A practical high-throughput screening system for feruloyl esterases: Substrate design and evaluation

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**Abstract**

Feruloyl esterases (FAEs) are a group of important industrial enzymes, which could hydrolyze the ester bonds between hydroxycinnamic acids and arabinose or galactose in hemicellulose present in plant cell walls. To establish a practical high-throughput screening system of feruloyl esterases, two new substrates with modified chromo- or fluoro-phore, 2-chloro-4-nitrophenyl ferulate (CNPF; 1b) and umbelliferyl 5-O-feruoyl-α-L-arabinofuranoside (UFA; 2a), were designed and synthesized. Both substrates provided significantly improved signal-to-noise ratio compared with previously known structural analogues. The chromogenic substrate CNPF could be readily adapted to the high-throughput screening of feruloyl esterase A from *Aspergillus niger*, one of the most studied FAEs, with the coefficient of variance of as low as 10.3% as determined for a single sequence library.

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1. Introduction

Feruloyl esterases (FAEs, EC 3.1.1.73) consist of a diverse group of hydrolyses catalyzing the cleavage of ester bonds between arabinose or galactose and hydroxycinnamic acid, and act as the integral part of the enzyme system involved in the complete hydrolysis of hemicellulose. The last decade, in particular, has seen considerable efforts to the application of FAEs in biomass degradation, food and pharmaceutical industries [1]. One of the most extensively studied FAEs, feruloyl esterase A (AnFaeA) from *Aspergillus niger* [2], has widely been applied to enhance the yield of sugar products from lignocellulose [3,4].

FAEs generally display broad substrate specificities with the requirement of phenylalkanoate substrates but have different specificity on a specific group at a specific location [5]. However, they always have specificity on monomeric ferulate moiety and are active on its synthetic substrate analogues. The assay of FAEs activity commonly depends on HPLC, using enzymatic hydrolysis of a variety of natural and synthetic compounds, such as hydroxycinnamic esterified polysaccharide, methyl ferulate and their analogues [6–9]. Recently, several chlorogenic substrates of FAEs have been reported for quantitative assays, such as 4-nitrophenyl ferulate [10,11] (Fig. 1), 2-O- and 5-O-feruloylated 4-nitrophenyl-α-L-arabinofuranosides [12], feruloylated p-nitrophenyl α-L-arabinofuranosides and β-D-xylpyranosides [13], and 2-chloro-4-nitrophenyl 5-O-hydroxycinnamoyl-α-L-arabinofuranoside (Fig. 1) [14] as well as substrates based on (2-chloro-4-nitrophenyl)-1,2,4-butanetriol that require additional non-enzymatic reactions of periodate oxidation and beta-elimination to trigger the liberation of the chromophore [15]. A microplate screening based on the pH indicator, 4-nitrophenol, has also been reported [16]. However, the establishing and evaluation of a reliable and practical high-throughput screening system is still required, which would otherwise facilitates the directed evolution of FAEs, as well as the novel enzyme mining from environmental microorganisms [17,18].

In the current work, we designed and synthesized two new mimic substrate 2-chloro-4-nitrophenyl ferulate (CNPF) and umbelliferyl-5-O-hydroxycinnamoyl-α-L-arabinofuranoside (UFA) (Fig. 1), and developed a high-throughput screening system for FAEs. Both of them and their individual structural analogue reported were evaluated with feruloyl esterase A from *A. niger* as a model enzyme. The result shows that the substrate CNPF could be readily obtained and applied to the high-throughput screening of FAEs with desirable sensitivity, while the substrate UFA is difficult to synthesize but has a higher sensitivity, which may be used

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Fig. 1. The substrates of FAES: structures of 1a/1b (A), and synthesis of 2a/2b (B). Compounds 1b and 2a were newly designed substrates. 1a: 4-nitrophenol ferulate (pNPF); 1b: 2-chloro-4-nitrophenol ferulate (CNPF); 2a: umbelliferyl-5-O-feruloyl-L-arabinofuranoside (UFA); 2b: 2-chloro-4-nitrophenol 5-O-feruloyl-L-arabinofuranoside (CNPF). Reagents and conditions: (i) BF₃·Et₂O/TEA, 7-hydroxy-2H-chromen-2-one (for 2a); or BF₃·Et₂O, 2-chloro-4-nitrophenol (for 2b), −10°C; (ii) TEA/CH₃OH, reflux (for 2a), or NaOCH₃, CH₃OH, rt (for 2b); (iii) Pyridine, 4-acetyl feruloyl chloride, toluene, 0°C − rt; 4h; (iv) pyrrolidine/CH₂Cl₂ (1:12), rt. The enzymatic cleavage sites of the substrates are marked with dotted lines.

for the detection of trace amount of FAES existing in biological samples.

