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# Fingerprint lipolytic enzymes with chromogenic *p*-nitrophenyl esters of structurally diverse carboxylic acids

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ABSTRACT

A series of structurally diverse chromogenic esters, including a new compound (4-nitrophenyl 2-methylpentanoate), has been synthesized, constituting an array of 17 substrates which could be applied to rapidly fingerprint the activity of lipases or esterases to reveal their substrates specificity and functional characteristics. Combined with genetic technology such as "data mining" and directed evolution, such fingerprints might be a promising platform for discovery of potentially useful enzymes in industrial application. The fingerprint of commercially available Lipase-B from *Candida antarctica* as a model enzyme was first measured to confirm the reliability of this method. Then three new enzymes mined from genomic libraries were successfully fingerprinted, revealing the functional characteristics of those enzymes. Among them, the enzyme SrfAD was founded with specific substrate preference towards cycloalkyl carboxylic esters and aromatic esters, making it more promising in synthetic utilities than other tested enzymes.

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## 1. Introduction

Lipase (EC 3.1.1.3) and esterase (EC 3.1.1.1), belonging to α/β hydrolases because of their three dimensional structures with the characteristic α/β fold [1], are considered as the most important group of biocatalysts for industrial applications. Furthermore, novel applications have been successfully established using lipases or esterases for the synthesis of biopolymers and biodiesel, as well as the production of enantiopure pharmaceuticals, agrochemicals, and flavor compounds [2,3].

With the rapid progress in sequencing technology and the dramatic increase of genomic data, an increasing number of lipases and esterases of industrial interest were currently obtained based on published genomic information [4,5]. Unfortunately, the activity of these putative enzymes cannot be deduced accurately from their structure because of the limited predictive value of chemical theories. Therefore, characterization of a (bio)catalyst is an experimental exercise that amounts to recording its activity fingerprint across a broad range of substrates [6]. In addition, the analysis of enzyme activity will be particularly facilitated if the enzyme activity on different substrates can be measured simply in a high-throughput format [7]. So far, there have been a range of high-throughput screening methods developed to measure the activity of lipases and esterases, such as the assays with chromogenic [8,9] or fluorogenic [10,11] substrates, pH indicators [12], or fluorescence dyes coupling reactions [13]. Currently, the general trend beyond the development of enzyme assays is to assemble libraries of chromogenetic or fluorogenic substrates, rather than single substrates, to provide an analytical tool to characterize the reactivity fingerprint of certain enzymes [14,15]. But in those researches, the substrate arrays consisting of one or several groups of substrates [16,17] were not diverse enough to characterize the activity profile of lipases and/or esterases in detail.

Here in, we constructed a chromogenic substrate array (Fig. 1) consisting of four groups of *p*-nitrophenyl esters: (I) the linear aliphatic esters with various chain lengths, (II) the branched aliphatic esters with different position of branched chain, (III) the cycloalkyl carboxylic esters, and (IV) the aromatic esters. With this substrate array as a tool for the rapid fingerprinting of lypolytic enzymes on structurally diverse substrates, characterization of the substrates specificity of any newly discovered enzymes becomes very easy and fast, which will be very helpful for functional classification of new enzymes and for discovering industrially relevant enzymes.

## 2. Experiments

### 2.1. Chemicals and enzymes

All chemicals were purchased from Alfa Aesar or TCI, and of analytical grade. Lipase B from *Candida antarctica* (CALB) was bought from Novozyme (Beijing, China). A putative lactamase, EstF4K, was

