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Efficient Production of (*S*)-Naproxen with (*R*)-Substrate Recycling Using an Overexpressed Carboxylesterase BsE-NP01

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Abstract An (*S*)-enantioselective esterase from *Bacillus subtilis* ECU0554, named BsE-NP01, has been cloned and over-expressed in a heterologous host *Escherichia coli* BL21. BsE-NP01 was shown to be a carboxylesterase with a molecular mass of about 32 kDa, and temperature and pH optima at 50 °C and 8.5, respectively. It could catalyze the selective hydrolysis of the (*S*)-enantiomer of racemic naproxen methyl ester, giving optically pure (*S*)-naproxen with 98% enantiomeric excess. A mechanic-grinding approach to substrate dispersion was also reported, which was considered to be an alternative to take the place of deleterious surfactants such as Tween-80, with improved performance of the hydrolysis reaction. Batch production of (*S*)-naproxen was repeatedly carried out in a solid-water biphasic system at 2-L scale, achieving an average total yield of about 85% after ten runs with complete recycling of (*R*)-substrate.

Keywords (*S*)-naproxen · *Bacillus subtilis* carboxylesterase · Enantioselective hydrolysis · Naproxen methyl ester

Introduction

As the development of chiral separation technologies, as well as the strong emphasis upon the search of therapeutic benefits with the goal of developing safer and more effective drugs, drug chirality has become a major theme in the design, discovery, development, launching, and marketing of new drugs. The high degree of stereoselectivity of many biological processes implies that when a given racemic mixture is administered as a drug both enantiomers will not be equally potent, giving the fact that one plays a positive role while the other may contribute to undesirable effects [1–3]. Asymmetric synthesis or enantiomeric resolutions are feasible processes for the preparation of drugs in enantiopure

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form. Kinetic resolution appears particularly convenient when recycling of distomer occurs readily, thus, rendering the preparative process attractive in terms of chemical yield [4].

Carboxylesterases (E.C. 3.1.1.1) represent a diverse group of hydrolases which catalyze the cleavage, formation, or transfer of ester bonds. In addition to their wide distribution in plants, animals, and microorganisms, they also show high regio- and stereoselectivity making them attractive biocatalysts for production of optically pure compounds in fine chemicals synthesis [5]. Many bacterial esterases have been cloned and overexpressed during the last decades in order to assess their enantioselective properties [6–11].

Naproxen, i.e., (+)-(*S*)-2-(6-methoxy-2-naphthyl)propionic acid, is a member of the 2-aryl propionic acids, which are also known as non-steroidal anti-inflammatory drugs. Like other 2-aryl propionic acids, for instance, ketoprofen and ibuprofen, the anti-inflammatory activity of naproxen was believed to reside in its (*S*)-enatiomer, showing activity of *ca.* 28 times more than its (*R*)-enantiomer, which might also promote unwanted gastrointestinal disorders, giving the necessity of getting enantiopure (*S*)-naproxen in the industrial production [6]. Various efforts have therefore been made to obtain optically pure (*S*)-naproxen, especially using the powerful tool of biocatalyst engineering, which is generally considered to be highly efficient, highly specific and environmentally friendly. Lipases from *Candida rugosa* and *Carica papaya*, and an esterase from *Bacillus subtilis* (carboxylesterase NP) have been used for kinetic resolution of racemic naproxen esters to produce (*S*)-naproxen [6, 12, 13].

