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Enantioselective properties of extracellular lipase from *Serratia marcescens* ES-2 for kinetic resolution of (*S*)-flurbiprofen

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Abstract

Serratia marcescens ES-2 excreting a catalytic lipase for the enantioselective hydrolysis of (*R*,*S*)-flurbiprofen ethyl ester to (*S*)-flurbiprofen was newly screened, and the excreted lipase was purified by a three-step procedure. The molecular mass was measured to be 64.9 kDa, a relatively high value among known bacterial lipases, and the optimal temperature was determined to be 37 °C. The enantioselectivity was significantly enhanced by the inorganic ion Ca^{2+} and surfactant Triton X-207, yet seriously inhibited by EDTA and SDS. The kinetic resolution of 50 mM (*R*,*S*)-flurbiprofen ethyl ester to optically pure (*S*)-flurbiprofen was carried out using 100 U lipase/mmol (*R*,*S*)-flurbiprofen ethyl ester in an aqueous phase reaction system supplemented with 10 mM Ca^{2+} and 1% Triton X-207. A high conversion, corresponding to an enantiomeric excess of 98.5% and conversion yield of 45.1%, was achieved after 24 h, along with a very high *E*-value of 332. An enantioselective lipase from *S. marcescens* ES-2 seems to be practically applicable for the production of optically active (*S*)-flurbiprofen.
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Keywords: Lipase; *Serratia marcescens* ES-2; (*R*,*S*)-Flurbiprofen ethyl ester; (*S*)-Flurbiprofen; Kinetic resolution

1. Introduction

The profen family, including flurbiprofen {(*R*,*S*)-[2-(2-fluoro-4-phenyl) phenyl] propionic acid}, is one of the major classes of commercially available non-steroidal anti-inflammatory drugs. The family exists in two stereo-isomeric forms, yet only an (*S*)-enantiomer exhibits pharmacological activity through the inhibition of the cyclooxygenase system, while an (*R*)-enantiomer is not only biologically inactive but also shows negative effects, such as gastrointestinal toxicity and chiral inversion [1,2]. Nonetheless, the majority of these compounds are still marketed as a racemic mixture, therefore, the kinetic resolution of an optically pure (*S*)-enantiomer form is urgently required [3,4].

The kinetic resolution of (*S*)- or (*R*)-flurbiprofen has mainly been conducted by chemical methods, such as the asymmetric synthesis of an (*S*)-enantiomer or the use of chiral chromatography and stereoselective crystallization [5–7], however, these methods entail expensive manufacturing processes and are complex for industrial application. Therefore, the use of biochemical processes for the kinetic resolution of an optically active (*S*)-enantiomer from its corresponding racemic molecule using biocatalysts from a microbial origin has recently drawn much attention [8,9].

Lipases (triacylglycerol acylhydrolase, E.C. 3.1.1.3) are most commonly used as the biocatalyst for the enzymatic resolution of an (*S*)-enantiomer [10,11]. For example, a lipase from *Candida rugosa* was found to have a relatively high enantioselective activity toward the (*S*)-flurbiprofen ester compared to other known lipases or esterases, however, the level of the enantiomeric excess was unsatisfactory [12,13]. An immobilized lipase from *Candida antarctica* Novozyme 435 has also been used for the catalytic resolution of (*R*,*S*)-flurbiprofen into (*S*)-flurbiprofen through an esterification reaction in an organic medium [14,15]. Plus, the esterase PF1-K from the newly screened *Pseudomonas* sp. KCTC 10122BP was found to effectively hydrolyze (*S*)-flurbiprofen ethyl ester into optically pure (*S*)-flurbiprofen [13].

In previous work [16–19], the current authors developed a lipase-catalyzed dispersed aqueous phase reaction system, in which cyclodextrin (CD) or its derivative was added as a dispenser or chiral selector to increase the solubility of an immiscible (*R*,*S*)-profen and the optical resolution of a lipase. An

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enantiomeric excess of 98.0% and conversion yield of 48.0% were achieved when the above system contained chiral succinyl β-CD for the kinetic resolution of (*S*)-flurbiprofen using a lipase from *C. rugosa* [16,17]. Nonetheless, despite the significantly enhanced enantiomeric excess and conversion yield, tailor-made biocatalysts suitable for the kinetic resolution of (*R*,*S*)-profen ethyl ester into (*S*)-profen still need to be developed [18,19].