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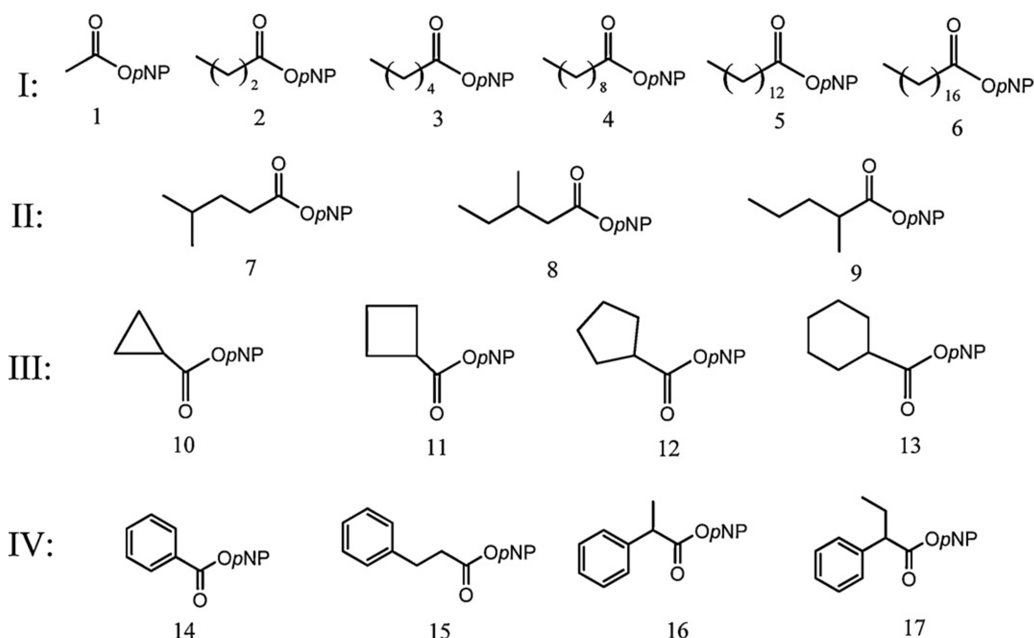


**Fig. 1.** Four groups of chromogenic substrates synthesized for fingerprinting lipolytic enzymes. (I) Linear aliphatic esters; (II) branched aliphatic esters; (III) cycloalkyl carboxylic esters; (IV) aromatic esters.

mined from water metagenomic library, and esterases PnbA and SrfAD were cloned from *Bacillus subtilis* 168. Except EstF4K which was purified, whereas all the other enzymes used were in crude form (cell free extract).

### 2.2. General procedure for synthesis of p-nitrophenol esters

*p*-Nitrophenol (2.8 g, 20 mmol), carboxylic acid (16 mmol), 4-dimethylamiopryidine (0.05 g, 0.4 mmol), and *N*,*N*-dicyclohexylcarbodiimide (DCC) (4.2 g, 20 mmol) were dissolved in 30 mL of dimethyl sulfoxide. The solution was stirred continuously for 8 h at room temperature. The reaction mixture was collected and filtered by Buchner funnel, and the filtrate was evaporated under reduced pressure to yield a crude product. The later was dissolved in 50 mL ethyl acetate, then washed successively with saturated sodium bicarbonate (50 mL × 3), cold water, and saturated sodium chloride, and dried over anhydrous sodium sulfate. The crude product was finally purified by column chromatography using the mixture of petroleum ether and ethyl acetate (at proper ratios) as an eluent. The organic solvent of eluted solution was evaporated under reduced pressure and the purified product was dried under vacuum.

All reactions were real-time monitored by TLC. $^1$H NMR and $^{13}$C NMR spectra of refined products were recorded with a Bruker AVANCE 500 spectrometer.

#### 2.2.1. 4-Nitrophenyl acetate (**1**)

The reaction was carried out following the general procedure starting with 16 mmol of acetic acid, yielding 2.5 g (86.3%) of **1**. Pale yellow solid; TLC: $R_f$ = 0.35 (petroleum ether/ethyl acetate, 20/1, v/v); $^1$H NMR (500 MHz, $CDCl_3$): $\delta$/ppm 8.28 (d, 2H, $J$ = 9.1 Hz), 7.29 (d, 2H, $J$ = 9.1 Hz), 2.35 (s, 3H).

#### 2.2.2. 4-Nitrophenyl butyrate (**2**)

The reaction was carried out following the general procedure starting with 16 mmol of butyric acid, yielding 2.7 g (81.5%) of **2**. Pale yellow oil; TLC: $R_f$ = 0.59 (petroleum ether/ethyl acetate, 20/1, v/v); $^1$H NMR (500 MHz, $CDCl_3$): $\delta$/ppm 8.27 (d, 2H, $J$ = 9.0 Hz), 7.28 (d, 2H, $J$ = 9.1 Hz), 2.61–2.58 (t, 2H, $J$ = 7.36 Hz), 1.84–1.77 (m, 2H), 1.07–1.04 (t, 3H, $J$ = 7.40 Hz).