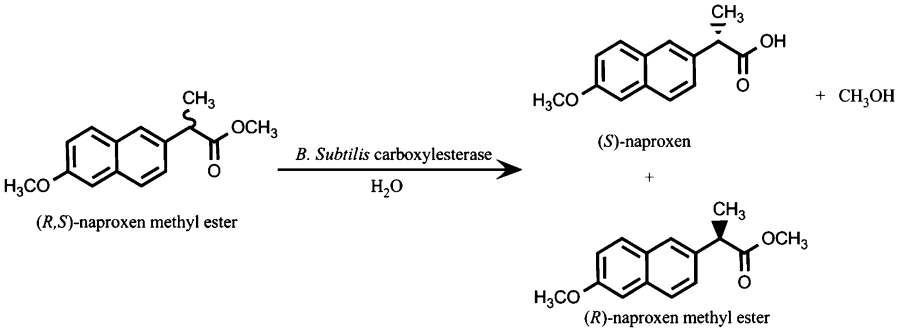
Among these successful examples, the carboxylesterase NP from *B. subtilis* ThaiI-8 is well-known in chiral separation for its excellent enantioselectivity in biocatalytic hydrolysis of (*R,S*)-naproxen esters to produce (*S*)-naproxen [6]. In the presence of Tween-80 for helping substrate dispersibility, (*S*)-naproxen was yielded with excellent enantioselectivity of 99% *ee* (enantiomeric excess). Recently, Steenkamp and Brady [14] made further progress on the way of industrial production of (*S*)-naproxen using carboxylesterase NP under optimized conditions. Although the addition of Tween-80 helped to increase the dispersion of hydrophobic substrate, thus resulting in somewhat better enantioselectivity, it becomes more difficult to get medically pure (*S*)-naproxen through conventional separation.

Based on the development of bioinformatics, as well as the genome project of *B. subtilis* 168 [15], which has provided an attractive alternative approach to find alternative biocatalysts for rational drug preparation, we succeeded in cloning and expressing an esterase BsE-NP01 from *B. subtilis* ECU0554, which was previously isolated from soil and showed excellent performance on *l*-menthyl acetate but no enantioselectivity towards naproxen ester using wild-type enzymes. Fortunately, the recombinant esterase, BsE-NP01, showed high enantioselectivity (>98% *ee*) towards naproxen esters as carboxylesterase NP. This recombinant esterase was therefore optimized and employed for repeated production of (*S*)-naproxen (Scheme 1).

Experimental Section

Chemicals

(*R*,*S*)-Naproxen was provided by Baju Pharmaceutical Co., Ltd (Jiangsu, China). Synthesis of naproxen methyl ester was conducted according to the previous report [16]. All *p*-nitrophenyl esters were synthesized in our laboratory using the method described previously [17]. All other reagents or chemicals used were obtained commercially and of analytical grade.



Scheme 1 Enzymatic resolution of (*R,S*)-naproxen methyl ester using recombinant *Bacillus subtilis* esterase BsE-NP01

Bacterial Strains, Plasmids, and Culture Conditions

B. subtilis ECU0554 was previously isolated in our laboratory as a *l*-menthyl ester hydrolase producer for enantioselective hydrolysis of *dl*-menthyl acetate to *l*-menthol [18]. *Escherichia coli* DH5α and BL21 (DE3) were host strains used in the gene cloning and protein expression respectively, employing pMD 18-T vector (Takara, China) and pET-11a (Novagen, Germany) as plasmids.

Wild-type and recombinant *E. coli* cells were cultured regularly in Luria-Bertani (LB) medium at 37 °C and an appropriate amount of ampicillin (100 μg/ml) was added for cultivation of recombinant *E. coli* cells when needed. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was also added to the culture medium for induction of the target enzyme. The cell growth was determined by measuring the optical density at 600 nm (OD_{600}) using a spectrophotometer.

Cloning and Overexpression of *B. subtilis* Carboxylesterase in *E. coli*

DNA manipulation and transformation were carried out according to standard methodologies [19]. A 903-bp DNA fragment containing the gene of esterase BsE-NP01 was amplified by polymerase chain reaction (PCR) using the genomic DNA from *B. subtilis* ECU0554 as template. Primers were designed according to the reported gene sequence of carboxylesterase NP from *B. subtilis* ThaiI 8 [6]:

Forward, 5′-TTC***CATATG***TCAAACCATTCATCTAGTATTCCCG-3′;
Reverse, 5′-***GGATCC***TTACCGTGAAATGCCTGTTTCTGCATTG-3′.