Accordingly, this work isolated a strain excreting a novel enantioselective lipase suitable for the enzymatic resolution of (*R*,*S*)-flurbiprofen ethyl ester into (*S*)-flurbiprofen. The excreted lipase from the newly isolate strain *Serratia marcescens* ES-2 was purified by a three-step procedure, then the catalytic properties of the purified lipase were compared with other known lipases. The kinetic resolution of (*R*,*S*)-flurbiprofen ethyl ester into (*S*)-flurbiprofen was carried out using the enantioselective lipase from *S. marcescens* ES-2 to evaluate its potential for industrial application.

2. Materials and methods

2.1. Isolation of strain producing enantioselective lipase

Strains excreting an enantioselective lipase were primarily selected on a solid LB medium containing Rhodamine B that can be used for monitoring lipolytic activity by the irradiation of UV light at 350 nm. The selected strains were re-cultivated in 10 ml of an LB medium supplemented with 10 mM flurbiprofen ethyl ester at 30 °C for 3 days, then the (*R*)-, (*S*)-, and (*R*,*S*)-flurbiprofen ethyl ester produced in the culture broth were analyzed by HPLC, resulting in the selection of strain ES-2. The isolate was classified according to *Bergey's Manual of Systematic Bacteriology* and the partial 16S rDNA sequence.

2.2. Cultivation of S. marcescens ES-2 for lipase production

A newly isolated strain *S. marcescens* ES-2 was cultivated for the excretive production of lipase in a liquid medium composed of 1.5% tryptone, 0.5% polypeptone, 0.5% NaCl, 0.1% K_2HPO_4, and 0.1% (w/v) olive oil at 30 °C, pH 7.5, for 3 days.

2.3. Measurements of lipase activity

The lipase activity was measured by the copper soap colorimetric method [20] using 100 mM tricaprin emulsified by sonication in a 50 mM Tris–HCl buffer (pH 7.5) containing 0.3% (w/v) gum arabic at 37 °C, 200 rpm for 30 min. One unit of lipase was defined as the amount of lipase liberating 1 μmol capric acid/min.

2.4. Purification of enantioselective lipase from S. marcescens ES-2

The culture broth from *S. marcescens* ES-2 was concentrated using an Amicon PM-50 membrane, loaded on a hydrophobic Phenyl-Sepharose CL-4B chromatographic column (10 cm × 2.5 cm) equilibrated with 20 mM Tris–HCl (pH 7.5), then the absorbed proteins were eluted with 50 ml of 50% (v/v) ethylene glycol dissolved in 20 mM Tris–HCl (pH 7.5) and dialyzed against 20 mM Tris–HCl (pH 7.5) to remove the ethylene glycol. Next, the eluants were loaded on a DEAE Sephadex A50 column equilibrated with a 20 mM Tris–HCl buffer (pH 7.5), then washed with the same buffer and eluted with a linear gradient of a 0–1.0 M NaCl solution to collect the lipase activity fractions.

2.5. Determination of molecular mass

The molecular mass of the purified lipase was determined by SDS-PAGE, according to the method of Laemmli by staining with Coomassie brilliant blue R-250 [21]. The protein concentration was assayed using the method of Bradford with bovine serum albumin as the standard [22].

2.6. Effect of temperature and chemicals on enantioselective hydrolysis of (R,S)-flurbiprofen ethyl ester

The (*R*)- and (*S*)-flurbiprofen produced from the (*R*,*S*)-flurbiprofen ethyl ester were measured after the hydrolytic reaction of the (*R*,*S*)-flurbiprofen ethyl ester with 100 U lipase/mmol (*R*,*S*)-flurbiprofen ethyl ester in a 50 mM Tris–HCl buffer (pH 7.5) at 37 °C after 24 h to determine the effect of temperature, metal ions, inhibitors, and neutral surfactants.