#### 2.2.3. 4-Nitrophenyl hexanoate (**3**)

The reaction was carried out following the general procedure starting with 16 mmol of hexanoic acid, yielding 3.0 g (79.1%) of **3**. Pale yellow oil; TLC: $R_f$ = 0.65 (petroleum ether/ethyl acetate, 20/1, v/v); $^1$H NMR (500 MHz, $CDCl_3$): $\delta$/ppm 8.27 (d, 2H, $J$ = 9.0 Hz), 7.28 (d, 2H, $J$ = 9.1 Hz), 2.62–2.59 (t, 2H, $J$ = 7.50 Hz), 1.80–1.74 (m, 2H), 1.41–1.26 (m, 4H), 0.95–0.92 (t, 3H, $J$ = 6.92 Hz).

#### 2.2.4. 4-Nitrophenyl decanoate (**4**)

The reaction was carried out following the general procedure starting with 16 mmol of decoic acid, yielding 5.85 g (80.1%) of **4**. Pale yellow solid; TLC: $R_f$ = 0.66 (petroleum ether/ethyl acetate, 20/1, v/v); $^1$H NMR (500 MHz, $CDCl_3$): $\delta$/ppm 8.27 (d, 2H, $J$ = 9.0 Hz), 7.28 (d, 2H, $J$ = 9.2 Hz), 2.61–2.58 (t, 2H, $J$ = 7.50 Hz), 1.78–1.75 (m, 2H), 1.43–1.29 (m, 12H), 0.90–0.87 (t, 3H, $J$ = 6.75 Hz).

#### 2.2.5. 4-Nitrophenyl tetradecanoate (**5**)

The reaction was carried out following the general procedure starting with 16 mmol of tetradecanoic acid, yielding 4.1 g (73.3%) of **5**. Pale yellow solid; TLC: $R_f$ = 0.69 (petroleum ether/ethyl acetate, 20/1,v/v); $^1$H NMR (500 MHz, $CDCl_3$): δ/ppm 8.27 (d, 2H, $J$ = 9.1 Hz), 7.27 (d, 2H, $J$ = 11.1 Hz), 2.61–2.58 (t, 2H, $J$ = 7.50 Hz), 1.78–1.75 (m, 2H), 1.43–1.26 (m, 20H), 0.90–0.87 (t, 3H, $J$ = 6.86 Hz).

#### 2.2.6. 4-Nitrophenyl stearate (**6**)

The reaction was carried out following the general procedure starting with 16 mmol of octadecanoic acid, yielding 4.8 g (73.9%) of **6**. Pale yellow solid; TLC: $R_f$ = 0.71 (petroleum ether/ethyl acetate, 20/1, v/v); $^1$H NMR (500 MHz, $CDCl_3$): $\delta$/ppm 8.27 (d, 2H, $J$ = 9.0 Hz), 7.28 (d, 2H, $J$ = 10.1 Hz), 2.61–2.58 (t, 2H, $J$ = 7.49 Hz), 1.79–1.73 (m, 2H), 1.41–1.26 (m, 28H), 0.89–0.87 (t, 3H, $J$ = 6.82 Hz).

#### 2.2.7. 4-Nitrophenyl 4-methylpentanoate (**7**)

The reaction was carried out following the general procedure starting with 16 mmol of 4-methyl pentanoic acid, yielding 3.2 g (84.4%) Pale yellow solid; TLC: $R_f$ = 0.58 (petroleum ether/ethyl

acetate, 20/1, v/v); $^1$H NMR (500 MHz, $CDCl_3$): δ/ppm 8.27 (d, 2H, J = 9.1 Hz), 7.28 (d, 2H, J = 9.3 Hz), 2.62–2.59 (t, 2H, J = 7.48 Hz), 1.68–1.66 (m, 3H), 0.97 (d, 6H, J = 6.05 Hz).

