Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Molecular Catalysis B: Enzymatic 27 (2004) 237–241
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

# Occurrence of ofloxacin ester-hydrolyzing esterase from *Bacillus niacini* EM001

Hyung Kwoun Kim [a], Hyeon Seob Na [b], Min Soon Park [b], Tae Kwang Oh [c], Tae Suk Lee [b,*]

[a] *Division of Biotechnology, The Catholic University of Korea, San 43-1, Yokkok 2-Dong, Wonmi-Gu, Puchon 420-743, South Korea*
[b] *EnzyChem Co., Ltd., 7106, KAIST High Tech Venture Hall, 373-1 Kusung-Dong, Yusung-Ku, Taejon 305-701, South Korea*
[c] *Microbial Genomics & Application Center, Korea Research Institute of Bioscience & Biotechnology, P.O. Box 115, Yusung, Taejon 305-600, South Korea*

Received 25 June 2003; received in revised form 10 November 2003; accepted 10 November 2003

**Abstract**

A *Bacillus niacini* strain (EM001) producing an ofloxacin ester-enantioselective esterase was isolated from the soil samples collected near Taejon, Korea. The cloned gene showed that the esterase EM001 composed of 495 amino acids corresponding to a relative molecular weight ($M_r$) of 54,098 kDa. Based on the $M_r$ and the protein sequence, the esterase EM001 was similar to *p*-nitrobenzyl esterase from *Bacillus subtilis* with an identity of 41.8%. The optimum temperature and pH of the purified His-tagged enzyme were 45 °C and 9.0, respectively. The purified esterase EM001 hydrolyzed preferably (*R*)-ofloxacin propyl ester than (*S*)-form ester at the initial reaction phase with an $ee_P$ of 67% until the conversion rate become up to 35%.


*Keywords:* Esterase; *Bacillus niacini*; Levofloxacin; Ofloxacin

## 1. Introduction

Esterases are hydrolases (EC 3.1.1.1) acting on the ester bonds of short chain fatty acid derivatives. They are ubiquitous enzymes that have been purified and studied from various sources, including animals, plants, and microorganisms. Each esterase has unique and useful characteristics: substrate specificity, regio-specificity, and chiral selectivity [1,2]. Therefore, they are important for the biotechnological applications in medicine and synthetic chemistry (e.g. synthesis of chiral compounds) [3–5].

A large group of chiral drugs are the pyridone carboxylic acid derivatives, which are widely used as bactericidal agents in clinical medicine [6–8]. One drug in this group is ofloxacin, 9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7H-pyrido[1,2,3-de]-1,4-benzoxazine-6-carboxylic acid (Fig. 1). The carbon in position $C_3$ is chiral which results in two enantiomers, *S*-(−)-ofloxacin and *R*-(+)-ofloxacin. (*S*)-form ofloxacin (that is, levofloxacin) is more potent antimicrobial agent than *R*-form against a broad range of bacterial pathogenic strains.

In addition, studies on pharmacokinetics in humans have shown some differences in the pharmacokinetic parameters and in the disposition of the (*S*)- and (*R*)-enantiomers [7]. By oral administration to man, the levofloxacin is extensively absorbed and rapidly eliminated renally with peak urinary concentration being significantly higher than the $MIC_{90}$ values for usual pathogens. Levofloxacin undergoes the limited metabolism and is mainly eliminated without any change.

It has been reported that levofloxacin was made from ofloxacin butyl ester by using an enantioselective animal esterase [9]. In this case, the porcine liver esterase hydrolyzed enantioselectively the (*S*)-ofloxacin ester.

In this paper, an ofloxacin ester-enantioselective esterase-producing *Bacillus niacini* (EM001) was screened from the soil samples and the esterase gene was cloned and the corresponding protein was expressed in high level. Its

*Abbreviations:* TBN, tributyrin; PNPC, *p*-nitrophenyl caprylate; PNPEs, *p*-nitrophenyl esters; PNBCE, *p*-nitrobenzyl carboxy esterase; PNPO, *p*-nitrophenyl ofloxacin

* Corresponding author. Tel.: +82-42-864-2845; fax: +82-42-864-2844.
*E-mail address:* tsleepc@orgio.net (T.S. Lee).