2.7. Enantioselective hydrolysis of (R,S)-flurbiprofen ethyl ester in aqueous phase reaction system for production of (S)-flurbiprofen

The substrate (*R*,*S*)-flurbiprofen ethyl ester was synthesized from a commercially available (*R*,*S*)-flurbiprofen (Kolon Co., Seoul, Korea) based on an esterification reaction in the authors' laboratory [17]. A total of 50 mM of the (*R*,*S*)-flurbiprofen ethyl ester dissolved in ethanol was suspended in a 50 mM Tris–HCl buffer (pH 7.5) containing 10 mM Ca^{2+} and 1% of Triton X-207, then 100 U of lipase from *S. marcescens* ES-2/mmol (*R*,*S*)-flurbiprofen ethyl ester. The hydrolysis reaction was carried out at 37 °C, 200 rpm for 24 h, and the resulting (*R*)- and (*S*)-flurbiprofen hydrolyzed from the (*R*,*S*)-flurbiprofen ethyl ester were monitored by HPLC.

2.8. Analytical methods

The (*R*,*S*)-flurbiprofen ethyl ester, (*S*)-, and (*R*)-flurbiprofen were determined by an HPLC (Gilson Inc., France) equipped with a UV (250 nm) detector using an RS-Tech TBB chiral column (0.46 cm × 25 cm) under the conditions of a mobile phase (*n*-hexane/methyl *tert*-butyl ether/acetate: 6/4/0.01) and flow rate of 2.0 ml/min. The enantiomeric excess (ee_p), conversion yield (c), and enantiomeric ratio of (E) to (*S*)-flurbiprofen were calculated as follows:

Enantiomeric excess, ee_p

$$= \frac{[(S)\text{-flurbiprofen}] - [(R)\text{-flurbiprofen}]}{[(S)\text{-flurbiprofen}] + [(R)\text{-flurbiprofen}]}$$

$$\text{Conversion yield, } c = \frac{[(S)\text{-flurbiprofen}] + [(R)\text{-flurbiprofen}]}{[\text{initial } (R, S)\text{-flurbiprofen ethyl ester}]}$$

$$\text{Enantiomeric ratio, } E = \frac{\ln[1 - c(1 + ee_p)]}{\ln[1 - c(1 - ee_p)]}$$

3. Results and discussion

3.1. Isolation and identification of microorganism producing enantioselective lipase for (S)-flurbiprofen

Strain ES-2, excreting an enantioselective lipase, was newly screened from waste water in a fishpond. The morphological, physiological, and biochemical characteristics of the new strain were investigated according to *Bergey's Manual of Systematic Bacteriology*, and found to be Gram-negative, rod-shaped, motile, facultative anaerobic, catalase positive, and oxidase negative, plus the strain exhibited an acid-forming capability from glucose and sucrose, yet not from lactose (data not shown). Taxonomically, the isolated ES-2 was classified within the genus *Serratia* belonging to the Enterobacteriaceae family.

The 16S rDNA sequence of the isolate ES-2 was determined and registered in GenBank (accession number, DQ357510). The phylogenic tree for the partial 16S rDNA sequence from the new isolate was constructed for comparison with 10 other species showing the high homology. As shown in Fig. 1, it showed a similarity of up to 98.4% with *S. marcescens*, and also revealed a strict enantioselectivity to (*S*)-flurbiprofen ethyl ester. As such, ES-2 was classified as *S. marcescens* ES-2 (KACC 91216).

3.2. Purification and molecular weight of lipase from S. marcescens ES-2

The lipase excreted from the newly isolated strain *S. marcescens* ES-2 was purified by a three-step procedure: ultrafiltration, hydrophobic Phenyl-Sepharose CL-4B, and DEAE Sephadex A50. As shown in Table 1, a 56-fold purification was ultimately achieved, and the final specific activity of the purified lipase was 117.6 U/mg proteins.

Table 1
Purification of enantioselective lipase excreted from *S. marcescens* ES-2

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Supernatant	190	399	2	100	1
Ultra-filtration	39	345	9	86	4
Phenyl-Sepharose CL-4B	11	132	12	33	6
DEAE Sephadex A50	0.5	59	118	15	56

SDS-PAGE also revealed a single band with a molecular weight of 64.9 kDa, as shown in Fig. 2, which is a relatively high value among other known lipases from bacteria. Yet, *S. marcescens* strains are known to produce lipases that have relatively large molecular masses, ranging from 52 to 65 kDa, and they excreted mainly the enzyme belong to the I.3 lipase family [23] such as the extracellular lipases from *S. marcescens* Sr41 8000 and SM6.