### 2.2.8. 4-Nitrophenyl 3-methylpentanoate (8)

The reaction was carried out following the general procedure starting with 16 mmol of 3-methyl pentanoic acid, yielding 3.4 g (89.7%) of **8**. Pale yellow oil; TLC: $R_f$ = 0.73 (petroleum ether/ethyl acetate, 20/1, v/v); $^1$H NMR (500 MHz, $CDCl_3$): δ/ppm 8.27 (d, 2H, J = 7.12 Hz), 7.27 (d, 2H, J = 9.1 Hz), 2.63–2.58 (m, 1H), 2.42–2.38 (m, 1H), 2.05–2.01 (m, 1H), 1.50–1.44 (m, 1H), 1.37–1.32 (m, 1H), 1.05 (d, 3H, J = 6.69 Hz), 0.98–0.95 (t, 3H, J = 7.43 Hz).

### 2.2.9. 4-Nitrophenyl 2-methylpentanoate (9)

The reaction was carried out following the general procedure starting with 16 mmol of 2-methyl pentanoic acid, yielding 3.4 g (89.7%) of **9**. Pale yellow oil; TLC: $R_f$ = 0.63 (petroleum ether/ethyl acetate, 20/1, v/v); $^1$H NMR (500 MHz, $CDCl_3$): δ/ppm 8.27 (d, 2H, J = 9.0 Hz), 7.27 (d, 2H, J = 8.9 Hz), 2.75–2.71 (m, 1H), 1.83–1.77 (m, 1H), 1.60–1.53 (m, 1H), 1.49–1.41 (m, 2H) 1.31 (d, 3H, J = 7.0 Hz), 0.99–0.96 (t, 3H, J = 7.30 Hz); $^{13}$C NMR (500 MHz, $CDCl_3$): δ/ppm 174.99, 156.29, 145.81, 125.75, 123.05, 40.04, 36.31, 20.99, 17.40, 14.53.

### 2.2.10. 4-Nitrophenyl cyclopropanecarboxylate (10)

The reaction was carried out following the general procedure starting with 16 mmol of cyclopropanecarboxylic acid, yielding 2.4 g (72.7%) of **10**. Pale yellow solid; TLC: $R_f$ = 0.51 (petroleum ether/ethyl acetate, 10/1, v/v); $^1$H NMR (500 MHz, $CDCl_3$): δ/ppm 8.27 (d, 2H, J = 9.1 Hz), 7.29 (d, 2H, J = 9.1 Hz), 1.90–1.85 (m, 1H), 1.23–1.19 (m, 2H), 1.11–1.08 (m, 2H).

### 2.2.11. 4-Nitrophenyl cyclobutanecarboxylate (11)

The reaction was carried out following the general procedure starting with 16 mmol of cyclobutanecarboxylic acid, yielding 3.0 g (84.8%) of **11**. Pale yellow solid; TLC: $R_f$ = 0.54 (petroleum ether/ethyl acetate, 40/1, v/v); $^1$H NMR (500 MHz, $CDCl_3$): δ/ppm 8.28 (d, 2H, J = 9.1 Hz), 7.28 (d, 2H, J = 9.1 Hz), 3.46–3.39 (m, 1H), 2.49–2.33 (m, 4H), 2.14–1.98 (m, 2H).

### 2.2.12. 4-Nitrophenyl cyclopentanecarboxylate (12)

The reaction was carried out following the general procedure starting with 16 mmol of cyclopentanecarboxylic acid, yielding 3.2 g (85.1%) of **12**. Pale yellow oil; TLC: $R_f$ = 0.63 (petroleum ether/ethyl acetate, 20/1, v/v); $^1$H NMR (500 MHz, $CDCl_3$): δ/ppm 8.27 (d, 2H, J = 9.1 Hz), 7.27 (d, 2H, J = 9.0 Hz), 3.05–2.99 (m, 1H), 2.07–1.92 (m, 4H), 1.81–1.66 (m, 4H).