1381-1177/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2003.11.007

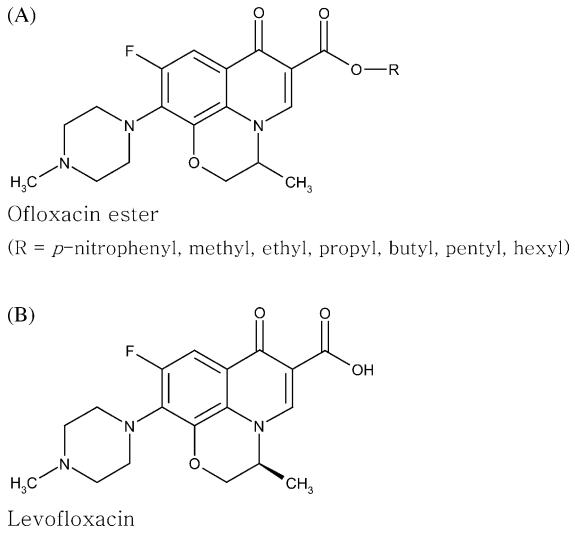


Fig. 1. Structures of ofloxacin esters (A) and levofloxacin (B).

biochemical properties were characterized and the protein was used to produce levofloxacin from racemic ofloxacin esters.

## 2. Materials and methods

### 2.1. Materials

Ofloxacin was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ofloxacin *p*-nitrophenyl ester (OPNE) and ofloxacin methyl (ethyl, propyl, butyl, pentyl, hexyl) esters were chemically synthesized. Ofloxacin (7.22 g, 20 mmol) was dissolved in 36 ml of chloroform and 1,1′-carbonyldiimidazole (4.008 g, 24 mmol). The mixture was refluxed for 2 h. Each of alcohols (30 mmol, methanol, ethanol, propanol, butanol, pentanol, hexanol, *p*-nitrophenol) and catalytic amounts of 60%-NaH (oil dispersed) were added to the reaction mixture and then refluxed for 3 h. The reaction mixture was extracted with 30 ml of $H_2O$, 10 ml of sat-$NaHCO_3$ and 30 ml of methylene chloride. The organic layer was dried using $MgSO_4$ and then evaporated in vacuo. The residue was crystallized from 30 ml of ethyl acetate to obtain ofloxacin esters as 70–80% yield. All other chemicals and solvents were analytical reagents obtained from the common commercial sources.

### 2.2. Isolation of ofloxacin ester-hydrolyzing bacteria

Soil samples collected near Taejon, Korea were suspended in sterile water and spread onto tributyrin (TBN-LB) plate, an esterase-screening plate prepared as follows: a tributyrin (TBN) emulsion was made by emulsifying 5 ml of TBN in 45 ml of an gum arabic solution (200 mM NaCl, 10 mM $CaCl_2$, and 5% gum arabic) for 2 min in a Waring blender. This TBN emulsion (50 ml) was mixed with 450 ml of a LB agar medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar). Strains forming clear zones on the screening plate were selected after 24 h incubation at 37 °C.

*p*-Nitrophenyl ofloxacin (PNPO) was used as a substrate for the second screening (Fig. 1), and then, various chain lengths (methyl, ethyl, propyl, butyl, pentyl, hexyl) of ofloxacin esters (Fig. 1) were subsequently used as substrates to isolate ofloxacin ester-enantioselective bacterial strains. The detailed assay methods were described in the Section 2.5.

### 2.3. Cloning and expression of the esterase EM001 gene

Genomic DNA from the isolated strain (*B. niacini* EM001) was prepared, completely digested with *Hin*dIII, ligated into pUC19 vector, and used to transform *Escherichia coli* XL1-Blue. A colony forming a clear halo on TBN-LB plate containing ampicillin (50 μg $ml^{-1}$) was selected. Recombinant plasmid (pH1) was purified from the transformant and the DNA sequence inserted was analyzed.