The PCR product was then ligated to pMD 18-T vector (Takara, China) and transformed to *E. coli* DH5α. Positive colonies were identified by PCR and subjected to sequence analysis. The plasmids containing the 903-bp inserted fragment extracted from the positive colonies were digested with *Nde*I and *BamH*I, and cloned into pET-11a vector to generate the expression plasmids. Finally, the expression plasmids were transformed to *E. coli* BL21 cells.

The *E. coli* BL21 strain harboring the expression plasmids was firstly grown at 37 °C and then induced at 25 °C in LB medium. In both cases, the medium was supplemented with 100 μg/ml ampicillin. The expression of the esterase was induced for 4 h at an OD_{600} of 0.6 by the addition of 0.5 mM IPTG. The bacterial cells were harvested by centrifugation. The cells were washed twice with physiological saline and broken by

sonication. The lysate was removed by centrifugation and the supernatant was subjected to enzyme assay and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue R-250 staining.

Acquisition of large quantities of the enzyme was achieved by fermentation of the recombinant *E. coli* in a 5-L fermentor (Shanghai Baoxing Bioengineering Equipment Co. Ltd, China). After growing in LB medium overnight, 10% (*v*/*v*) of the seed culture was inoculated to 3 L fermentation medium with the following composition (*w*/*v*): glucose 0.5%, tryptone 2%, yeast extract 1%, K_2HPO_4 0.4%, $MgSO_4$ 0.05% and NaCl 1%. The cells were firstly grown for 3 h at 37 °C and then induced for 9 h at 25 °C, with aeration and agitation rates of 1 vvm and 500 rpm, respectively. The cells were harvested by centrifugation, washed twice with physiological saline and then disrupted in 100 mM pH 8.5 H_3BO_3-$Na_2B_4O_7$ by high pressure homogenizer (ATS Engineering Inc, Italy). The crude enzyme was harvested after removal of cell debris by centrifugation.

Enzymatic Resolution of (*R,S*)-naproxen Methyl Ester

Effects of temperature, pH, and substrate concentration on the hydrolysis activity of the recombinant esterase towards naproxen methyl ester were investigated. The main product (*S*)-naproxen and co-product methanol were produced in the resolution reaction. Therefore, the possible inhibition of the esterase activity by different (*S*)-naproxen and methanol concentrations was also examined. The lyophilized powder of cell-free extract was used for the activity assay.

Repeated Batch Process for (*S*)-naproxen Production

2-L Scale Production of (S)-naproxen Using Esterase BsE-NP01

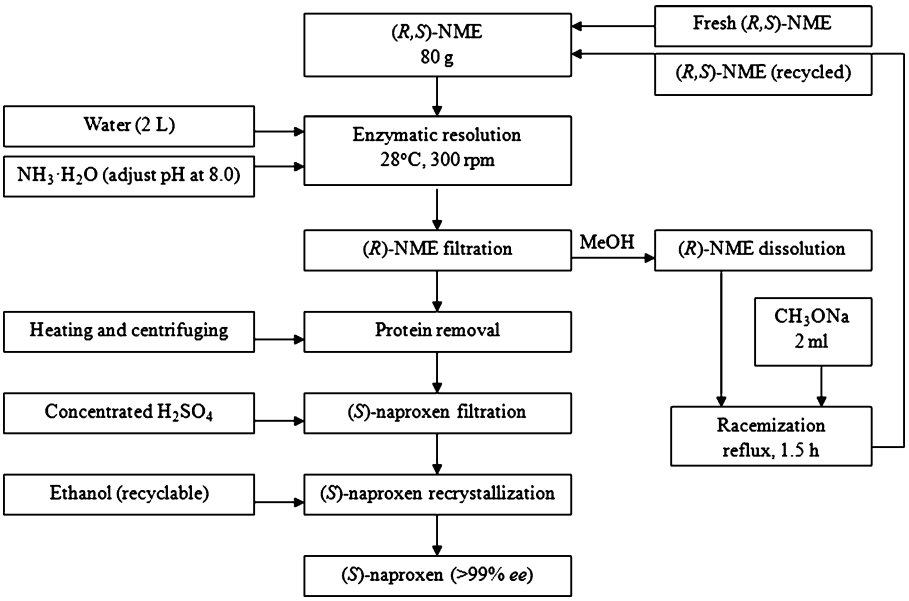
The flow diagram was shown in Scheme 2. Eighty grams of (*R,S*)-naproxen methyl ester, pretreated by a high speed mixer, was added to 2-L aqueous solution including 5 mM boric buffer, pH 8.0. After the addition of 600 U of BsE-NP01, the reaction was performed at 28 °C and 300 rpm with a constant pH of 8.0 auto-titrated by concentrated ammonia. The reaction was stopped after 5 h by removing the unreacted (*S*)-substrate through filtration. Then the filtrate was heated to 70 °C for 30 min and centrifuged to remove the proteins. The crude product (*S*)-naproxen was precipitated after acidization, filtered, washed, and dried. The crude product was refined by recrystallization in ethanol.