### 2.2.13. 4-Nitrophenyl cyclohexanecarboxylate (13)

The reaction was carried out following the general procedure starting with 16 mmol of cyclohexanecarboxylic acid, yielding 3.6 g (90.4%) of **13**. Pale yellow oil; TLC: $R_f$ = 0.76 (petroleum ether/ethyl acetate, 20/1, v/v); $^1$H NMR (500 MHz, $CDCl_3$): δ/ppm 8.27 (d, 2H, J = 9.1 Hz), 7.26 (d, 2H, J = 9.0 Hz), 2.63–2.57 (m, 1H), 2.09–2.06 (m, 2H), 1.86–1.82 (m, 2H), 1.72–1.7 (m, 1H), 1.64–1.58 (m, 2H), 1.42–1.28 (m, 3H).

### 2.2.14. 4-Nitrophenyl benzoate (14)

The reaction was carried out following the general procedure starting with 16 mmol of benzoic acid, yielding 3.3 g (84.9%) of **14**. Pale yellow solid; TLC: $R_f$ = 0.50 (petroleum ether/ethyl acetate, 20/1, v/v); $^1$H NMR (500 MHz, $CDCl_3$): δ/ppm 8.34 (d, 2H, J = 9.1 Hz), 8.21 (d, 2H, J = 7.3 Hz), 7.71–7.68 (m, 1H), 7.57–7.54 (m, 2H), 7.43 (d, 2H, J = 9.1 Hz).

### 2.2.15. 4-Nitrophenyl 3-phenylpropanoate (15)

The reaction was carried out following the general procedure starting with 16 mmol of 3-phenylpropanoic acid, yielding 3.5 g (80.7%) of **15**. Pale yellow solid; TLC: $R_f$ = 0.54 (petroleum ether/ethyl acetate, 20/1, v/v); $^1$H NMR (500 MHz, $CDCl_3$): δ/ppm 8.25 (d, 2H, J = 9.1 Hz), 7.35–7.32 (m, 2H), 7.27–7.25 (m, 3H), 7.19 (d, 2H, J = 9.1 Hz), 3.10–3.07 (t, 2H, J = 7.57 Hz), 2.96–2.92 (m, 2H, J = 7.57 Hz).

### 2.2.16. 4-Nitrophenyl 2-phenylpropanoate (16)

The reaction was carried out following the general procedure starting with 16 mmol of 2-phenylpropanoic acid, yielding 3.8 g (87.6%) of **16**. Pale yellow solid; TLC: $R_f$ = 0.49 (petroleum ether/ethyl acetate, 20/1, v/v); $^1$H NMR (500 MHz, $CDCl_3$): δ/ppm 8.22 (d, 2H, J = 9.0 Hz), 7.39 (d, 4H, J = 4.3 Hz), 7.34–7.31 (m, 1H), 7.18 (d, 2H, J = 9.0 Hz), 4.02–3.97 (m, 1H), 1.63 (d, 3H, J = 7.1 Hz).

### 2.2.17. 4-Nitrophenyl 2-phenylbutanoate (17)

The reaction was carried out following the general procedure starting with 16 mmol of 2-phenylbutanoic acid, yielding 3.5 g (76.8%) of **17**. Pale yellow oil; TLC: $R_f$ = 0.59 (petroleum ether/ethyl acetate, 20/1, v/v); $^1$H NMR (500 MHz, $CDCl_3$): δ/ppm 8.24 (d, 2H, J = 9.1 Hz), 7.41–7.38 (m, 4H), 7.34–7.32 (m, 1H), 6.88 (d, 2H, J = 9.1 Hz), 3.74–3.71 (t, 1H, J = 7.68 Hz), 2.26–2.20 (m, 1H), 1.96–1.90 (m, 1H), 1.01–0.98 (t, 3H, J = 7.36 Hz).

## 2.3. Enzyme assay

All enzyme samples were prepared by weighing the fresh solid sample and diluting into a solution of 100 mg $mL^{-1}$ in KPB (aq. 100 mM potassium phosphate, pH 7.0). The protein concentration was measured by Coomassie Brilliant Blue method. Based on the protein concentration, the solution of enzyme samples was diluted to a stock solution of 1 mg protein $mL^{-1}$. All substrates were diluted with DMSO from 100 mM stock solutions. The solutions could be stored at 4 °C for several months without notable decomposition.