2. Materials and methods

2.1. Chemicals

Ferulic acid, 2-chloro-4-nitrophenol and umbellifereone were purchased from Alfa Aesar (Tianjin, China). 4-Nitrophenol ferulate (pNPF) (1a) was synthesized with established method [11]. 1,2,3,5-Tetra-O-acetyl-α-L-arabinofuranose (3) was synthesized from l-arabinose according to the literature [19]. Spectra of 1b, 2a, 2b, 4a, 4b, 5a, 5b are provided in the Supplementary information.

2.2. Synthesis of 2-chloro-4-nitrophenyl ferulate (1b)

CNPF was synthesized with established method [11] except that 2-chloro-4-nitrophenol was used instead of 4-nitrophenol. The product was purified by column chromatography to give 1b as a light yellow solid (2.79 g, 80%). 1H NMR (CDCl₃, 600 MHz) δ 3.97 (s, 3H), 6.50 (d, 1H, J 15.8 Hz), 6.97 (d, 1H, J 8.1 Hz), 7.11 (d, 1H, J 1.7 Hz), 7.18 (dd, 1H, J 1.7 Hz and J 8.1 Hz), 7.44 (d, 1H, J 9.1 Hz), 7.88 (d, 1H, J 15.8 Hz), 8.21 (dd, 1H, J 2.7 Hz and J 9.1 Hz), 8.39 (d, 1H, J 2.7 Hz); ESI-HRMS exact mass: m/z calcld. for C₁₆H₁₂CINaO₅ [M+Na] 372.0245; found 372.0243.

2.3. Synthesis of umbelliferyl-5-O-feruloyl-α-L-arabinofuranoside (2a)

Umbelliferyl 2,3,5-tri-O-acetyl-α-L-arabinofuranoside (4a): To a solution of 3 (1.0 g, 1.34 mmol) in CH₂Cl₂ (5 mL), 7-hydroxy-2H-1-benzopyran-2-one (0.25 g, 1.57 mmol), Et₃N (0.24 mL, 1.57 mmol) and BF₃·OEt₂ (1 mL, 7.85 mmol), were successively added. The reaction mixture was stirred for 19 h at 0°C, then diluted with CH₂Cl₂ (20 mL) and washed with a 5% solution of aqueous Na₂CO₃ until the solution was completely colourless. The aqueous layers were extracted with CH₂Cl₂, and the product was purified by flash chromatography to yield 4a as a white foam (0.71 g, 52%); [α]D20 = −96 (c 20.6, CHCl₃); 1H NMR (600 MHz, CDCl₃) δ 2.11, 2.16, 2.17 (3 × s, 9H, 3× CH₃O); 4.27−4.30 (m, 1H, H-5a), 4.40−4.45 (m, 2H, H-4, H-5b), 5.14 (d, 1H, J 3.2 Hz, H-3), 5.40 (s, 1H, H-1), 5.78 (s, 1H, H-1), 6.29 (d, 1H, J 9.2 Hz, H=CH=CHO), 6.98 (dd, 1H, J 8.5 Hz, J 2.2 Hz, H-Ar), 7.42 (d, 1H, J 8.5 Hz, H-Ar), 7.67 (d, 1H, J 9.5 Hz, H=CH=CHO); 13C NMR (150 MHz, CDCl₃): δ 20.6 (CH₃O), 20.7 (CH₃O), 20.9 (CH₃O), 62.9 (C-5), 67.8, 81.4, 81.9 (C-2, C-3, C-4), 103.9 (C-1), 105.6 (HC=CHO), 113.7 (C-Ar), 113.9 (C-Ar), 114.1 (C-Ar), 128.8 (C-Ar), 143.1 (C-Ar), 155.4 (C-Ar), 158.8 (C-Ar), 160.7 (HC=CHO), 169.5 (CH₃O), 170.0 (CH₃O); ESI-HRMS exact mass: m/z calcld for C₂₀H₂₀NaO₁₀ [M+Na] 443.0954; found 443.0950.

Umbelliferyl α-L-arabinofuranoside (5a): A mixture of 4a (0.15 g, 0.347 mmol) and Et₃N (3.5 mL, 0.5 M in MeOH) was refluxed for 2 h. The reaction mixture was left overnight at room temperature, and was neutralized with silica. The crude product was purified by flash column chromatography to give compound 5a as a white foam (0.082 g, 80%); [α]D20 = −195 (c 0.87, CH₂Cl₂); 1H NMR (600 MHz, CDCl₃): δ 3.68 (dd, 1H, JH₂,H₃A 4.8 Hz, JH₃B, B 12.1 Hz, H-5a), 3.78 (dd, 1H, J₅B, J₅A 4.3 Hz, J₆B,J₆A 6.8 Hz, H-5b), 4.01 (dd, 1H, J₂, J₅B 4.1 Hz, J₃, J₃₂ 6.2 Hz, H-3), 4.05−4.08 (m, 1H, H-4), 4.28 (dd, 1H, J₂, J₃₂ 4.0 Hz, H-2), 5.63 (d, 1H, J₁₂, J₁₉ 1.9 Hz, H-1), 6.26 (d, 1H, J 9.5 Hz, H=CH=CHO), 7.02−7.04 (m, 2H, H-2, H-5), 7.53 (d, 1H, J 8.4 Hz, H-Ar), 7.87 (d, 1H, J 9.5 Hz, H=CH=CHO); 13C NMR (150 MHz, CDCl₃): δ 20.9, 20.7, 20.9 (CH₃O), 62.9 (C-5), 67.8, 81.4, 81.9 (C-2, C-3, C-4), 103.9 (C-1), 105.6 (HC=CHO), 113.7 (C-Ar), 113.9 (C-Ar), 114.1 (C-Ar), 128.8 (C-Ar), 143.1 (C-Ar), 155.4 (C-Ar), 158.8 (C-Ar), 160.7 (HC=CHO), 169.5 (CH₃O), 170.0 (CH₃O); ESI-HRMS exact mass: m/z calcld for C₂₀H₂₀NaO₁₀ [M+Na] 443.0954; found 443.0950.