As shown in Table 2, the general physicochemical properties of lipase from *S. marcescens* ES-2 were compared to those

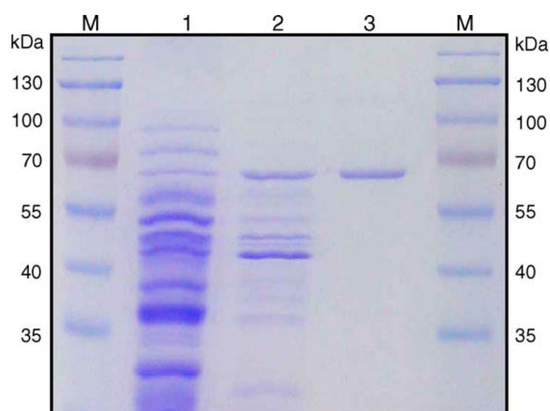

Fig. 2. SDS-PAGE of purified lipase from *S. marcescens* ES-2. M: standard protein marker; lane 1: culture supernatant; lane 2: Phenyl-Sepharose CL-4B; lane 3: purified lipase.

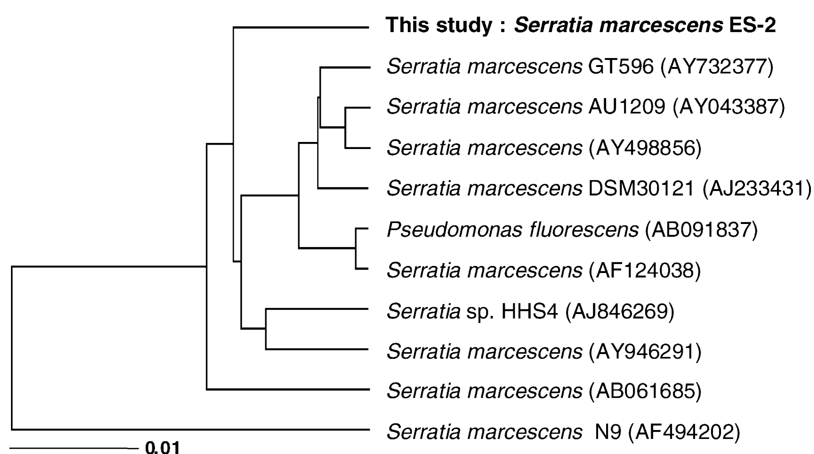

Fig. 1. Phylogenetic tree of 16S rDNA from new isolate ES-2. The phylogenetic analysis was performed using the neighbor-joining method from www.genebee.msu.su. The scale bar indicates 0.01 substitutions per site, while the numbers at the nodes are the percentage of bootstrap confidence values based on 1000 replicates.

Table 2
Comparison of physicochemical properties of lipases from *S. marcescens* ES-2 with those from other known *S. marcescens* strains

Lipase	Molecular mass	Optimum pH	Optimum temperature (°C)	Substrate specificity	Kinetic resolution	Reference
S. marcescens Sr41 8000[a]	64.9	8	45	C_4–C_8	MPGM	[24,25]
S. marcescens SM6	64.8	6–10	N.D.	N.D.	IPG, 2-phenyl-1-propanol	[26,27]
Psychotrophic *S. marcescens*	52	8	37	N.D.	N.D.	[28]
S. marcescens ES-2	64.9	7	37	C_8–C_{12}	Flurbiprofen	This work

[a] The primary structure of *S. marcescens* Sr41 8000 is given in a database with an accession number PIR No. JS0763.

of lipases other *S. marcescens* strains. The optimum pH of the purified lipase from the *S. marcescens* ES-2 was 7.0, which is the lowest among lipases from other *S. marcescens* strains. The optimum temperature was 37 °C, similar to a lipase from psychotropic *S. marcescens*, however, lower than that from *S. marcescens* Sr41 8000 and SM6 showing a similar molecular mass.