Assay was carried out in 96-well clear-bottom polystyrene microtiter plates. A KPB solution of 180 μL containing the enzyme at an appropriate concentration was first placed in the wells. The appropriate concentration of enzyme was obtained by pre-measurements, in which the initial reactivity velocity of enzyme samples could be tested accurately. The microtiter plate was incubated at 30 °C for 2 min. Then the substrate solutions (20 μL) were added simultaneously into each well. The microtiter plate was immediately placed in a Microplate Spectrophotometer (BioTek® PowerWave XS2) and the reaction was monitored at 405 nm for over 3 min. The spontaneous hydrolysis of each substrate was measured in absence of enzyme under the same condition.

The concentration of p-nitrophenol is linear under the absorption at 405 nm. The calibration equation is $A_{405} = 8.00 \times [pNP] + 0.055$, in which $A_{405}$ represents the absorption at 405 nm and the [pNP] represents the concentration of p-nitrophenol with unit of mmol $L^{-1}$. Therefore, the primary Abs-versus-time data were first converted into product-versus-time using the calibration equation. The steepest linear portion (<10% conversion) of each curve was then used to calculate the apparent initial reaction rate in each well. Given the spontaneous hydrolysis of substrates, the real initial reaction rates of enzymes were obtained by subtraction of the spontaneous hydrolysis rate from the apparent rate.

## 3. Results and discussion

### 3.1. Composing of substrate array

Esters of nitrophenol have been known for many years as chromogenic substrates for lipases and esterases. Although assays based on such substrates cannot enable direct testing of the reaction of a particular substrate of interest, they are by far the most reliable, the easiest method to directly realize high-throughput screening [11,14,15]. Therefore, we decided to construct a library of chromogenic derivatives as probes for fingerprinting lypolytic enzymes, including lipases and esterases.

This substrate array consists of 17 esters of *p*-nitrophenol (Fig. 1) prepared by acylation with corresponding carboxylic acids, which can be divided into four groups of *p*-nitrophenyl esters: (I) the linear aliphatic esters with various chain lengths (**1**–**6**), (II) the branched aliphatic esters with different position of branched chain (**7**–**9**), (III) the cycloalkyl carboxylic esters with different ring sizes (**10**–**13**), and (IV) the aromatic esters (**14**–**17**). This substrate array has more diversity in carboxylic acid part of esters than those reported previously [6,16]. It is obvious that the activity fingerprints of lipases and esterases recorded by our substrate array reveal more comprehensive and systematical profile of substrates specificity. In future, we would add the optically pure substrates into the array so as to record the stereoselectivity of lipases or esterases in a high-through-put mode.

### 3.2. Fingerprints of lypolytic enzymes

The B-lipase from *C. antarctica* (CALB) is one of the most widely used lipases, because of its high activity and stability [18]. The amino acid sequence and 3D structure of CALB have been resolved by Uppenberg [23]. Furthermore, the catalytic characteristics, such as specific activity and selectivity, have also been well studied. So the CALB was used as the model enzyme to verify whether the fingerprint recorded by this substrates array could predict the substrate preference and potential application of the tested enzyme.

The fingerprint of CALB (Fig. 2A) revealed that the short fatty acid ester (C2, compound **1**) was relatively poor substrate whereas other middle and long straight-chain aliphatic esters were accepted almost equally well (C6–C18, compounds **3**–**6**). In addition, the branch-chained aliphatic esters reacted slowly, especially the one with branched chain in the vicinity of ester bond (compounds **8** and **9**). These results agreed with the previous studies [19,20]. The reason could be possibly explained by the fact that the catalytically active serine residue of CALB is located at the bottom of a narrow and deep pocket approximately 10 × 4 Å wide and 12 Å deep [21], which also could be responsible for unreactivity of the substrates with the bulky groups close to the enzyme-activity ester bond such as the aromatic esters, compounds **14**, **16** and **17**, and the cycloalkyl ester compound **13**. Palomo et al. also found that aromatic esters were 100-fold less active than aliphatic esters against CALB [18]. The enzyme CALB also showed apparent reactivity against compound **15**, which might result from that the aromatic group was not proximity to the ester bond. The compound **11** with four-member ring and the compound **12** with five-member ring reacted well against CALB.

