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Molecular Cloning and Functional Expression of *esf* Gene Encoding Enantioselective Lipase from *Serratia marcescens* ES-2 for Kinetic Resolution of Optically Active (*S*)-Flurbiprofen

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Abstract An enantioselective lipase gene (*esf*) for the kinetic resolution of optically active (*S*)-flurbiprofen was cloned from the new strain *Serratia marcescens* ES-2. The *esf* gene was composed of a 1,845-bp open reading frame encoding 614 amino acid residues with a calculated molecular mass of 64,978 Da. The lipase expressed in *E. coli* was purified by a three-step procedure, and it showed preferential substrate specificity toward the medium-chain-length fatty acids. The *esf* gene encoding the enantioselective lipase was reintroduced into the parent strain *S. marcescens* ES-2 for secretory overexpression. The transformant *S. marcescens* BESF secreted up to 217 kU/ml of the enantioselective lipase, about 54-fold more than the parent strain, after supplementing 3.0% Triton X-207. The kinetic resolution of (*S*)-flurbiprofen was carried out even at an extremely high (*R,S*)-flurbiprofen ethyl ester [(*R,S*)-FEE] concentration of 500 mM, 130 kU of the *S. marcescens* ES-2 lipase per mmol of (*R,S*)-FEE, and 1,000 mM of succinyl β-cyclodextrin as the dispenser at 37°C for 12 h, achieving the high enantiomeric excess and conversion yield of 98% and 48%, respectively.

Key words: Enantioselective lipase, *esf* gene, *Serratia marcescens* ES-2, molecular cloning and expression, kinetic resolution, (*S*)-flurbiprofen

Because of its versatile biocatalytic action for the kinetic resolution of optically active (*S*)-enantiomer from a corresponding racemic mixture, the lipase [triacyl glycerol acylhydrolase (E.C. 3.1.1.3)] has been widely used for the kinetic resolution of the optically active pharmaceuticals [13, 19, 22, 23, 28]. Flurbiprofen {(*R,S*)-[2-(2-fluoro-4-phenyl) phenyl] propionic acid}, belonging to the profen family, is one of the typical examples of the commercially available nonsteroidal anti-inflammatory drugs (NSAIDs). Yet, only the (*S*)-form of flurbiprofen that inhibits the cyclooxygenase (COX) system has pharmacological activity, whereas the (*R*)-form enantiomer is biologically inactive and even has negative side effects, such as gastrointestinal toxicity and chiral inversion [7, 10].

The Gram-negative bacterium *Serratia marcescens* is one of the commonly known lipase producers, and its extracellular lipase is widely used for the stereoselective hydrolysis of chiral racemic substrates [24]. The enzymatic properties of *S. marcescens* lipase have already been characterized intensively; however, only two studies have dealt with the nucleotide sequence of the lipase gene [1, 17]. Specifically, the lipases from *S. marcescens* and *Pseudomonas fluorescens*, belonging to the subfamily I.3, lack the usual N-terminal signal sequence but contain a C-terminal motif [9, 21] that can be recognized by ABC exporters for the secretory production of the corresponding proteins [2, 6, 27].

In our previous work [5], *S. marcescens* ES-2 strain secreting a lipase suitable for the kinetic resolution of (*S*)-flurbiprofen was newly screened, and the secreted lipase showed excellent enantioselectivity for (*S*)-flurbiprofen, achieving an enantiomeric excess of 98.5% and conversion yield of 45.1%. In addition, a novel dispersed aqueous phase reaction system was developed, in which a succinyl β-CD (suβ-CD) was supplemented to increase the chiral selectivity and solubility of water-immiscible (*R,S*)-FEE [25]. In this reaction system, the high enantiomeric excess of 98.0% and conversion yield of 48.0% were achieved even at an extremely high (*R,S*)-FEE concentration, and even using a commercial lipase from *Candida rugosa* [26].

