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ORIGINAL PAPER

A cold-active esterase of *Streptomyces coelicolor* A3(2): from genome sequence to enzyme activity

Sameh H. Soror · V. Verma · Ren Rao · Shafaq Rasool · S. Koul · G. N. Qazi · John Cullum

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Abstract The genome sequence of *Streptomyces coelicolor* A3(2) contains 51 putative lipase and esterase genes mostly of unknown function. The gene *estB* (locus SCO 6966) was expressed as a His-tagged protein in *E. coli*. Esterase B was active at low temperatures exerting its maximum activity at 30°C and retaining more than 25% of its activity at 4°C. The optimum pH was 8–8.5. The enzyme was active against short synthetic *p*-nitrophenylesters (C_2–C_{10}) with maximum activity towards the acetate ester (C_2). The esterase was tested on 13 series of racemic esters of potential interest for the synthesis of chiral pharmaceutical compounds. 4 of the series were substrates and a modest degree of enantioselectivity was observed (enantiomeric ratios of 1.1–1.9).

Keywords *Streptomyces coelicolor* · Esterases · Cold-active enzyme · Enantioselectivity

Introduction

Lipolytic enzymes play an ever-increasing role in chemical synthesis with chiral-specific reactions being especially important [1]. Although lipolytic enzymes are common in *Streptomyces* species [2–4], only very few have been studied so far [5–11]. *Streptomyces* are very active in secondary metabolism, which makes it likely that they will contain lipolytic enzymes with interesting novel properties. However, the fact that they might only be expressed under special growth conditions makes traditional screening difficult. An interesting alternative is to mine genome sequences for novel enzymes. The genome sequence of *Streptomyces coelicolor* A3(2) was published in 2002 [12] and 51 putative esterase or lipase genes were annotated on the basis of similarity searches using the FASTA algorithm [13]. Although a distinction is made between esterases (EC.3.1.1.1), which hydrolyse smaller substrates and lipases that hydrolyse larger substrates (EC.3.1.1.3), there is no fundamental biochemical difference and classifications based on sequence similarities (e.g. [14]) do not separate the two classes of enzymes.

Many biotechnological processes are expedited by the use of higher temperatures and this generated a lot of research into thermostable enzymes. However, more recently there has been great interest in cold-adapted enzymes for transformations in which substrate and product stabilities require the use of low temperatures. Such psychrophilic enzymes are important in many fields including the detergent, textile and food industries, as well as for a variety of biocatalysis reactions [15, 16]. For most enzymes, lowering the temperature by 10°C decreases the rate of reaction by two to threefold, resulting in 15–80 fold lower activity at 0°C compared to 37°C [17]. A better retention of activity in the cold can be achieved through a decrease in the activation energy resulting from an increase in protein flexibility [18].

Because *S. coelicolor* A3(2) is a mesophilic soil organism which would often encounter temperatures below 10°C in nature, it seemed likely that it would be a good source for enzymes that are active in the cold. Ten genes for putative lipolytic enzymes were chosen for characterisation by

S. H. Soror · R. Rao · J. Cullum (✉)
LB Genetik University of Kaiserslautern,
Postfach 3049, 67653 Kaiserslautern, Germany
e-mail: cullum@rhrk.uni-kl.de

V. Verma · S. Rasool · S. Koul · G. N. Qazi
Biotechnology Regional Research Laboratory (CSIR),
Canal Road, JammuTawi 180001, India

expression in *E. coli* using a His-tag vector for easy protein purification (S.H. Soror and J. Cullum, in preparation). In this paper, we report the expression of the gene *estB* (locus SCO 6966, EMBL accession number CAB89027), which encodes a cold-active esterase. The enzyme was tested for chiral-selective activity using substrates of interest for the preparation of intermediates for pharmaceutical products.