Combining all together, the fingerprint obtained by our substrate array could correctly indicate the substrate preference of tested enzyme, and can also be applied for the prediction of industrial applications.

Three home-made enzymes in our laboratory were measured by this substrate array. We wanted to find some potential applications in organic synthesis of these enzymes.

A putative lactam hydrolase, EstF4K, minded from metagenomic library, was found with high lipolytic activity in our research,

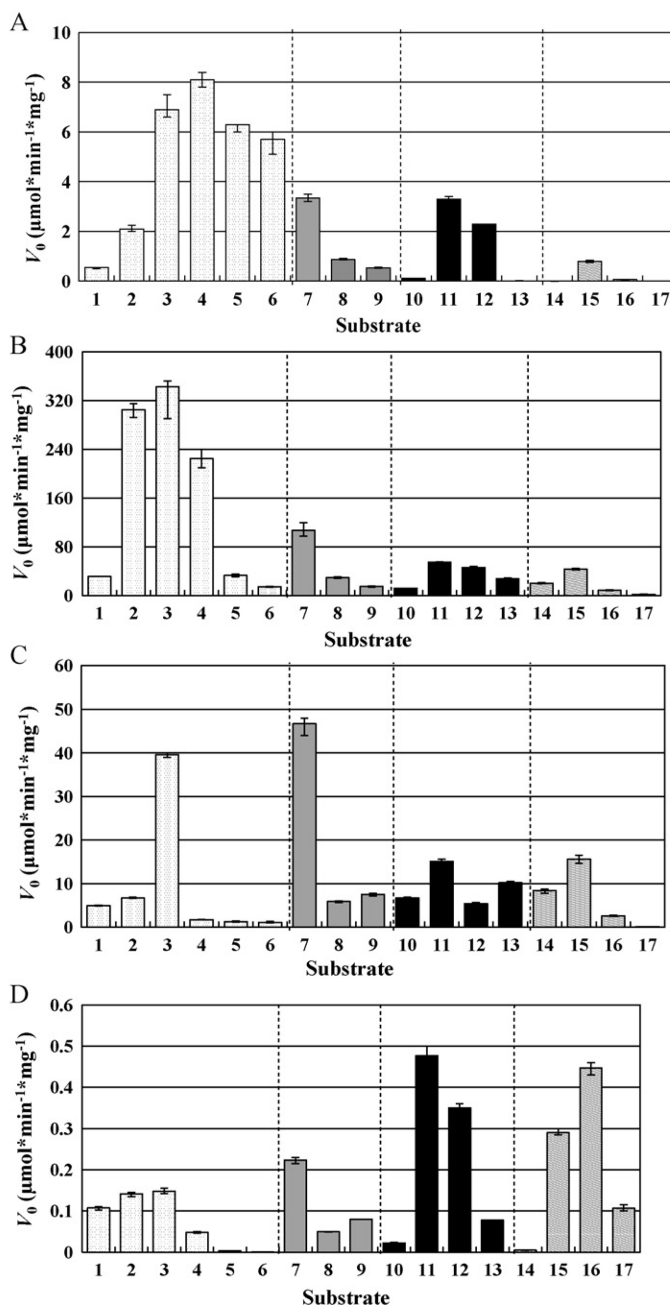


**Fig. 2.** Activity fingerprints of enzymes with array of chromogenic esters of *p*-nitrophenol and various carboxylic acids, as illustrated in Fig. 1. (A) Activity fingerprint of lipase B from *Candida antarctica* (CALB, Novozyme). (B) Activity fingerprint of EstF4K, which was predicted as a putative lactam hydrolase but no lactamase activity was detected with ampicillin as a substrate. (C) Activity fingerprint of PnbA, which was identified a robust biocatalyst for enzymatic resolution of *dl*-methyl acetate [23]. (D) Activity fingerprint of SrfAD, which was one of the surfactin synthetase subunits [24]. Reaction conditions: 100 mM KPB, pH 7.0, 100 μM substrate, 10% (v/v) DMSO, 30 °C. The concentration of enzyme samples against each substrate was obtained by pre-measurements (CALB: 0.1–10 μg mL$^{-1}$, EstF4K: 0.01–1 μg mL$^{-1}$, PnbA: 0.05–5 μg mL$^{-1}$, SrfAD: 5–200 μg mL$^{-1}$). Reactions (0.2 mL each) were followed spectrophotometrically at 405 nm over 3 min in 96-well plates using a microtiter plate reader. The steepest linear portion (<10% conversion) of each time curve was used to calculate the apparent reaction rate according to a calibration curve with pure *p*-nitrophenol. The spontaneous reaction rates in the absence of enzyme were measured under the same conditions, and subtracted from the apparent rate to obtain the real initial reaction rates.