In the current work, a new *esf* gene encoding an enantioselective lipase was newly cloned from *S. marcescens*

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ES-2 strain, and the nucleotide and amino acid sequences were aligned with other lipases from various sources. The cloned *esf* gene was reintroduced into the parent strain *S. marcescens* ES-2 for secretory overexpression, and the transformant was cultivated using various surfactants as the carbon sources and inducers. The enantioselective lipase was applied as a new biocatalyst for the kinetic resolution of (*S*)-flurbiprofen in a dispersed aqueous phase reaction system containing suβ-CD.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Media

The strains and plasmids used in this study are listed in Table 1. The parent strain *S. marcescens* ES-2 (KACC 91216) was cultivated in a lipase production (LP) medium (pH 7.5) composed of 1.5% tryptone, 0.5% polypeptone, 0.5% NaCl, 0.1% K_2HPO_4, 0.05% $CaCl_2$, and 0.1% (w/v) olive oil at 30°C for 24 h. The recombinant *E. coli* EESF was cultivated in Luria-Bertani (LB) medium with ampicillin (50 μg/ml) and chloramphenicol (34 μg/ml) as the selection markers, and the transformant *S. marcescens* BESF in the LP medium with kanamycin (200 μg/ml).

Measurements of Lipase Activity

The lipase activity was determined by a spectrophotometric method using *p*-nitrophenyl (*p*-NP) caprate as the substrate. Ten μl of the enzyme solution was mixed with 480 μl of 50 mM Tris-HCl (pH 7.5) buffer, and then 10 μl of 10 mM *p*-NP-caprate dissolved in acetonitrile was added as the substrate. After incubation at 37°C for 10 min, the enzyme reaction was terminated by the addition of 500 μl of 1 M acetone, and the resulting *p*-nitrophenol measured at 405 nm. One unit of lipase activity was defined as the amount of enzyme liberating 1 μmol of *p*-nitrophenol per min.

Construction of Genomic Library and Screening of Enantioselective Lipase Gene

The isolated genomic DNA of *S. marcescens* ES-2 was mechanically sheared, and then blunt-ended by T4 DNA polymerase and T4 polynucleotide kinase consecutively (Stratagene, U.S.A.). Thereafter, the blunt-ended DNA fragments were ligated with HincII-digested pUC19 vector to construct a genomic library, and transformed into *E. coli* XL1-Blue. The constructed recombinants were incubated on a 1% tributyrin agar plate containing X-gal at 37°C for 24 h, and the positives forming clear halos selected as potential clones. The selected colonies were recultivated in LB medium for another 24 h, the sonicated cell lysates reacted with 10 mM (*R,S*)-FEE, and the produced (*S*)- and (*R*)-flurbiprofen analyzed using HPLC to select the highest enantioselective strain for (*S*)-flurbiprofen.

Nucleotide and Amino Acid Sequence Analysis

The whole DNA fragment cloned in the plasmid pUESF was sequenced based on the dideoxy nucleotide chain-termination method using an Automatic Sequencer ABI 377 (PE Applied Biosystems, U.S.A.). The deduced amino acid sequence of the lipase gene from *S. marcescens* ES-2 was then aligned with other lipases using the BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and Clustal W programs.

Subcloning and Expression of Enantioselective Lipase Gene

The lipase gene in pUESF was amplified by *pfu* DNA polymerase (Stratagene, USA) using the following set of primers: lipF (5'-GGCCAGGCGGCATAATTC-3') and lipR (5'-GGCCAACACCACCTGATCG-3'). The amplified PCR product was subcloned into the EcoRV site of pET20b(+) to construct pEESF, and then transformed into the expression host *E. coli* BL21(DE3). The recombinant *E. coli* EESF carrying the *esf* gene was cultivated in LB medium at 37°C until the A_{600} reached 0.5, and then IPTG

Table 1. Bacterial strains and plasmids used in this study.

Strain/plasmid	Relevant characteristics	Source/reference
Strains		
S. marcescens ES-2	Wild-type; lipase gene producer	[5]
S. marcescens BESF	*S. marcescens* ES-2/pBESF	This study
E. coli XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44* [F' *proAB lacI*^{q}ZΔ15 Tn*10*(Tet^r)	Novagen
E. coli BL21(DE3)	F$^-$ *ompT hsdSB* (r_B^- m_B^-) *gal dcm* (*lcIts857 ind1 Sam7 nin5 lacUV5-T7 gene1*)	Novagen
E. coli EESF	*E. coli* BL21(DE3)/pEESF	This study
Plasmids		
pUC19	ColE1 Amp^r *lacI* ϕ80d*lacZ*	Stratagene
pUESF	2.5-kb fragment inserted into HincII site of pUC19, Amp^r	This study
pET20b(+)	*E. coli* expression vector, Amp^r	Novagen
pEESF	*esf* gene cloned into EcoRV site of pET20b(+), Amp^r	This study
pBBRIMCS2	Broad-host-range shuttle vector, Kan^r	[15]
pBESF	*esf* gene cloned into *Eco*RV site of pBBRIMCS2, Kan^r	This study

was added up to 0.1 mM and the cultivation continued at 25°C for 8 h for overexpression and secretion of the lipase.