Materials and methods

Chemicals

All chemicals were analytical grade. *p*-nitrophenyl esters, α-naphthylacetate, *p*-nitrophenol and fast blue RR salt were purchased from Sigma–Aldrich, Germany and DTT from Böhringer Mannheim, Germany. Other chemicals were purchased from Roth, Germany. The racemic substrates were either prepared or purchased commercially and the authenticity of the racemic compounds was confirmed by spectroscopic analysis including NMR, LC/MS and that of the resolved enantiomers by optical rotation and chiral chromatographic methods. The optical rotation was measured with a Perkin–Elmer 241 polarimeter. Chiral HPLC analysis was carried out on a Shimadzu LC10 AT model. TLC was run on 0.25 mm silica gel F_{254} plates (E Merck) using UV light or ceric sulphate solution for the detection of spots. The enantiomeric ratio of 1-(6-methoxy-2-naphthyl)-ethanol,1-(3,4-methylene-dioxyphenyl)-ethanol, 1-(3,4-methylenedioxyphenyl)-pentanol and 2-(6-methoxy-2-naphthyl)-propanol was determined on a Chiralcel OD-H HPLC column (5 µm) using hexane/2-propanol (19:1) with a flow rate of 0.5 ml/min. The enantiomeric ratio of ethyl 3-hydroxy-3-phenyl propionate was determined on Chiradex column using methanol: water: triethylamine (1:1:0.01) as mobile phase (0.8 ml). The enantiomeric ratio of ethyl 2-hydroxy-4-phenylbutanoate was determined by comparison of the optical rotation with the standard value $[\alpha]_D$-8.4 (c 1.15 , EtOH, R-ester ee ~99%). The absolute configuration was determined on the basis of the sign of specific rotation.

Strains and growth conditions

The wild type *S. coelicolor* A3(2) strain 1147 was used as a source of DNA for the cloning experiment. The *Streptomyces* strain was grown in YEME medium with 34% sucrose [19]. The *E. coli* strains TOPO 10 (Invitrogen, Germany), XL-1-BLUE (Stratagene) [20] and BL21(DE3) [21] were used. *E. coli* strains were grown at 37°C on LB liquid medium and LB agar with 2% agar with addition of ampicillin or X-gal when needed [22, 23].

DNA methods

Total DNA was prepared from *S. coelicolor* A3(2) [19]. Ten nanogram of DNA was used for a PCR reaction with the following primers: SCO6966-F (5′-CTCGAGGACAA ACGCCCGGC-3′, *Xho*I site underlined) and SCO6966-R (5′-CGGAGGACATATGGCCGAGGC-3′, *Nde*I site underlined). The PCR reaction mixture (50 pM of each primer, 2.5 mM $MgCl_2$, 200 µM of each dNTP, 5% DMSO and 5U *Taq* DNA polymerase (Fermentas, Germany) in 50 µl reaction volume) was amplified in an Eppendorf thermocycler with the program: 30 cycles of: denaturation at 96°C for 30 s, annealing at 60°C for 45 s, extension at 72°C for 45 s followed by a final incubation at 72°C for 10 min. The amplified fragment was purified using a PCR purification kit (Qiagen, Germany), and cloned into vector PCR 4-TOPO using a TOPO TA cloning kit for sequencing (Invitrogen, Germany). The cloning mixture was transformed in one shot® TOPO 10 chemical competent cells. DNA sequencing used the chain termination method [24] with M13 standard universal and reverse primers and the walking primers SCO6966-WF (5′-GGGAGAGCGAGGCC GGGC-3′) and SCO6966-WR (5′-TGATCGCCTCCCG CTACGCC-3′). Sequencing was carried using an ABI PRISM BigDye™ terminator 1.1 sequencing kit (Applied Biosystems, USA) and an ABI capillary sequencer ABI 3100 at the Center for Nanostructure Technology and Biomolecular Technology at the University of Kaiserslautern.

DNA from pSHS810 and pET-16 b were digested with enzymes *Nde*I and *Xho*I (Fermentas) using conditions recommended by the manufacturer. The digested DNA was purified by phenol-chloroform extraction. The pSHS810 DNA was subjected to a further digestion with the enzyme *Dra*I to digest the vector and prevent recovery of pSHS810. Ligation and electroporation were as in [23, 25–27]. The positive clones were verified by sequencing using T7 primer.

Protein methods

Protein estimation used the Bradford method [28] with bovine serum albumin as a standard. Induction of the cloned lipase gene was carried out according to the Novagen pET manual 2002. Exponential phase cultures (OD_{600} 0.6–1.0) were induced by adding 0.25 mM IPTG and harvested after 4 h induction at 30°C. The cells were harvested by centrifugation and 100 ml of culture was resuspended in 4 ml of buffer (0.05 M sodium phosphate/0.3 M NaCl) and treated with lysozyme (1 mg/ml) on ice for 45′. The lysate was centrifuged for 40 min at 12,000 rpm at 4°C in an Eppendorf centrifuge and the supernatant was filtered through at 0.45-µm filter. The proteins were purified by Ni-affinity chromatography on PROTINO resin (Macherey-Nagel) or for