and was identified as an esterase (unpublished data). The fingerprint of this enzyme supports this conclusion (Fig. 2B), and also indicated that this enzyme had broad substrate spectrum, with strong preference to the linear aliphatic esters with short- or middle-chain (C4–C10). So it might have wide application in industrial productions involving short- or middle-chain fatty acid. The EstF4K also had a relatively low reactivity against the branched esters, cycloalkyl carboxylic esters, especially the compound **10** with three-member ring, and aromatic esters. This suggests that further optimization of reaction condition may enable this enzyme to hydrolyze those kinds of esters.

An esterase, PnbA, cloned from *Bacillus subtilis*, was reported previously to have high activity against methyl esters [22]. From the fingerprint of enzyme PnbA (Fig. 2C), it was clearly proved that the C6-acid esters, compounds **3** and **7**, had the highest reactivity compared to other substrates. Furthermore, it was found interestingly that the branched C6-ester (compound **7**) was more reactive than straight C6-ester (compound **3**), although other branched C6-esters (compounds **9** and **10**) were much less reactive. It might be evident that the branched chain far away from ester bond in acyl group has less effect on the activity of PnbA. Against the cycloalkyl carboxylic and aromatic esters, the enzyme PnbA displayed quite similar reactivity behaviors with EstF4K. Therefore, both the two enzymes probably have a parallel potential for industrial application.

From the activity fingerprint of SrfAD (Fig. 2D), an interesting result was observed that SrfAD preferred cycloalkyl carboxylic esters and aromatic esters instead of aliphatic esters, which was opposite from the other two tested enzymes. In addition, this enzyme showed relatively high reactivity against the compound **16** which is an aromatic ester with methyl substitution on α-position, and also exhibited good reactivity against hard-to-react compound **17** which is an aromatic ester with ethyl substitution on α-position. So it was proved that this enzyme might have great potential applications in the hydrolysis of aromatic esters with substitutions on α-carbon. The esters with 4-membered ring (compound **11**) and 5-membered ring (compound **12**) reacted strongly against the SrfAD. Furthermore, the ester with 3- and 6-member rings showed reactivity over this enzyme. It recommends that this enzyme is possible to be used for the enzymatic hydrolysis of cycloalkyl carboxylic esters. From the activity of SrfAD against fatty acid esters, it was also clearly known that SrfAD preferred short- or middle-chain (C6–C10) esters, and the branched ester (compound **7**) reacted slightly faster than linear one (compound **3**) when the branched chain was not close to ester bond.

## 4. Conclusion

We have constructed a substrate array consisting of a set of *p*-nitrophenyl esters with various carboxylic acids to fingerprint lipases or esterases in high-throughput format. The data collected by this assay was then interpreted in terms of description of the enzyme substrate preferences which can be used to predict the application scope of enzyme in organic synthesis.

The activity fingerprint of CALB recorded by this method corresponds well to the substrate preference and application scope in organic synthesis studied previously, indicating the reliability of the assay using this high-throughput approach.

Three house-made enzymes in our laboratory were fingerprinted by the substrate array described herein. It was found that SrfAD had very different substrate preference from other tested enzymes, exhibiting a relatively high reactivity against the esters with bulky acids which are important chemicals in industry. It suggests that SrfAD has the greatest application potential among the tested enzymes and further investigations on the stereoselectivity of SrfAD are being carried out in our laboratory.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2011.07.010.

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