To express the recombinant esterase EM001, an *E. coli* expression vector, pET22b was used. First, two oligomeric primers (primer 1 and 2) and *B. niacini* genomic DNA (in pH1) were used to generate PCR product carrying a *Nde*I restriction site at its 5′ end (immediately upstream of the initiation codon ATG of the esterase EM001 gene) and a *Hin*dIII site at its 3′ end. The nucleotide sequences of the two primers are as follows: Primer 1: 5′-TT CAT ATG ACT AAA ACG ATT GTT GGA-3′ Primer 2: 5′-TT AAG CTT CCC TTT CAT CAC CAT GGA TAA-3′

After the digestion with *Nde*I and *Hin*dIII, the PCR product was ligated with pET22b vector. *E. coli* XL1-Blue was transformed with the ligation mixture and the resulting recombinant plasmid, pEST54, was obtained from a transformed *E. coli* cell. *E. coli* BL21(DE3) was transformed by an electroporation with the pEST54 plasmid.

### 2.4. Purification of esterase EM001

Transformed *E. coli* was cultivated in LB medium at 30 °C. After IPTG (1 mM) induction and ultrasonic cell lysis, total soluble proteins were recovered from the cell extract. They were attached to the Ni-NTA column and, after washing with 20 mM Imidazole, 300 mM NaCl, and 50 mM $NaH_2PO_4$ buffer (pH 7.0), the bound esterase EM001 was eluted with 200 mM Imidazole, 300 mM NaCl, and 50 mM $NaH_2PO_4$ buffer (pH 7.0). It was dialyzed against 50 mM Tris–HCl buffer (pH 8.0) and used to characterize its biochemical properties.

### 2.5. Esterase assay

Activity was measured with *p*-nitrophenyl caprylate (PNPC) or other *p*-nitrophenyl esters (PNPEs) [10]. The reaction mixture consisted of 0.01 ml of 10 mM substrate in acetonitrile, 0.04 ml of ethanol, and 0.95 ml of 50 mM

Tris–HCl buffer (pH 8.0) containing an appropriate amount of the enzyme. In the assay of revealing the biochemical properties of the purified enzyme, 0.05 mg ml$^{-1}$ of BSA was added to the enzyme solution as a stabilizer. The enzyme reaction was performed for 3 min at 35 °C, unless otherwise specified. The amount of *p*-nitrophenol liberated during the reaction was measured by its absorbance at 405 nm. One esterase unit is defined as the amount of enzyme liberating 1 μmol of *p*-nitrophenol per minute.

To detect the active esterase band on the polyacrylamide gel, zymographic analysis was done as follows [11]. SDS–PAGE (12%) was performed using slab gels and renaturation of the enzyme was done by soaking the gel in 50 mM Tris–HCl buffer (pH 8.0) for 1 h. Activity assay of the renatured enzymes was achieved by the attached gel to TBN agarose plate and incubating at 37 °C.

PNPO assay used for the second screening was as follows. The isolated strains were cultured in LB media for 24 h at 37 °C and then, the cultured cell (0.05 ml) was mixed with a reaction mixture (0.01 ml of 10 mM PNPO in acetonitrile, 0.04 ml of ethanol, and 0.90 ml of 50 mM Tris–HCl buffer (pH 8.0)). After 10 min incubation at 35 °C, the absorbance at 405 nm was measured.

The hydrolytic activity toward various ofloxacin esters was done as follows. The reaction mixtures (100 ml) contained the purified EM001 esterase (100 u), 10 mM of each ofloxacin esters, and 0.1 M sodium phosphate buffer, pH 6.8. The enzyme reactions were performed at 37 °C for 24 h with mechanical stirring (2.5 rps). After then, the reaction products were analyzed with RP-HPLC system.

The enzyme hydrolysis of ofloxacin propyl ester was done as follows. The reaction volume and condition were almost same with the above assay processes. The differences were that 25 mM of ofloxacin propyl ester was used as substrate and that the amounts of products produced were analyzed with time course.