smaller volumes using Ni-spin columns (Qiagen). The samples were desalted, concentrated and the buffer was changed to Tris–HCl 0.02 M pH 8 using the centrifugal filter devices Amicon ultra-4 10,000 MWCO (Millipore, Germany). The His-Tag was cut using factor Xa capture kit (Novagen, Germany) as recommended by the manufacturer and the sample was desalted, concentrated and the buffer was changed again to Tris–HCl 0.02 M pH 8 through ultra-filtration. SDS-PAGE was carried out using a 4% stacking gel and a 12% resolving gel [29] and the bands were detected with Coomassie brilliant blue. For activity staining the gels were run under native conditions and stained with 50 μg α-naphthylacetate and 10 mg fast blue RR salt in 100 ml HEPES 0.02 M pH 7 and incubation at 37°C 30–60′ until the bands were developed [30].

The standard esterase assay used *p*-nitrophenylacetate as the substrate at 25°C with pH 8.0 slightly modified from Hotta et al. and Tesch et al. [11, 31]. 1 mM *p*-nitrophenylacetate was added to buffer (0.02 M Phosphate buffer pH 8.0, 1% acetonitrile). 1 ml of the substrate solution was pre-incubated for 1 min at 25°C, 2–3 μl esterase (3U) preparation was added and the reaction mixture incubated for 5 min. The release of *p*-nitrophenol was measured at 410 nm using a model 4054 UV/visible spectrophotometer (LKB, Pharmacia). Controls without enzyme showed no significant non-enzymatic hydrolysis. One unit of lipase activity was defined as the amount of activity releasing 1 μM *p*-nitrophenol per minute. The standard assay was modified to test the effect of different parameters on enzyme activity. A temperature range of 4–50°C was tested. The effect of temperature on enzyme stability was determined by incubating 15U of the esterase in 0.02 M Tris–HCl buffer pH 8.0 at the selected temperature for 1 h with the residual activity being determined at 25°C. The effect of pH on enzyme activity was determined using acetate buffer (pH 3–5.5), phosphate buffer (pH 6–8.5) and carbonate buffer (pH 9–11.0) and the reaction time was reduced to 2 min. The effect of pH on enzyme stability was determined by incubation at 15°C for 24 h in the appropriate buffer and the residual activity was measured after pre-incubation in the standard buffer for 1 min. The substrate specificity was determined by using the appropriate *p*-nitrophenyl esters in the assay. The effect of metal ions, EDTA, DTT and PMSF was determined by pre-incubation of the enzyme for 1 h at 15°C in 0.02 M Tris pH 8.0. The effect of water miscible organic solvents on activity was determined adding 10% of each in the substrate solution, and their effect on stability was measured after pre-incubation in 20 and 50% solution. The K_m and V_{max} were determined with a Lineweaver–Burk plot using *p*-nitrophenylesters concentration 0.1–5 mM.

In the experiments to study stereoselectivity of the enzyme, the following substrates were used: acyl esters of 1-(3,4-methylenedioxyphenyl)-ethanol, 1-(3,4-methylenedioxyphenyl)-pentanol, bisnapthol, 1-(6-methoxy-2-naphthyl)-ethanol, 2-(6-methoxy-2-naphthyl)-propan-1-ol, 4-(*p*-flurophenyl)-3-hydroxymethyl-N-methyl-piperidine and ethyl 3-hydroxy-3-phenyl-propanoate. Alkyl esters of 2-(6-methoxy-2-naphthyl)-propanoic acid, 2-(*p*-(2-methylpropyl)-2-phenyl) propanoic acid, 2-(*p*-chlorophenyl)-3-methyl propanoic acid and 2-bromo propanoic acid. 2-hydroxy-4-phenyl butanoic acid and (indol-2-yl) formate were used as their ethyl esters. The reactions were carried out as described in Maqbool et al. [32]. The substrates at concentrations of 20–50 g/l were incubated in volumes of 2–5 ml with the enzyme (50–200 mg of lyophilised extract) at 25°C in 0.1 M phosphate buffer pH 7.0. The progress of the reaction was monitored by TLC for 8–240 h and the products extracted with ethyl acetate (4 × 15 ml). The organic layer was washed with water (2 × 5 ml), dried over anhydrous sodium sulfate and concentrated under reduced pressure on a rotavapour. The enantiomeric ratios were determined as described above.