Purification of Enantioselective Lipase and Zymogram Analysis

The enantioselective lipase expressed in the recombinant *E. coli* EESF was purified using a three-step procedure: ultrafiltration, hydrophobic Phenyl-Sepharose CL-4B (Sigma Co., U.S.A.), and DEAE-Sephadex A50 column, as described previously [5]. The homogeneity and molecular mass were confirmed by SDS-PAGE gel staining with Coomassie brilliant blue R-250. The ester-hydrolyzing activity was assayed by zymographic analysis based on the formation of a clear band, after transferring the native gel containing the lipase onto a 0.5% agarose gel containing 1% Tween 80 and 0.001% $CaCl_2$.

pH, Temperature, and Chain Length Specificity of Purified Lipase

The optimal pH of the lipase was determined after incubation with 50 mM sodium acetate (pH 4 to 6), 50 mM potassium phosphate (pH 6 to 8), 50 mM Tris-HCl (pH 7 to 9), and 50 mM glycine-NaOH buffers (pH 8 to 11) at 30°C, and the pH stability measured after preincubation at 30°C for 30 min. The optimal temperature and thermal stability were measured within a range of 20–80°C in 50 mM Tris-HCl buffer (pH 7.5).

Two groups of substrates, triacylglycerols [including triacetin (C_2), tributyrin (C_4), tricaproin (C_6), tricaprylin (C_8), tricaprin (C_{10}), trilaurin (C_{12}), tripalmitin (C_{16}), and tristearin (C_{18})] and *p*-nitrophenyl esters (PNPEs) [including acetate (C_2), butyrate (C_4), caproate (C_6), caprylate (C_8), caprate (C_{10}), laurate (C_{12}), palmitate (C_{16}), and stearate (C_{18})], were used to determine the substrate specificities. The relative activities were determined by measuring the amount of fatty acids produced from 100 mM of the triacylglycerols emulsified with 0.5% (w/v) of gum arabic [5], and measuring the *p*-nitrophenol concentration liberated from 50 mM of the PNPEs.

Construction of Transformant *S. marcescens* for Overexpression of Enantioselective Lipase

The amplified 1.8-kb fragment of the *esf* gene in pEESF using *pfu* DNA polymerase was cloned into the EcoRV site of the broad host range shuttle vector pBBRIMCS2. The constructed plasmid pBESF was then transformed into the parent *S. marcescens* ES-2 by electroporation, and the transformant *S. marcescens* BESF amplifying the *esf* gene was cultivated in the LP medium using several Tweens and Triton Xs as the carbon sources and inducers at 30°C for 24 h.

Kinetic Resolution of (*S*)-Flurbiprofen in Dispersed Aqueous Phase Reaction System Containing Suβ-CD

The kinetic resolution in a dispersed aqueous phase reaction system containing suβ-CD was already carried out as in our previous work [25]. The (*R,S*)-FEE (50 mM) was dissolved in 50 mM Tris-HCl buffer (pH 7.5) supplemented with suβ-CD (100 mM), sonicated to form an inclusion complex, and then 130 kU of crude Esf lipase per mmol of (*R,S*)-FEE was added. The enantioselective hydrolysis was carried out at 37°C for 12 h with stirring (300 rpm). The reaction conditions [the amount of lipase, molar mixing ratio between immiscible (*R,S*)-FEE and suβ-CD, and concentration of the substrate (*R,S*)-FEE] were changed accordingly.

Analytical Methods

The (*R,S*)-FEE, (*S*)-flurbiprofen, and (*R*)-flurbiprofen were analyzed by HPLC (Gilson Inc., France): RS-Tech TBB chiral column (0.46×25 cm), UV (250 nm) spectrometer, (n-hexane/methyl *tert*-butyl ether/acetate: 6/4/0.01) as the mobile phase, and flow rate of 2.0 ml/min. The enantiomeric excess (ee) and conversion yield (C) to (*S*)-flurbiprofen were calculated as follows:

Enantiomeric excess (ee)
=[(*S*)-profen−(*R*)-profen]/[(*S*)-profen+(*R*)-profen]
Conversion yield (C)=[(*S*)-profen
+(*R*)-profen]/[initial (*R,S*)-profen ethyl ester]

Nucleotide Sequence Accession Number

The nucleotide sequence of the *esf* gene was deposited in the GenBank under the accession number DQ841349.