The purified lipase from *S. marcescens* ES-2 hydrolyzed preferably the medium-chain length triacylglycerides (C_8–C_{12}), while the lipase from *S. marcescens* Sr41 8000 toward a relatively short-chain length triacylglycerides (C_4–C_8). The lipase from *S. marcescens* ES-2 seems to be the first example reporting the kinetic resolution of (*S*)-flurbiprofen among those from *S. marcescens* strains, except for the lipase from *S. marcescens* SM6 that had been used for the kinetic resolution of other racemic mixtures such as racemic isopropylideneglycerol acetate (IPG) and 2-phenyl-1-propanol, however, the enantiomeric excess was lower than around 20–35% [27].

3.3. Effect of temperature on kinetic resolution of (R,S)-flurbiprofen ethyl ester

The effect of temperature is shown in Fig. 3, where the conversion yield and enantiomeric excess are compared at different reaction temperatures. A relatively high conversion yield occurred between 30 and 50 °C, yet decreased rapidly at temperatures higher than 50 °C. A high enantiomeric excess was also maintained at temperatures between 20 and 42 °C, yet decreased significantly at higher temperatures. The optimal temperature for the kinetic resolution of (*R*,*S*)-flurbiprofen ethyl ester to (*S*)-flurbiprofen was 37 °C. The selectivity toward optically inactive (*R*)-form enantiomer seems to increase at a high temperature over than 42 °C due to conformational change of enzyme as reported by Yoon et al. [29] who investigated the resolution of 1,3-dioxolane derivatives using a lipase from *Acinetobacter junii* SY-01.

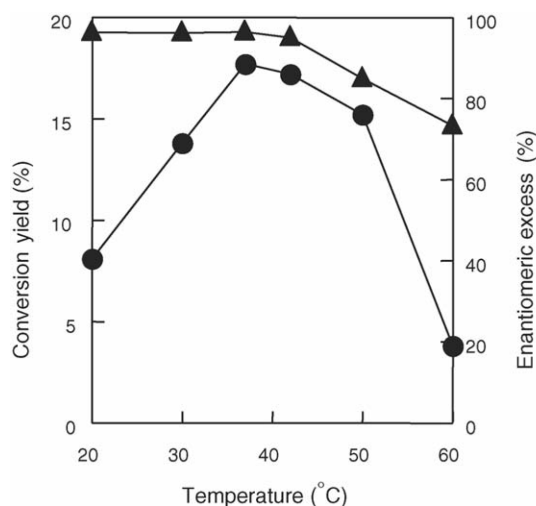

Fig. 3. Effect of temperature on kinetic resolution of (*R*,*S*)-flurbiprofen ethyl ester into (*S*)-flurbiprofen. Reaction was carried out at 100 U lipase/mmol (*R*,*S*)-flurbiprofen ethyl ester, 5 mM (*R*,*S*)-flurbiprofen ethyl ester, 50 mM Tris–HCl buffer (pH 7.5), 37 °C, and 200 rpm for 24 h. Conversion yield (●) and enantiomeric excess (▲).

3.4. Effect of metal ions, reagents, and surfactants on enantioselective hydrolysis of (R,S)-flurbiprofen ethyl ester

The enzymatic resolution of the chiral compounds ester hydrolase family is known to be partially or significantly affected by the presence of a cofactor divalent metal ion [30,31]. As shown in Table 3, most divalent metal ions had a negative effect, except for Ca^{2+} and Mg^{2+}, in particular, the Ca^{2+} ion enhanced the conversion yield up to 4.6-fold at 10 mM. The lipases from *S. marcescens* are already known to contain a Ca^{2+} binding site

Table 3
Effects of metal ions and reagents on the enantioselective hydrolysis of (*R*,*S*)-flurbiprofen ethyl ester into (*S*)-flurbiprofen

Reagents	Concentration (mM)	Conversion (%)	ee_p (%)	*E*
None		9.2	96.4	60
$CaCl_2$	1	23.5	96.7	80
	5	31.1	96.6	89
	10	42.6	96.8	133
	20	40.2	96.9	125
$MgCl_2$	1	8.7	97.2	77
$MnCl_2$	1	10.0	88.2	18
$CoCl_2$	1	12.8	78.8	9
$CuCl_2$	1	1.7	43.9	3
$ZnCl_2$	1	5.5	37.2	2
EDTA	10	1.9	62.9	4
SDS	10	2.5	90.5	21
DTT	10	12.8	90.5	23
β-Mercaptoethanol	10	10.1	92.7	29
PMSF	10	9.9	93.5	33