Results and discussion

The DNA sequence of the *estB* gene corresponds to a peptide with 269 amino acid residues, belonging to family V of Arpigny and Jaeger [14]. The *estB* gene was amplified by PCR from total DNA of *S. coelicolor* A3(2). Many expression vectors are designed to create an in-frame fusion using an *Nde*I restriction site in the start codon. It was, therefore, necessary to introduce an *NdeI* site into the start codon of *estB* by including mismatches in the upstream primer. The downstream primer included an *Xho*I site, so that the *estB* gene could later be obtained as an *Nde*I–*Xho*I fragment. A PCR fragment of the expected size (810 bp) was amplified and cloned into the T-vector PCR4-TOPO, using a cloning system based on vaccinia virus DNA topoisomerase [33]. The cloning mixture was transformed into *E. coli* strain TOPO 10 and the transformation mixture was plated onto agar containing kanamycin and X-gal. More than 30 white colonies were obtained and restriction analysis showed that 6 plasmids contained inserts of the correct size. The insert in one of the clones was sequenced to confirm the correct PCR product. This plasmid was named pSHS810.

The *Nde*I–*Xho*I fragment containing the *estB* gene was purified from pSHS810 and ligated into the expression vector pET-16b [34–36]. The ligation mixture was transformed into *E. coli* strain XL-1-BLUE. Plasmid DNA was isolated from one of the ampicillin resistant clones and the presence of the correct insert was confirmed by restriction analysis and sequencing. The recombinant plasmid was named pSHS811. pSHS811 was later transformed into the expression host strain BL21(DE3). In pET-16b the insert is transcribed under the influence of a T7-polymerase promoter

and induced by IPTG. Preliminary experiments showed that *p*-nitrophenylacetate was hydrolysed by the clone (pSHS811) and this substrate was used to monitor the induction. Once suitable induction conditions were established, the enzyme was purified from the cell extract by affinity chromatography on Ni-agarose. Native PAGE with activity staining gave a single band with the purified enzyme (data not shown). SDS-PAGE also gave a single band (Fig. 1, lane 2) of the correct size (31.9 kD) for the recombinant protein including the His-tag (predicted MW 31.443 kD). Attempts to remove the His-tag by digestion with factor Xa were unsuccessful (Fig. 1, lane 3). The purified fractions were stored at −20°C, and were used to study the enzyme properties.

The influence of pH on enzyme activity was measured (Fig. 2). The enzyme preferred alkaline conditions with an optimum activity at pH 8.0–8.5 and very little activity (<20%) at a pH lower than 6.5. The enzyme retained substantial activity at pH values up to 9.0. The enzyme was stable on storage at pH values between 7.5 and 11 for 24 h at 15°C (Fig. 2). The influence of temperature on enzymatic activity was determined (Fig. 3). Maximum activity was at 30°C, but the enzyme retained more than 25% of its activity at 4°C. In order to assess its thermostability, the enzyme was pre-incubated at different temperatures for 1 h before assaying the residual activity. Figure 3 shows that there was a gradual loss of activity above 30°C with practically no activity after incubation for one hour at 50°C. Esterase B not only showed a good activity at low temperatures, but also had good thermostability compared with other cold-adapted enzymes e.g. the low-temperature lipase from psychotropic *Pseudomonas sp*. strain KB700A [37]. Although high catalytic activity at low temperature tends to be associated with thermosensitivity [38], directed evolution studies to improve the thermostability of cold adapted enzymes revealed that there is not a strict correlation [17, 18].

The effect of pre-incubation with different metal ions and other chemicals on the enzymatic activity was tested (Fig. 4). None of the metal ions tested stimulated enzyme activity and the insensitivity to EDTA suggested that no divalent cations are needed for enzyme activity. The enzyme was sensitive to Hg^{2+} and there was a slight stimulation of activity with DTT, which suggests that one or more thiol groups are important for the activity. All of the metal ions showed negative effects on activity at higher concentrations. The enzyme was inactivated by PMSF, which is explained by the fact that esterase B, like other lipolytic enzymes, has a serine residue in the active site in a conserved pentapeptide G-X-S-X-G [39, 40]. A 10% concentration of water-miscible organic solvents reduced the enzyme activity significantly (Table 1). However, the enzyme was fairly stable in 20% solutions of most of the solvents tested compared to a large loss of activity observed with 50% solutions. This is in contrast to some lipolytic enzymes, which were activated by pre-incubation in organic solvents [41, 42].