Substrate Recycling

Recycling of the unreacted naproxen methyl ester (NME) was achieved by racemization, employing sodium methoxide as a catalyst. The unreacted NME separated from the reaction mixture was firstly dissolved in methanol. Then the mixture was refluxed for 1.5 h in 0.5% (*w*/*v*) sodium methoxide under stirring. Finally, the mixture was neutralized with sulfuric acid and the residual methanol was removed by vacuum evaporation. The recycled NME was then put back into a new round of batch reaction.

Enzyme Assay and Protein Determination

Esterase activity was routinely determined by a *p*-nitrophenyl butyrate (*p*NPB) assay to measure the amount of *p*-nitrophenol formed from *p*NPB as described previously [20].



Scheme 2 Block flow diagram of repeated batch process for (*S*)-naproxen production from (*R*,*S*)-naproxen methyl ester (NME) with (*R*)-substrate recycling in a stirred tank reactor with 2 L of working volume

Hydrolytic activities on various *p*-nitrophenyl esters were also measured by a modified lipase assay method in order to determine its substrate specificity to various *p*-nitrophenyl esters. One unit of esterase activity was defined as the amount of enzyme releasing 1.0 μmol of *p*-nitrophenol per minute under such conditions.

Protein concentration was determined both by the Bradford method [21] and by gel electrophoresis on a SDS-PAGE as described by Laemmli [22].

Analytical Methods

The *ee* of the residual (*R*)-NME and the produced (*S*)-naproxen were determined by HPLC using a chiral column (Chiralcel OD, Φ 0.4×25 cm, Daicel, Japan), eluted with hexane/isopropanol/acetic acid (97:3:0.2, by volume) at a flow rate of 1.0 ml/min and detected at UV 254 nm. The retention times of (*R*)-NME, (*S*)-NME, (*R*)-naproxen, and (*S*)-naproxen were 6.7, 7.8, 16.5, and 18.9, respectively. The *ee* of NME (ee_s) and naproxen (ee_p) were calculated as follows: $ee_p = ([(S)\text{-NAP}]-[(R)\text{-NAP}])/([(S)\text{-NAP}] + [(R)\text{-NAP}]$ [13]

Results

Cloning and Overexpression of *B. subtilis* Carboxylesterase BsE-NP01 in *E. coli*

B. subtilis carboxylesterase gene was PCR-amplified from genomic DNA of *B. subtilis* ECU0554. The amplified gene was flanked by restriction sites *Nde*I and *BamH*I. After digested with *Nde*I and *BamH*I, the DNA fragment with digested esterase gene was ligated

to pET-11a vector and then transformed in *E. coli* BL21 cells. Successful cloning and expression was confirmed by sequencing of the positive clone and by esterase activity assay on a tributyrin agar plate [23].