Results and Discussion

Cloning and Sequence Analysis of *esf* Gene Encoding Enantioselective Lipase from *S. marcescens* ES-2

A genomic library of *S. marcescens* ES-2 was constructed, and one active clone exhibiting the highest enantioselectivity for (*S*)-flurbiprofen was selected finally from 150 potential clones forming a clear-halo zone. The selected active clone harboring plasmid pUESF included a DNA fragment of approximately 2.5 kb as a single open reading frame. The new enantioselective *esf* gene for (*S*)-flurbiprofen (GenBank Accession No. DQ841349) was composed of 1,845-bp nucleotides encoding a protein of 614 amino acids with a predicted molecular mass of 65 kDa, a similar value estimated in our previous work that was carried out using a purified lipase from the parent strain *S. marcescens* ES-2 [5]. The active-site consensus sequence GX_1SX_2G, which is common in many bacterial lipases [12, 14], was also identified.

Comparison of Amino Acid Sequences and Identification of Extreme C-Terminal Motif

The amino acid sequence of the enantioselective Esf lipase from *S. marcescens* ES-2 was compared with other known lipases from various sources, and it showed 97%, 96%,

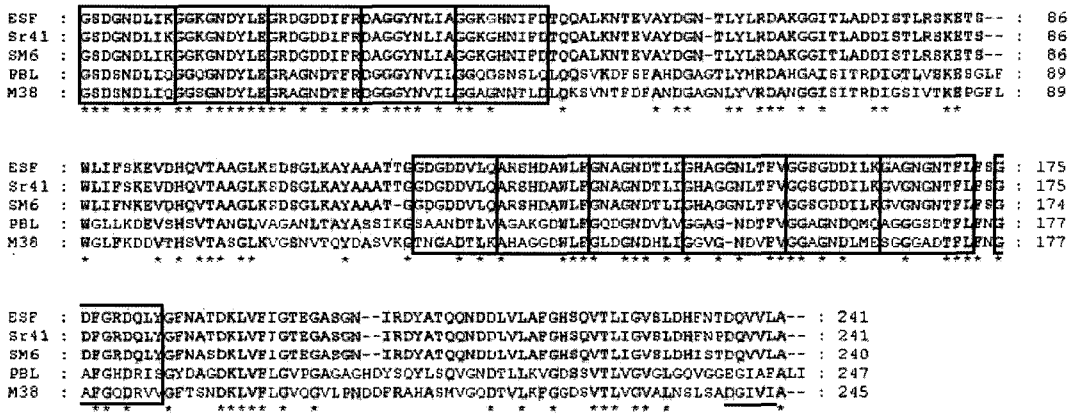

Fig. 1. Multiple alignment of C-terminal region in the enantioselective Esf lipase from *S. marcescens* ES-2 (ESF) with those of *S. marcescens* Sr41 8000 (Sr41), *S. marcescens* SM6 (SM6), *P. brassicacearum* (PBL), and *Pseudomonas* sp. MIS38 (M38).
Nine-residue GGXGXDXXX sequence motifs and C-terminal signal motif are boxed and underlined, respectively. Amino acid residues common to three aligned sequences are shaded. Identical residues are marked by an asterisk.

61%, and 61% identity with those of *S. marcescens* SM6 [17], *S. marcescens* Sr41 8000 [1], *Pseudomonas brassicacearum* [8], and *Pseudomonas* sp. MIS38 [3], respectively.

As shown in Fig. 1, the amino acid sequence for the C-terminal region of the *esf* gene was aligned with other lipases showing the higher homology. The Esf lipase from *S. marcescens* ES-2 did not possess an N-terminal signal sequence commonly required for the secretion of lipase, but it contained an extreme C-terminal motif consisting of a hydrophobic five-residue sequence that can be recognized by an ABC-transporter system correlated to a signal peptide-independent mechanism for the secretion of protein in Gram-negative bacteria [6, 27]. It indicates that the new strain *S. marcescens* ES-2 also secretes the enantioselective Esf lipase via an ABC exporter, as reported by Akatsuka *et al.* [2] who investigated the functional role of the ABC-transporter genes (*lipBCD*) from *S. marcescens* Sr41 8000.