### 2.6. Chiral ligand-exchange RP-HPLC system

The Shimadzu HPLC system was used. The analytical column in use was packed with $C_{18}$ stationary phase (ODS-A 25 cm × 4.6 mm, YMC-Pack). The solution of chiral mobile phase additive (CMPA) was made up of 9 mM L-isoleucine mixed with 3 mM $CuSO_4$ in water. The mobile phase consisted of CMPA solution–methanol (85:15). The UV detector was operated at 330 nm. The flow-rate of mobile phase was set at 1.0 ml min$^{-1}$. Chromatographic assay was carried out at 30 °C.

### 2.7. Temperature and pH effects on esterase EM001

The optimum temperature of the esterase EM001 was measured by assaying their hydrolytic activities of PNPC at various temperatures (10–80 °C). To examine the thermostability of the esterase EM001, the enzyme was incubated at various temperatures (20–55 °C) for 30 min in a 50 mM Tris–HCl buffer (pH 8.0) containing 0.05 mg ml$^{-1}$ of BSA and then the residual activity was measured at 35 °C and pH 8.0.

The optimum pH of the esterase EM001 was measured by assaying at various pHs (pH 6.5–12). GTA buffer (100 mM 3,3-dimethylglutaric acid, 100 mM Tris, 100 mM 2-amino-2-methyl-1,3-propanediol), an universal buffer, was used in this experiments. And to find out the pH stability of the enzyme, it was incubated at various pHs (pH 4–12) for 30 min in the presence of 0.05 mg ml$^{-1}$ BSA and the residual esterase activities were measured at 35 °C and pH 8.0. In this case, 0.1 M sodium acetate (pH 4–6), potassium phosphate (pH 6–7.5), Tris–HCl (pH 7.5–9), KCl–glycine–KOH (pH 9–10), $Na_2HPO_4$–NaOH (pH 10–11), and $Na_2CO_3$–NaOH (pH 11–12) buffers were used.

## 3. Results and discussion

Among about 50 microbial strains isolated on TBN-LB plate, just four strains had hydrolytic activity towards PNPO. Among them, a strain (EM001) showed the highest activity for the enantioselective hydrolysis of ofloxacin propyl ester.

The selected strain EM001 was Gram-positive, rod-shaped, endospore-forming, aerobic, and catalase-positive. API 50CHB test, which was manufactured by Biomerieux Industry (USA) for the identification of various *Bacillus* strains, showed that it was very similar with *B. niacini* (data not shown). In addition, the determined 16S rRNA sequence showed the highest homology (99.1%) with that of *B. niacini*. So, the selected strain EM001 was designated as *B. niacini* EM001 and deposited to the Korean Collections for Type Cultures under a deposition number of KCTC0965BP. *B. niacini* species has been known to be a group of *Bacillus* strains able to grow on nicotinate (niacin) as a sole source of carbon, nitrogen, and energy. And no esterase enzyme has been reported from any *B. niacini* strain [12,13].

The isolated *B. niacini* EM001 strain showed two different cell-bound lipolytic activities (54 and 37 kDa) on SDS–PAGE gel by zymographic analysis (Fig. 2B). As mentioned above, because no esterase enzyme had been reported from this strain, these seemed to be novel enzymes. So, gene cloning and gene expression were done to obtain and characterize the enzyme.

The chromosomal DNA library of *B. niacini* EM001 was prepared and used to transform *E. coli* XL1-Blue. An *E. coli* transformant formed a clear halo on TBN-LB plate after 24 h incubation. The recombinant plasmid isolated from this transformant had a 3.3 kb-sized insert DNA. The nucleotide sequence showed one major open reading frame of 1488 bp, which encoded a polypeptide of 495 amino acid sequences and corresponds to a relative molecular mass of 54,098. The nucleotide sequence was submitted to the GenBank under accession number of AF417207. The predicted amino acid sequence was compared with the protein sequences in the Swissprot data bank using BLAST program. The most

similar enzymes were *p*-nitrobenzyl carboxy esterase (PNBCE) from *Bacillus subtilis* [14], which had a 41.8% of identity. PNBCE has been known to be an useful enzyme catalyzing the hydrolysis of several β-lactam antibiotic PNB esters, in which PNB esters served as protecting groups on intermediate in the manufacture of the antibiotics.