The nucleotide sequence of the inserted DNA was determined and submitted to GenBank under the accession number GQ868652. It showed an ORF of 903 bp encoding a polypeptide of 300 amino acid residues, designated as BsE-NP01, which shares 99% identities on both DNA level and amino acid level with those of *B. subtilis* ThaiI 8 [6] and *B. subtilis* strain 168 [15].

SDS-PAGE of the sonicates of induced cells revealed a highly over-expressed protein of the expected molecular weight (~32 kDa) of the recombinant esterase BsE-NP01. As shown in Fig. 1, the recombinant BsE-NP01 expressed in soluble fraction was estimated to be more than 50% of the total proteins in the cell-free extract.

Large quantity of enzyme production was obtained by over-expression of BsE-NP01 in the 5-L fermentor. The maximum esterase activity (800 U/L for *p*NPB) was achieved after 8 h induction at 25 °C. The resultant crude recombinant esterase was used for the biocatalytic resolution of racemic naproxen esters without further purification.

Substrate Specificity Towards Various *p*-nitrophenyl Esters

To determine the substrate specificity towards *p*-NP esters, the activity of BsE-NP01 towards *p*-NP esters of varied carbon chain length was measured spectrometrically at 405 nm. The typical profile of chain length specificity of this esterase towards *p*-NP esters was shown in Fig. 2. We can conclude that BsE-NP01 displayed the highest activity towards *p*-NP butyrate. It also indicates that the recombinant BsE-NP01 was a typical carboxylesterase which preferred short-chain lipids in contrast to a typical lipase which prefers long-chain lipids.

Enzymatic Resolution of Naproxen Methyl Ester

Effects of Temperature and pH on the Activity of Esterase BsE-NP01

Effects of temperature and pH on the activity of BsE-NP01 were shown in Figs. 3 and 4, respectively. The enzyme showed the maximum activity towards naproxen methyl ester at

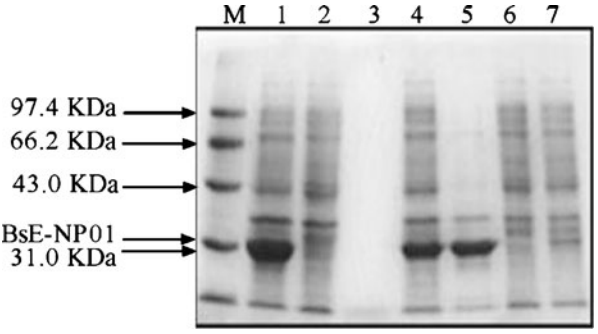


Fig. 1 SDS-PAGE (10% gel) analysis of polypeptides produced in the *E. coli* recombinant cells carrying the BsE-NP01 gene. *Lane M* molecular weight marker; *Lanes 1 and 2* cell lysates of the recombinant and native *E. coli* after induction; *Lane 3* culture supernatant of the recombinant *E. coli*, *Lanes 4 and 5* supernatant and precipitate of the recombinant *E. coli* cell lysates; *Lanes 6 and 7* cell lysates of recombinant and native *E. coli* before induction

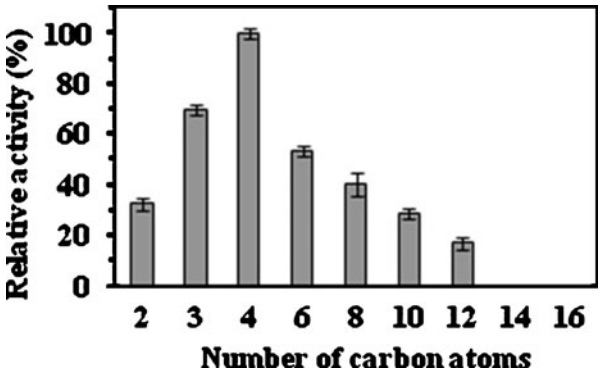


Fig. 2 Fatty acid specificity of the carboxylesterase BsE-NP01 towards *p*-nitrophenyl esters with varied length of aliphatic fatty acid chain. The activity towards *p*-nitrophenyl butyrate was taken as 100%. All assays were performed in triplicates

50 °C and pH 8.5, respectively, and the activity decreases significantly at a temperature over 50 °C and pH below 7.0. In spite of this, 28 °C and pH 8.0 were used for scaled-up preparation of (*S*)-naproxen considering the enzyme stability (data not shown).