S. marcescens ES-2 also contained a nine-residue GGXGXDXXX sequence motif in the upstream region of the C-terminal motif, known to form a parallel β-roll structure binding Ca^{2+} ions, enhancing the activity and stability of the lipase in *Pseudomonas* sp. MIS38 [3]. The kinetic resolution of (*S*)-flurbiprofen using the extracellular lipase from *S. marcescens* ES-2 was enhanced significantly, up to 4.6-fold, after adding 10 mM Ca^{2+} ions [5].

Expression of Enantioselective Lipase from *S. marcescens* ES-2 in Recombinant *E. coli*

The subcloned *esf* gene from *S. marcescens* ES-2 in pEESF was transformed into *E. coli* BL21(DE3). The constructed recombinant *E. coli* EESF was cultivated in LB medium at 25°C for 8 h after induction with 0.1 mM IPTG. The expressed lipase was mostly located in the intracellular fraction, and only a small portion of lipase was secreted into the culture broth (data not shown), even though the strong expression vector pET-20b(+) containing a secretory signal peptide was used for the secretory production. The secretion of the enantioselective Esf lipase seemed to be strongly controlled by a congruous ABC-transporter system in the parent strain *S. marcescens* ES-2 rather than the artificial secretory signal peptide in the expression vector pET-20b(+).

The expressed Esf lipase in recombinant *E. coli* EESF was purified by a three-step procedure: ultrafiltration, hydrophobic interaction with Phenyl Sepharose CL-4B, and anion exchange with DEAE-Sephadex A50, resulting in a 12.1-fold purification with an overall yield of 17% and the specific lipase activity of 58.4 kU/mg.

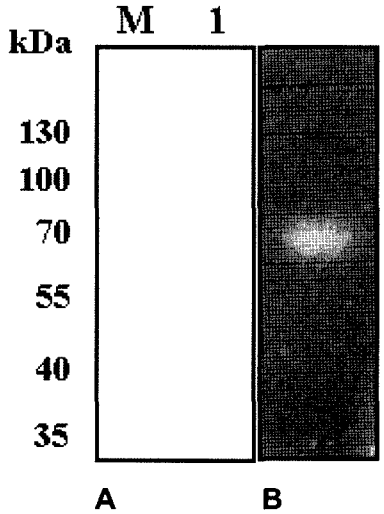

Fig. 2. SDS-PAGE (**A**) and zymogram (**B**) of purified Esf lipase from *S. marcescens* ES-2 expressed in recombinant *E. coli* EESF.
Lane M, standard protein marker; lane 1, purified Esf lipase.

Table 2. Chain length specificity of enantioselective Esf lipase of *S. marcescens* ES-2 expressed in recombinant *E. coli* EESF.

Substrates	Relative lipase activity (%)[a]	
	Triacylglycerides	PNPEs
Acetate (C_2)	0.0	1.0
Propionate (C_3)	0.0	3.8
Butyrate (C_4)	0.3	47.9
Caproate (C_6)	14.2	83.3
Caprylate (C_8)	54.7	100.0
Caprate (C_{10})	100.0	65.6
Laurate (C_{12})	66.3	24.1
Myristate (C_{14})	19.8	18.4
Palmitate (C_{16})	7.8	6.3
Stearate (C_{18})	0.4	0.0

[a]Relative activities were expressed as percentage comparable to maximum value (100%).

Fig. 2 illustrates the homogeneity of the purified Esf lipase from *S. marcescens* ES-2 based on SDS-PAGE (Fig. 2A), and a zymogram exhibiting the ester-hydrolyzing activity (Fig. 2B). The molecular mass of 69 kDa estimated by SDS-PAGE analysis was somewhat larger than the 65 kDa measured with the purified lipase of *S. marcescens* ES-2 [5], because of the attached His-tag sequence in the pET-20b(+) vector. The molecular mass of 65 kDa is a value similar to most of the lipases belonging to the I.3 family, including those from *P. fluorescens* and *S. marcescens*, yet a relatively large value compared with the lipases belonging to the I.1 and I.2 families [4].

pH, Temperature, and Substrate Specificity of Enantioselective Lipase from *S. marcescens* ES-2

The optimum pH of the purified Esf lipase was determined to be 7.0, a relatively low pH compared with other known lipases from *S. marcescens* in the range of 8.0–10.0, and it was stable within a pH range of 6.0–8.0. The optimum temperature was 37°C, and the thermal activity remained at around 50% after 1-h incubation at 50°C.