By comparison with PNBCE and other esterases, the $H^{105}G^{106}$ of esterase EM001 was identified as the oxyanion hole and the Ser193 as the active site serine, which is located in the conserved Gly-X-Ser-X-Gly region [14,15].

In both *B. niacini* EM001 strain and *E. coli* XL1-Blue/pH1 cell, although the presence of esterase activity was observed on TBN-LB plate, the enzyme could not be purified from the cellular proteins because of its low level of production. Therefore, we tried to express the enzyme in *E. coli* BL21(DE3) strain with an expression vector, pET22b. Esterase EM001 gene was inserted into the vector and a recombinant vector, pEST54, was constructed. The recombinant protein was designed to contain C-terminal 13-extra amino acid containing six His residues.

Esterase activity in *E. coli* BL21(DE3)/pEST54 cell extract was measured to be 6.340 $U l^{-1}$ when PNPC ($C_8$) was used as a substrate. That is, as much as 6,340 u of esterase enzyme were obtained from one liter-cultured cell. The enzyme was purified homogeneously on SDS–PAGE gel (Fig. 2) and the specific activity was estimated to be 123 $U mg^{-1}$.

The optimum temperature of esterase EM001 for the hydrolysis of PNPC was measured to be 45 °C. The enzyme was relatively stable up to 30 °C (Fig. 3). And the optimum pH of the esterase was measured to be 9.0 and it was stable at a broad pH range of 4.5–11.0 (Fig. 3).

The enzyme showed high hydrolytic activity toward the short-chained *p*-nitrophenyl esters ($C_2$-$C_6$) and low activity toward the substrates having longer chain length ($C_8$–$C_{12}$) (Fig. 4A).

On the other hands, when the various chain lengths of ofloxacin esters were used as substrates and reacted for

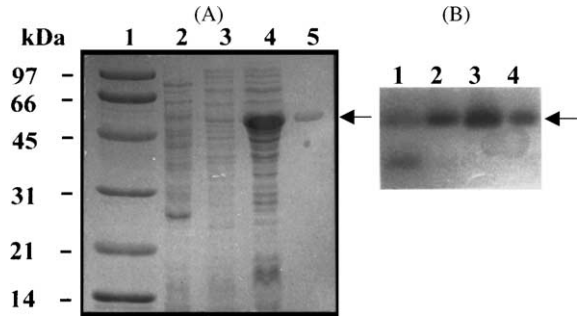


Fig. 2. SDS–PAGE of the esterase EM001. A. Lane 1 is standard protein marker. Lane 2 is cell extract of *B. niacini* EM001. Lane 3 is cell extract of *E. coli* XL1-Blue/pH1. Lane 4 is the cell fractions of *E. coli* BL21(DE3)/pEST54 cultivated at 37 °C for 4 h after IPTG was added. Lane 5 is the purified esterase EM001 by Ni-NTA column chromatography. B. After SDS-PAGE was done, zymographic analysis was carried out with TBN-agarose gel. Lanes 1–4 correspond to lanes 2–5 in A panel, respectively. Arrows indicate the esterase EM001.

