)-1-phenylethyl acetate. After process optimization of the enzymatic resolution of (±)-1-phenylethyl acetate, under the optimum reaction conditions for the preparation of (*R*)-1-phenylethanol: 10 mM (±)-1-phenylethyl acetate, 40 mg/mL immobilized extracellular proteases, pH 7.5 (Tris-HCl), 5% (*v*/*v*) methanol and 45 °C for 2 h, the (*R*)-1-phenylethanol was obtained with the $e.e._p$ being over 97%, conversion being 27%, and yield being 53%, respectively, and under the optimum reaction conditions for the preparation of (*S*)-1-phenylethyl acetate: 10-mM (±)-1-phenylethyl acetate, 360 mg/mL immobilized extracellular proteases, pH 6.0 (PB), 5% (*v*/*v*) DMSO and 35 °C for 1.5 h, the (*S*)-1-phenylethyl acetate was generated with the $e.e._s$ being over 99%, conversion being 60%, and yield being 79%, respectively. Notably, we noticed that some organic solvents, such as methanol or DMSO, may improve the stereoselectivity during the process of enzymatic kinetic resolution. In conclusion, the extracellular proteases of *Bacillus* sp. DL-1 were efficient and cost-effective deep-sea biocatalysts with high industrial potentials in the preparation of optically pure (*R*)-1-phenylethanol and (*S*)-1-phenylethyl acetate as well as of other chiral chemicals.

Disclosure statement

The authors all declare that they have no conflict of interest.

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