As shown in Table 2, the chain length specificity of the purified Esf lipase was investigated using triacylglycerides and PNPEs as substrates. The highest hydrolyzing activity was obtained against the medium-chain length fatty acids from tricaprlyate (C_8) to trilaurin (C_{12}), in contrast to the lipases from *S. marcescens* Sr41 8000 [20] as well as *Pseudomonas* sp. MIS38 [3] that preferred a relatively short-chain length from tributyrin (C_4) to tricaprylin (C_8). It also preferably hydrolyzed the medium-chain esters PNPEs from *p*-NP caproin (C_6) to *p*-NP caprylin (C_8).

Overexpression of Enantioselective Lipase in Transformant *S. marcescens* BESF Enforcing *esf* Gene

The enantioselective *esf* gene was reintroduced into the parent *S. marcescens* ES-2 to overcome the insufficient

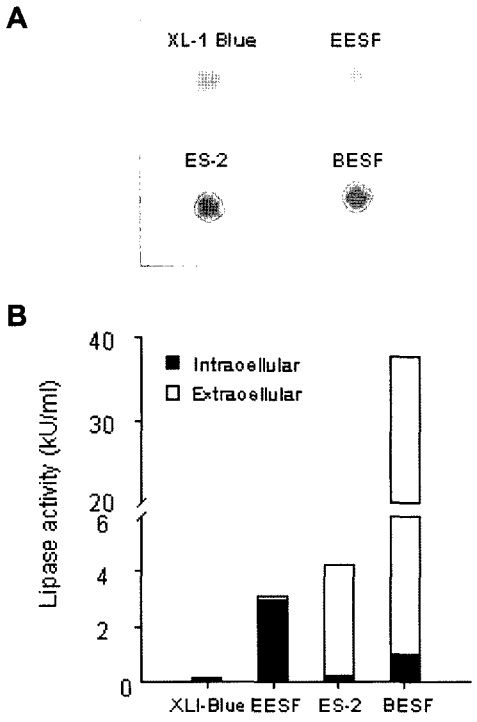

Fig. 3. Expression of enantioselective Esf lipase in parent *S. marcescens* ES-2, recombinant *E. coli* EESF, and transformant *S. marcescens* BESF.
Clear halo zones on tributyrin agar plates (**A**), and intra- and extracellular lipase activities of each strain cultivated in the LP medium for 24 h (**B**).

expression in the recombinant *E. coli* EESF, and then cultivated in the LP medium using olive oil as the carbon source for 24 h. Fig. 3 compares the clear halo zones formed on LB agar plate containing tributyrin detecting the lipase activity (Fig. 3A) and the intra- and extracellular lipases in *S. marcescens* ES-2, recombinant *E. coli* EESF, and transformant *S. marcescens* BESF (Fig. 3B).

As shown in Fig. 3B, the recombinant *E. coli* EESF produced the Esf lipase only at a 74% level compared with the parent strain, and nearly 95% of the expressed lipase was located in the intracellular fraction. The expression of the Esf lipase was increased significantly after enforcing the *esf* gene, increasing about 8.9-fold from 4,222 U/ml to 37,681 U/ml in the transformant *S. marcescens* BESF. In addition 97% of the expressed Esf lipase in the transformant was secreted extracellularly, and only a small portion of lipase remained inside the transformant.

The transformant *S. marcescens* BESF was also cultivated in the LP medium after substituting olive oil with the surfactant Tween and Triton Xs series known as the carbon sources and inducers. As shown in Table 3, the most promising result was obtained from Triton X-207 among the surfactant series, contrasting to the results by other investigators that showed the preference to Tween 80 during the cultivation of *S. marcescens* [11, 18]. The

Table 3. Effect of surfactants on secretion of enantioselective Esf lipase in transformant *S. marcescens* BESF overexpressing the *esf* gene.