The kinetic resolution was carried out at 100 U lipase/mmol (*R*,*S*)-flurbiprofen ethyl ester, 10 mM (*R*,*S*)-flurbiprofen ethyl ester, 50 mM Tris–HCl buffer (pH 7.5), 37 °C, and 200 rpm for 24 h.

Table 4
Effects of surfactants on the enantioselective hydrolysis of (*R*,*S*)-flurbiprofen ethyl ester into (*S*)-flurbiprofen

Surfactants	Conversion (%)	ee_p (%)	*E*
None	9.0	96.3	58
Tween 20	5.6	92.6	27
Tween 40	8.7	93.9	35
Tween 60	14.5	96.3	62
Tween 80	9.1	95.3	46
Triton X-45	4.4	89.2	18
Triton X-100	5.1	92.7	28
Triton X-102	11.4	95.1	45
Triton X-165	21.1	97.4	98
Triton X-207	30.0	98.0	150
Triton X-305	28.6	98.0	146

The kinetic resolution was carried out at 100 U lipase/mmol (*R*,*S*)-flurbiprofen ethyl ester, 10 mM (*R*,*S*)-flurbiprofen ethyl ester, 1% surfactants, 50 mM Tris–HCl buffer (pH 7.5), 37 °C, and 200 rpm for 24 h.

near the active site that affect the stability and activity of the lipases, plus the addition of the Ca^{2+} ion drastically enhances the lipase activities [25,32].

The chelating agent EDTA reduced not only the conversion yield but also the enantiomeric excess, suggesting that the purified enantioselective lipase is a metaloenzyme. However, the catalytic activity of the lipase from *S. marcescens* ES-2 was not seriously influenced by either PMSF, known as a specific inhibitor of serine, or β-mercaptoethanol and DTT, known as reducing agents.

Surfactants have been widely applied to lipase-catalyzed reactions of insoluble substrates to increase the lipid–water interfacial area, which in turn enhances the enantioselectivity as well as the reaction rates of the kinetic resolution [33]. In addition, surfactants are also involved in hydrogen bonds and hydrophobic action between lipases and surfactants, which can change the enantioselectivity of the lipase toward (*S*)-profen ester [34,35].

As shown in Table 4, the surfactant Triton Xs series showed a better result compared to the Tweens series. In particular, surfactant Triton X-207 increased the enantiomeric excess value from 96.3 to 98.0% as well as the conversion yield from 9.0 to 30.0%, thereby enhancing the *E*-value up to 2.6-fold. The positive effect of Triton Xs on enhancing both the conversion yield and the enantiomeric excess was also reported by Kim et al. [34] when using an esterase from *Pseudomonas* sp. S34 to study the chiral resolution of (*R*,*S*)-ketoprofen ethyl ester to (*S*)-ketoprofen.

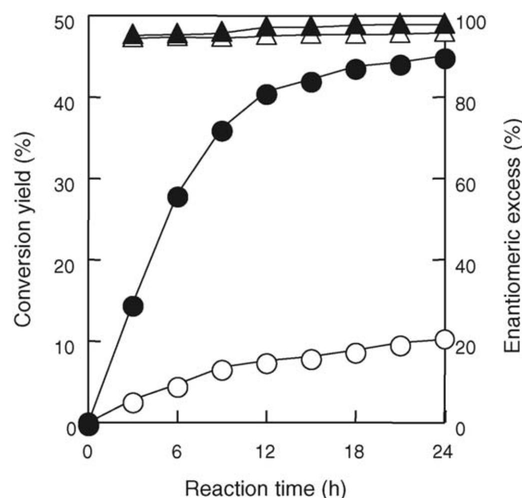

Fig. 4. Comparison of progress of resolution reactions in aqueous phase reaction system and without 10 mM of Ca^{2+} and 1% of Triton X-207. The kinetic reaction was carried out at 100 U lipase/mmol (*R*,*S*)-flurbiprofen ethyl ester, 50 mM (*R*,*S*)-flurbiprofen ethyl ester, Tris–HCl buffer (pH 7.5), 37 °C, and 200 rpm for 24 h. Conversion yield (○ and ●) and enantiomeric excess (△ and ▲) without and with 10 mM of Ca^{2+} and 1% of Triton X-207, respectively.