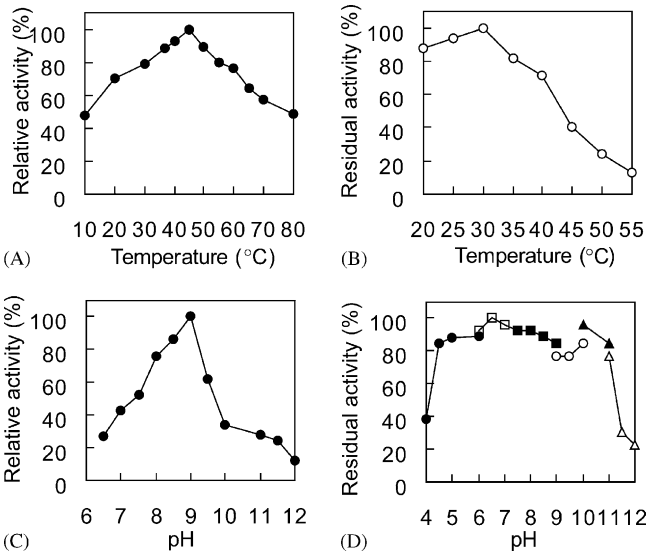


Fig. 3. Effects of temperature and pH on the esterase EM001. (A) The esterase activity was measured at various temperatures (10–80 °C). (B) Enzyme was pre-incubated at various temperatures for 30 min at pH 8.0 and the residual activity was measured at 35 °C, pH 8.0. (C) The esterase activity was measured at various pH 6.5–12.0 at 35 °C. (D) Enzyme was pre-incubated at various pH buffers for 1 h at 30 °C and the residual activity was measured at 35 °C, pH 8.0.

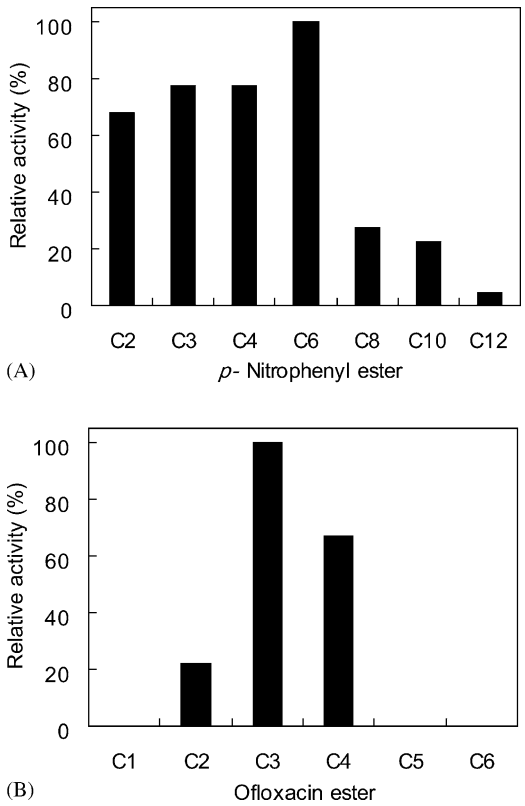


Fig. 4. Chain length specificity of the esterase EM001. (A) The hydrolytic activity was measured toward *p*-nitrophenyl esters ($C_2$–$C_{12}$). (B) The hydrolytic activity was measured toward various ofloxacin esters ($C_1$–$C_6$).

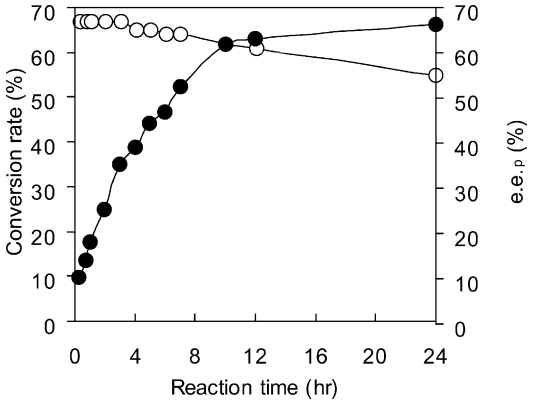


Fig. 5. Time course changes of conversion rate and enantiomeric excess. The initial concentration of ofloxacin propyl ester was 25 mM. The two symbols, (●) and (○), mean the conversion rate and the enantiomeric excess of product, respectively. The $ee_P$ was calculated from the ratio (%) of [(*R*-from) − (*S*-form)]/[(*R*-from) + (*S*-form)].