Effect of Substrate Dispersion

It has been observed that Tween-80 had a positive influence on esterase enantioselectivity in the resolution of NME [14]. Inclusion of only 1% (*w*/*v*) Tween-80 in the reaction medium could have a strong positive influence both on the reaction rate and enantioselectivity in the resolution of NME (data not shown). It is commonly believed that substrate dispersion is an important factor affecting the hydrolysis reaction. Based on this knowledge, here we explored a fresh way to displace the addition of surfactant Tween-80, which had adverse impact on downstream separation as well as product purity. A high-speed mixer (Joyoung

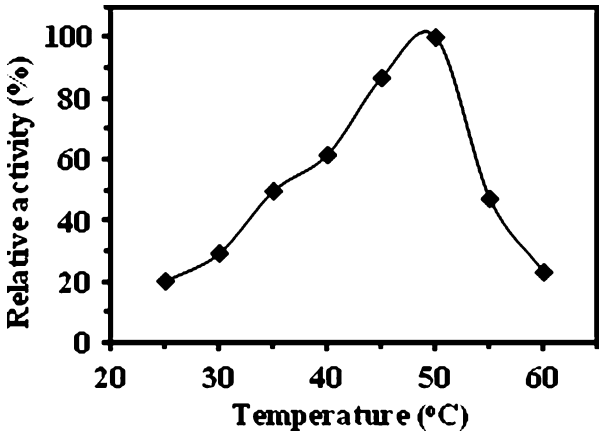


Fig. 3 Effect of temperature on esterase activity. Into 0.5 ml solution were added 20 mM NME and 1% (*w*/*v*) Tween-80. The mixture was incubated at different temperatures for 10 min in a thermomixer (Eppendorf Co., Germany) at 1,000 rpm after ultrasonic treatment, and the reaction mixture was extracted with ethyl acetate and analyzed by chiral HPLC. All assays were performed in triplicates

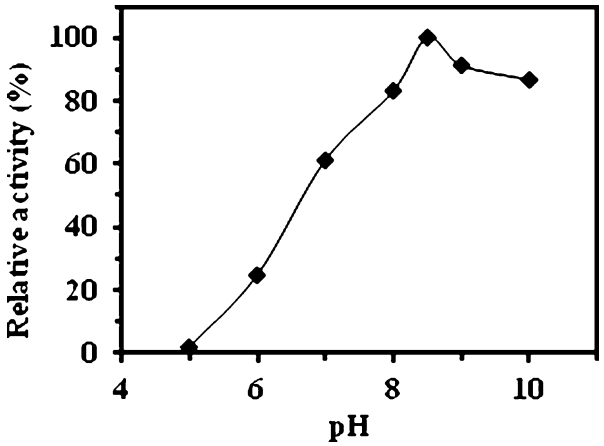


Fig. 4 Effect of pH on esterase activity. The reaction conditions were the same as that of temperature optimization except for the buffer, for which HAc-NaAc buffer (pH 5.0), KH_2PO_4-Na_2HPO_4 (pH 6.0–7.5), Tris-HCl buffer (pH 8.0), and Glycine-NaOH buffer (pH 8.5-10.5) were used. All assays were performed in triplicates

Co. Ltd., China) was employed to facilitate the dispersion of solid NME by cutting it into extremely small particles. Fortunately, the terminal enantioselectivity could reach a high value of more than 97% *ee* without any surfactants. This method was then used for 2-L scale preparation of (*S*)-naproxen.