3.5. Kinetic resolution of (R,S)-flurbiprofen ethyl ester to (S)-flurbiprofen in aqueous phase reaction system supplemented with Ca^{2+} and Triton X-207

Fig. 4 compares the changes in the conversion yield and enantiomeric excess during the kinetic resolution of 50 mM (*R*,*S*)-flurbiprofen ethyl ester to (*S*)-flurbiprofen using the enantioselective lipase from *S. marcescens* ES-2 in an aqueous phase reaction system before and after supplementation with the Ca^{2+} ion and surfactant Triton X-207. The conversion yield increased from 10.2 to 45.1%, while the enantiomeric excess increased from 96.5 to 98.5% after supplementation with Ca^{2+} and Triton X-207. The final *E*-value considering both the conversion yield and enantiomeric excess concomitantly was calculated to be 332, a very high value among known lipases for the kinetic resolution of a chiral drug.

3.6. Comparison of enantioselective property of lipase from S. marcescens ES-2 and other lipases

The efficiency of the enantioselectivity of the lipase from *S. marcescens* ES-2 was compared with that of other known

Table 5
Comparison of enantioselective properties of lipase from *S. marcescens* ES-2 and other lipases for kinetic resolution of (*R*,*S*)-flurbiprofen ethyl ester into (*S*)-flurbiprofen

Lipase	Amount of lipase (U)	Reaction time (h)	Conversion (%)	ee_p (%)	*E*	Reference
Novozyme 435	100	24	11.5	14.6	1	[14]
Candida rugosa	100	24	7.6	64.2	5	
Acinetobater sp. ES-1	100	24	13.6	82.4	12	[18]
Candida rugosa + Suβ-CD[a] (100 mM)	2000	96	48.4	98.1	345	[17]
Serratia marcescens ES-2	100	24	45.1	98.5	332	This work

The kinetic resolution was carried out with 50 mM (*R*,*S*)-flurbiprofen ethyl ester and different amounts of lipase/mmol (*R*,*S*)-flurbiprofen ethyl ester in 50 mM Tris–HCl buffer (pH 7.5), 37 °C, and 200 rpm for 24 h.
[a] Suβ-CD: succinyl β-cyclodextrin.

lipases used for the kinetic resolution of chiral drugs, such as a lipase from *Acinetobacter* sp. ES-1 [18] that is isolated for the kinetic resolution of (*R*,*S*)-ketoprofen ethyl ester to (*S*)-ketoprofen, a lipase B from *C. antarctica* Novozyme 435 [14,15], and a lipase from *C. rugosa* (Sigma Co., St. Louis, USA) [16,17,33]. The enantioselective lipase excreted from *S. marcescens* ES-2 exhibited the highest conversion yield and enantiomeric excess compared to other three lipases as presented in Table 5. Recently, Chung and coworkers [13] has also reported an recombinant esterase from *Pseudomonas* sp. KCTC 10122BP showing with an enantiomeric excess of 99% during the enantioselective hydrolysis of 600 mM (*R*,*S*)-flurbiprofen ethyl ester to (*S*)-flurbiprofen.

In our previous work, the enzymatic resolution of (*R*,*S*)-flurbiprofen ethyl ester to (*S*)-flurbiprofen in a dispersed aqueous phase reaction system supplementing a chiral succinyl β-CD as the dispenser and chiral selector was carried out using a lipase from *C. rugosa* [17]. A high enantiomeric excess of 98.0% and conversion yield of 48.0% was achieved.

The enantioselective lipase excreted from *S. marcescens* ES-2 seems to be practically applicable for the production of optically pure (*S*)-flurbiprofen not only in the conventional aqueous phase reaction system but also in the dispersed aqueous phase reaction system containing succinyl β-CD even with a lower amount of the lipase and short reaction time.

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