24 h, the enzyme showed a little different hydrolytic activity. That is, the highest hydrolytic activity was shown toward ofloxacin propyl ester. Ofloxacin butyl and ethyl esters were hydrolyzed slowly and none of ofloxacin methyl, pentyl, and hexyl esters were hydrolyzed (Fig. 4B).

The enzyme hydrolyzed preferably (*R*)-ofloxacin propyl ester than (*S*)-form ester at the initial reaction phase with an $ee_P$ of 67% and the value was sustained until the bioconversion rate become up to 35% (Fig. 5). After then, the $ee_P$ slightly decreased. When the reaction is continued for 24 h, the bioconversion rate and the $ee_P$ become 67 and 55%, respectively. As described previously, porcine liver esterase, on the contrast, hydrolyzed enantioselectively (*S*)-ofloxacin butyl ester with an $ee_P$ of 60% [9]. In that case, they had to recover the resulting (*S*)-ofloxacin by two additional steps of the concentration of the reaction mixture and the chiral separation process.

On the other hands, EM001 esterase hydrolyzed (*R*)-ofloxacin propyl ester more rapidly and, as a result, enriched (*S*)-form ester in the reaction mixture. Therefore, organic solvent extraction of the remaining esters, chiral separation, and acid-catalyzed hydrolysis could make (*S*)-ofloxacin. The merit of using EM001 esterase in the preparation of (*S*)-ofloxacin is the convenience in extraction and concentration of the enriched (*S*)-form ofloxacin ester.

Because only (*S*)-ofloxacin (levofloxacin) is an important compound with a potent antibiotic activity, this bioconversion process using esterase EM001 seems to be very important in the pharmaceutical industry.

## References

[1] K.E. Jaeger, S. Ransac, B.W. Dijkstra, C. Colson, M. van Heuvel, O. Misset, FEMS Microbiol. Rev. 15 (1994) 29–63.
[2] R.D. Schmid, R. Verger, Angew. Chem., Int. Ed. 37 1998 pp. 1608–1633.
[3] K.E. Jaeger, M.T. Reetz, TIBTECH 16 (1998) 396–403.
[4] K.E. Jaeger, B.W. Dijkstra, M.T. Reetz, Annu. Rev. Microbiol. 53 (1999) 315–351.
[5] J.S. Dordick, Enz. Microb. Technol. 11 (1989) 194–211.
[6] I. Hayakawa, S. Atarashi, M. Imamura, S. Yokohama, N. Higashihashi, K. Sakano, M. Ohshima, US patent 51,420,46, 1992.
[7] F.A. Wong, S.J. Juzwin, S.C. Flor, J. Pharma. Biomed. Anal. 15 (1997) 765–771.
[8] S. Zeng, J. Zhong, L. Pan, Y. Li, J. Chromato. B728 (1999) 151–155.
[9] S.Y. Lee, B.H. Min, S.H. Hwang, Y.M. Koo, C.K. Lee, S.W. Song, S.Y. Oh, S.M. Lim, S.L. Kim, D.I. Kim, Kor. J. Biotechnol. Bioeng. 15 (2000) 313–317.
[10] B.C. Oh, H.K. Kim, J.K. Lee, S.C. Kang, T.K. Oh, FEMS Microbiol. Lett. 179 (1999) 385–392.
[11] H.K. Kim, S.Y. Park, T.K. Oh, J. Microbiol. Biotechnol. 7 (1997) 37–42.
[12] M. Nagel, J.R. Andreesen, Int. J. Sys. Bacteriol. 41 (1991) 134–139.
[13] K. Goto, T. Omura, Y. Hara, Y. Sadaie, J. Gen. Appl. Microbiol. 46 (2000) 1–8.
[14] J. Zock, C. Cantwell, J. Swartling, R. Hodges, T. Pohl, K. Sutton, P.D. McGivray, S. Queener, P. Rosteck Jr., Gene 151 (1994) 37–43.
[15] H.K. Kim, H.J. Choi, M.H. Kim, C.B. Sohn, T.K. Oh, Biochim. Biophys. Acta 1583 (2002) 205–212.