Substrate specificity of esterase B was initially investigated using *p*-nitrophenyl esters of different alkyl chain length. The enzyme showed a high activity towards short chain fatty acids (C2–C6), it exerted the maximum activity against the acetate ester (Table 2). A similar specificity was found for the cold adapted lipase of *Pseudomonas* sp. strain B11-1 [42]. The K_m and V_{max} were also estimated on different substrates (Table 2) and showed a similar pattern to that observed with activity measurements. An important application of lipolytic enzymes is to produce chiral precursors

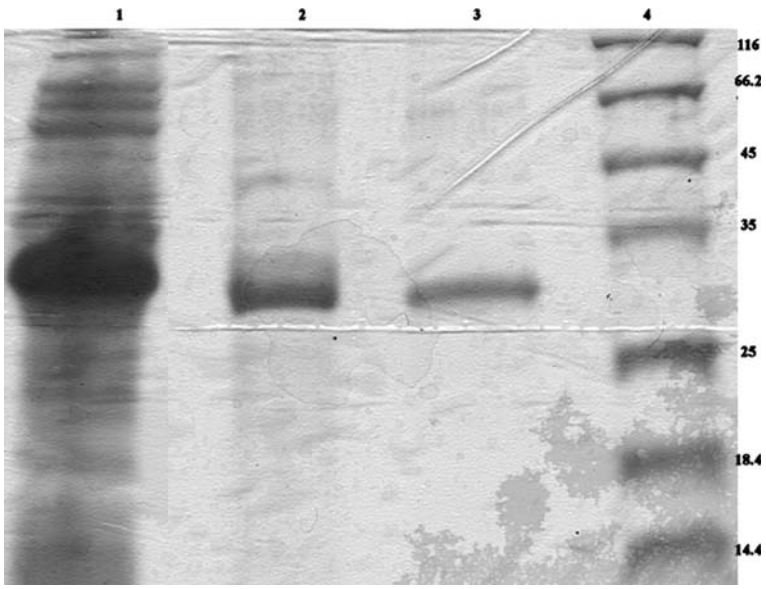


Fig. 1 SDS PAGE of Esterase B. *Lane 1* total cell protein, *lane 2* after Ni column. *Lane 3* after digestion with factor Xa, *lane 4* Marker (116 kDa *E. coli* *β*-galactosidase, 66.2 kDa bovine serum albumin, 45 kDa chicken ovalbumin, 35 kDa porcine muscle lactate dehydrogenase and 25 kDa *E. coli* restriction endonuclease Bsp981, 18.4kDa *β*-lactoglobulin bovine, 14.4 kDa lysozyme chicken egg)

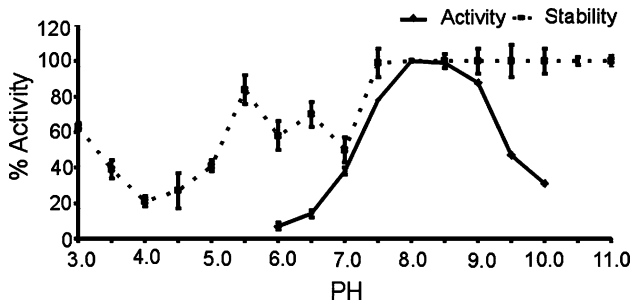


Fig. 2 Effect of pH on activity (*filled diamond*) and stability (*filled square*). Activities are shown as percentages of the maximum activity. Stability was measured after 24 h incubation in different buffers at 20°C. Standard deviations were derived from two different experiments each with four replicates of the His-tagged protein. SD are indicated as *error bars*. The absence of an *error bar* indicates a deviation less than the symbol size

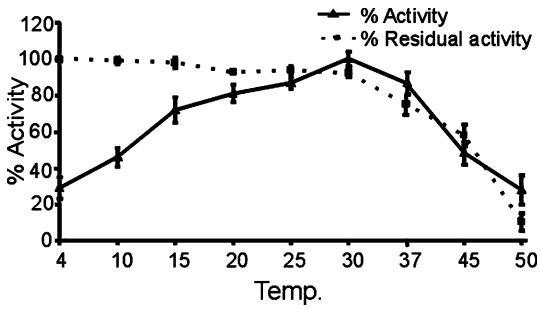


Fig. 3 Effect of temperature on activity and stability. Activity (*filled triangle*) is represented as percentage of the maximum activity. Stability (*filled square*) was measured by incubation for 1 h at the stated temperature with determination of the residual activity at 37°C. Standard deviations are derived from two different experiments each with four replicates of the His-tagged protein