Influence of Substrate Concentration

Results of the substrate concentration effect indicated that the initial rate (of racemic NME) did not significantly decrease with the increase of substrate concentration, suggesting that there was no substrate inhibition in this esterase-catalyzed reaction under the substrate saturated condition (data not shown). This is reasonable since the actual concentration of dissolved substrate (NME) in water or buffer is very low [12].

Inhibition of Products

The influence of (*S*)-naproxen concentration on the activity of esterase BsE-NP01 was shown in Fig. 5. The esterase activity decreased with the increase of (*S*)-naproxen added. Nearly 80% of the initial activity was lost when 2.5 mM of (*S*)-naproxen was added to the reaction system suggesting that the (*S*)-naproxen produced will be unfavorable to the function of the esterase and to sustainable production. Nevertheless, this problem could be solved by auto-titration with aqueous ammonia solution to keep the pH of reaction system at 8.5, which converts majority of the product from an acid into a salt. The ammonium salt of (*S*)-naproxen seems to be much benign to the enzyme's function though there is actually an equilibrium between the salt and the acid forms.

The effect of methanol as a co-product on the esterase activity was investigated between 0 and 10% (*v*/*v*) of methanol. The results (data not shown) showed that hardly any inhibition on the esterase activity was observed even at a high methanol concentration.

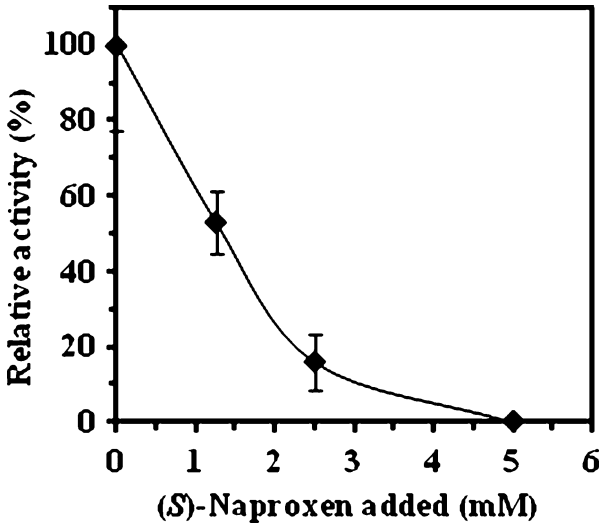


Fig. 5 Inhibition of BsE-NP01 activity by (*S*)-naproxen. The reactions were carried out at 50 °C, pH 8.5 and 200 rpm in a 10-ml reaction system containing 50 mM naproxen methyl ester and 1% of Tween-80. All assays were performed in duplicates

Repeated Batch Process for (*S*)-naproxen Production

A 2-L scale reaction was done under reaction conditions optimized in a 100-ml reaction system (data not shown). In order to make full use of the substrate, we explored a way of substrate recycling through racemization. By adding some fresh substrate to the recycled substrate, a new batch reaction was performed under the same condition, enabling the continual production of (*S*)-naproxen (Scheme 2). The results were listed in Table 1. After ten sequential recycling runs, a steady conversion of 39% and ee_p of 97% was achieved in

Table 1 2-L scale sequential runs of repeated batch reaction for producing (*S*)-naproxen with recycling of (*R*)-substrate.

Recycle No.	Fresh NME (g)	Conv. (%)[a]	ee_p (%)	(*S*)-NAP (g)[b]	Batch yield (%)[c]	Recycled NME (g)
1	80.0	33.9	96.2	24.0	76.0	46.5
2	33.5	39.4	90.8	25.8	85.7	48.1
3	31.9	33.8	87.2	23.7	70.5	44.3
4	35.7	32.2	86.1	21.3	85.2	53.5
5	26.5	39.4	94.5	27.0	87.5	47.2
6	32.8	35.9	94.4	27.0	91.7	48.8
7	31.2	36.6	97.1	27.4	87.3	46.7
8	33.3	38.4	96.9	27.0	87.1	47.1
9	32.9	39.2	96.7	27.7	86.7	46.1
10	33.9	39.1	97.0	27.6	88.2	n.d.