Surfactant (Conc., %)		Cell growth[a] (A_{600})	Relative activity (%)
None	(0.0)	5.64	28
Tween 20	(0.5)	5.24	102
Tween 40	(0.5)	5.88	29
Tween 60	(0.5)	6.58	16
Tween 80	(0.5)	7.11	145
Triton X-45	(0.5)	5.26	40
Triton X-100	(0.5)	4.74	112
Triton X-114	(0.5)	3.78	95
Triton X-207	(0.5)	6.02	222
Triton X-207	(1.0)	6.62	389
Triton X-207	(2.0)	6.86	500
Triton X-207	(3.0)	7.03	588
Triton X-207	(4.0)	6.92	469
Olive oil (Control)	(0.5)	6.38	100

[a]Transformant *S. marcescens* BESF was cultivated in LP medium containing various surfactants at 30°C for 24 h.

secretory production of the enantioselective Esf lipase was proportional to the Triton X-207 concentration of 3.0% achieving up to 217 kU/ml; around a 5.9-fold increment compared with the control medium containing olive oil, and 54-fold compared with the 4 kU/ml of the parent strain *S. marcescens* ES-2.

Kinetic Resolution of (*S*)-Flurbiprofen in Dispersed Aqueous Phase Reaction System Containing Chiral Suβ-CD at High (*R,S*)-FEE Concentration

The enantioselective hydrolysis of (*R,S*)-FEE to the optically active (*S*)-flurbiprofen was carried out in a novel dispersed aqueous phase reaction system supplementing suβ-CD as the chiral selector and dispenser [25]. The optimal kinetic resolution conditions were determined after changing the amount of the Esf lipase, the molar mixing ratio between immiscible (*R,S*)-FEE and suβ-CD, and the concentration of the substrate (*R,S*)-FEE, as illustrated in Fig. 4.

The conversion yield and enantiomeric excess were increased proportionally as the amount of the Esf lipase of *S. marcescens* ES-2 increased up to 130 kU/mmol of (*R,S*)-FEE. The optimal molar mixing ratio between immiscible (*R,S*)-FEE and suβ-CD was identified to be 1.0:2.0. Even at the extremely high immiscible substrate (*R,S*)-FEE concentration of 500 mM, the high enantiomeric excess of 98% and conversion yield of 48% were achieved, respectively, at 37°C after 12 h.

This implies that the enantioselective Esf lipase from *S. marcescens* ES-2 can be more potentially used as a biocatalyst for the kinetic resolution of (*S*)-flurbiprofen compared with the commercial lipase from *C. rugosa* [25], thereby facilitating the commercial production of a pharmacologically active (*S*)-profen.

Acknowledgments

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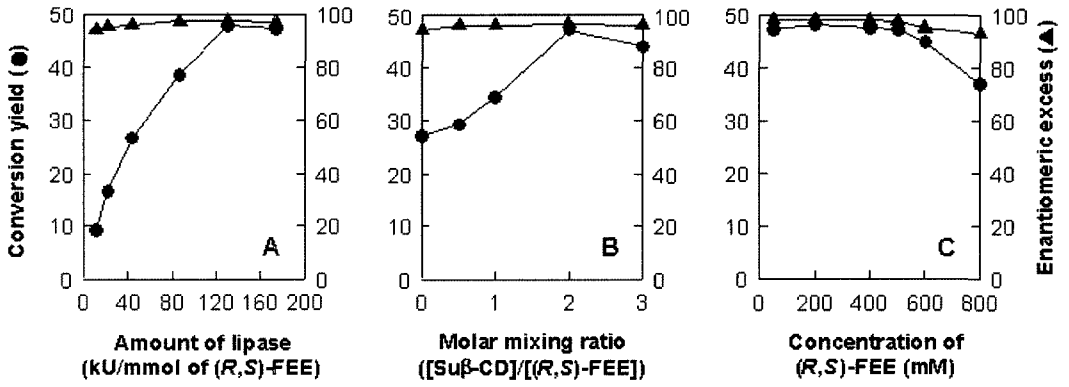

Fig. 4. Effect of reaction conditions on conversion yield and enantiomeric excess for kinetic resolution of (*S*)-flurbiprofen in a dispersed aqueous phase reaction system containing suβ-CD.

Reaction was carried out in 50 mM Tris-HCl buffer (pH 7.5), 37°C for 12 h with stirring (300 rpm). **A.** Amount of enantioselective Esf lipase; **B.** Molar mixing ratio between suβ-CD and 50 mM (*R,S*)-FEE; **C.** Concentration of substrate (*R,S*)-FEE.

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