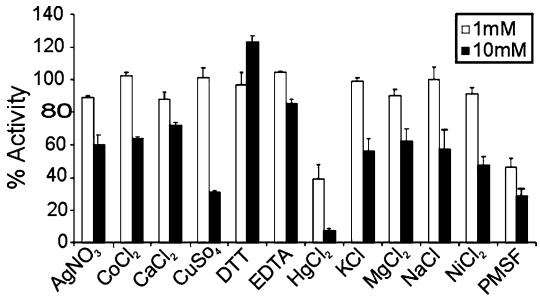


Fig. 4 Effects of metal ions and other reagents on activity of Esterase B. All reagents were tested using 1 mM (*open square* open bars) and 10 mM (*filled square* solid bars). The final concentration in reactions was always ≤2%. Standard deviations were derived from two different experiments each with four replicates of the His-tagged protein

for the synthesis of pharmaceutical products. Esterase B was tested for its ability to hydrolyse some interesting substrates for this purpose. In each case, a racemate was used as a substrate and the stereo-selectivity of the products was measured using a chiral HPLC column or an optical rotation method. Thirty-five racemic compounds comprising 13 sets of targets with different alkyl chain lengths (see Materials and methods) were tested against the enzyme. Esterase B showed activity with 4 of the target sets (Table 3) including ethyl-2-hydroxy-4-phenyl butanoate, which is an important precursor for ACE inhibitors. The enzyme did show some stereo-selectivity, but the entantiomeric ratios were low (less than 2 in all cases. An enantiomeric ratio above 10 would be necessary for a useful enzyme for chiral synthesis. However, directed evolution has been used to improve a lipase from a ratio of 1.1 to 25 [43]. As it is very difficult to find natural enzymes with suitably high ratios, it is likely that future applications will often use such strategies and the fact that Esterase B can achieve ratios above 1.5 makes it interesting.

Table 1 Stability and activity in different organic solvents; SD was derived from two different experiments each with four replicates

Organic solvent	Stability ± SD		Activity ± SD 10%
	20%[a]	50%[a]	
Acetonitrile	86 ± 2	7 ± 2	14 ± 4
Acetone	98 ± 1	13 ± 2	16 ± 3
DMF	94 ± 2	18 ± 1	15 ± 4
DMSO	96 ± 6	17 ± 5	37 ± 1
Isopropanol	54 ± 4	7 ± 2	11 ± 1
Ethanol	100 ± 1	13 ± 1	19 ± 3
Methanol	90 ± 4	10 ± 1	12 ± 1

[a] The concentration of the organic solvents in the reaction mixture was <1%

Table 2 Substrate specificity and kinetics; SD was derived from two different experiments each with four replicates

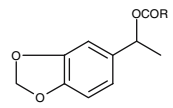
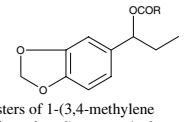
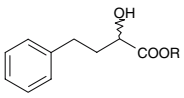
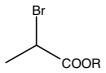
Substrate	% Activity ± SD	K_m ± SD (mM)	V_{max} ± SD (mM/min)
Acetate (C_2)	100 ± 8	0.89 ± 0.06	0.0077 ± 0.00004
Butyrate (C_4)	90 ± 9	0.93 ± 0.01	0.0050 ± 0.00028
Caproate (C_6)	75 ± 10	2.53 ± 0.51	0.0110 ± 0.00033
Caprylate (C_8)	33 ± 7	3.00 ± 0.28	0.0027 ± 0.00212
Caprate (C_{10})	5 ± 9	ND	ND

ND not determined

Conclusion

This study shows that it is possible to find interesting enzymes using the genomic sequences of *Streptomyces* as a starting point. The number of genomic sequences will increase rapidly in the next few years, giving large numbers of new enzyme genes. The main challenge will be to

Table 3 Enantio-selective activity of esterase B on acyl esters of primary and secondary alcohols and alkyl esters of alkanoic acids

Substrate	Enantiomeric ratio (E) of product alcohol				Excess product
	$R = CH_3$	$R = C_2H_5$	$R = C_3H_7$	$R = C_4H_9$	
 esters of 1-(3,4-methylene dioxyphenyl)-ethanol	1.5	1.7	1.9	–	*R*
 esters of 1-(3,4-methylene dioxyphenyl)-propan-1-ol	1.5	1.4	1.6	–	*R*
 Ethyl- 2-hydroxy-4-phenyl butanoate	–	1.5	–	–	*R*
 esters of 2-bromo propanoic acid	1	1.1	–	1.1	*S*

Measurements were carried out after conversion of 20–60% of the substrate

develop bioinformatics methods to identify the most interesting enzymes for expression.

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