[a] Conv. (%)=[(*R*)-NAP+(*S*)-NAP]/[(*R*)-NME+(*S*)-NME + (*R*)-NAP+(*S*)-NAP]*100, which was calculated based on their HPLC peaks area.

[b] Dry weight of the (*S*)-naproxen isolated.

[c] Batch yield (%)=([(*S*)-NAP]/230.26)/(80 - recycled NME)*244.36*100.

4 h, giving a productivity of 3.9 g/L/h. The average yield could reach almost 85% after ten batches, giving a stable batch yield of 88%, which was proved to be a very efficient production process of resolution reaction, as compared with the single batch reaction yield of less than 50% without substrate recycling.

Discussion

Currently, more than 50% of the organic pharmaceuticals are chiral [24]. However, most of the chiral drugs are obtained mainly by conventional chemical synthesis, which is considered to be environment unfriendly. Biocatalysis as an accessible alternative due to its high efficiency, specificity, and eco-friendliness has been attached more and more significance in the last decade. BsE-NP01 from *B. subtilis* was successfully cloned and expressed in *E. coli*, exhibiting excellent enantioselectivity towards (*S*)-naproxen methyl ester. With no big difference from carboxylester NP in amino acid or biochemical properties, BsE-NP01 was over-expressed as a soluble intracellular protein with a molecular weight of about 32 kDa and an optimum pH of 8.5 and temperature of 50 °C on the hydrolysis of naproxen methyl ester [14]. Both the enzymes were inhibited by (*S*)-naproxen at a low concentration, which was considered a burning question for (*S*)-naproxen production and probably could be solved only through genetic engineering tools.

Tween-80 was a widely used surfactant or additive in the hydrolase-catalyzed reactions, and often displayed a positive effect both on activity and enatioselectivity [25, 26]. In the case of the NME resolution by carboxylesterase NP, 1% (*w*/*v*) Tween-80 was also added for the same purpose [6, 14]. However, it can cause severe non-immunologic anaphylactoid reactions at a slight concentration inhaled by human [27]. Therefore, we developed a mechanical method to overcome this bottleneck. A high-speed blender (juice extractor) was employed to minimize the particle size of the insoluble substrate, which probably played an equal role of dispersing the substrate as Tween-80. As high as 97% *ee* of the (*S*)-naproxen was obtained in 39% conversion without Tween-80 or any other surfactants, and the enantiopurity could then be raised to 99% *ee* by subsequent recrystallization, which greatly simplified the downstream process of the product separation and purification.

Furthermore, isolation of the leftover ester involved simple filtration once the kinetic resolution reaction was complete, and efficient racemization allowed for the development of a repeated batch process. For substrate recycling, Steenkamp and Brady [14] used l,8-diazabicyclo(5,4,0)undec-7-ene (DBU) for racemization of NME, which was hardly removed and might have adverse effects on the resolution reaction or quality of the product. In our research, we employed sodium methoxide [16] instead of DBU, and the direct reuse of the racemized NME was feasible after vacuum evaporation of methanol, simplifying the repeated batch process without any adverse effect. As a consequence, in the esterase-catalyzed 2-L repeated batch process described herein, an average of approximately 85% yield and a steady value of 88% batch yield were achieved, implying that a larger scale and continual production of (*S*)-naproxen is of great potential.

In conclusion, we have developed an efficient process for kinetic resolution of naproxen methyl ester on a hundred-gram scale catalyzed by recombinant *B. subtilis* carboxylesterase BsE-NP01. The optimized batch operation hardly involves any organic solvents or surfactants, and the easy recycling of the undesired enantiomer extends this convenient procedure to manufacturing of pharmaceutically relevant (*S*)-naproxen, making the so-called white biotechnology more feasibly implemented in industrial production in the future.

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