2.4. Synthesis of 2-chloro-4-nitrophenyl 5-O-feruloyl-α-L-arabinofuranoside (2b)

2-Chloro-4-nitrophenyl 2,3,5-tri-O-acetyl-α-L-arabinofuranoside (4b): To a solution of 3 (1.3 g, 4.1 mmol) in dry CH₂Cl₂ (35 mL) containing fresh activated 4Å molecular sieves (1.3 g) was added
2-chloro-4-nitrophenol (0.72 g, 4.1 mmol). The reaction was then cooled to –20 °C and BF₃·Et₂O (3.3 mL, 25.9 mmol) was added dropwise. Then the mixture was slowly warmed up to room temperature and stirred for additional 4 h. The mixture was filtered through Celite, diluted with CH₂Cl₂, washed successively with 5% aq. Na₂CO₃ solution and H₂O, and dried with anhydrous Na₂SO₄. The crude product was purified by flash column chromatography to give compound 4b as a white foam (1.11 g, 63%); [α]D⁰^20 = −129 (c 2.0, CHCl₃); ¹H NMR (600 MHz, CDC1₃): δ 2.11, 2.15, 2.17 (3 s, 5H, 3 × CH₃COO); 4.29 (dd, 1H, J₃,5 6.8 Hz, J₅,6b 13.2 Hz, H-5a), 4.44–4.45 (m, 2H, H-4, H-5b), 5.12 (d, 1H, J₃,4 3.5 Hz, H-3), 5.46 (s, 1H, H-2), 5.86 (1H, s, H-1), 7.28 (d, 1H, J 9.1 Hz, H-Ar); 8.14 (dd, 1H, J 9.1, 2.7 Hz, H-Ar), 8.31 (d, 1H, J 2.7 Hz, H-Ar).

2-Chloro-4-nitrophenyl-α-l-arabinofuranoside (5b): Compound 4b (1.16 g, 2.7 mmol) was suspended in dry MeOH (8 mL), and NaOMe (17 mg, 0.31 mmol, 0.12 equiv.) was added at 0 °C. After 2 h, the reaction was neutralized with silica. The crude product was purified by flash column chromatography to give compound 5b as a yellowish solid (0.612 g, 77%); [α]D⁰^20 = −208 (c 0.2, CH₂OH); ¹H NMR (600 MHz, CD₂OD): δ 3.67 (dd, 1H, J₃,4 5.2 Hz, J₃,5b 12.1 Hz, H-5a), 3.78 (dd, 1H, J₃,4 3.3 Hz, J₃,5b 12.1 Hz, H-5b), 4.02 (dd, 1H, J₃,4 4.1 Hz, J₂,1 6.6 Hz, H-3), 4.07–4.10 (m, 1H, H-4), 4.39 (dd, 1H, J₂,1 1.9 Hz, J₃,4 4.4 Hz, H-2), 5.71 (d, 1H, J₁,2 1.5 Hz, H-1), 7.44 (d, 1H, J 9.1 Hz, H-Ar), 8.16 (dd, 1H, J 9.1, 2.9 Hz, H-Ar), 8.29 (d, 1H, J 2.5 Hz, H-Ar).

2-Chloro-4-nitrophenyl 5-O-feruloyl-α-l-arabinofuranoside (2b): The procedure was essentially the same as that for compound 2a. Under N₂ atmosphere, acid chloride (0.3 g, 7.28 mmol) in toluene (35 mL) was added dropwise to a stirring solution of 5b (0.45 g, 1.47 mmol) in pyridine (10 mL) at 0 °C. Once the addition was complete (approximately 25 min), the mixture was left undisturbed overnight. The resulting mixture was concentrated to a syrup and twice diluted with toluene and evaporated. The solid reaction product was dissolved in CH₂Cl₂ (9 mL), pyrrolidine (1.1 mL, 14.7 mmol, 10 equiv.) was added, and the reaction mixture was stirred at room. After 2 h, the reaction was complete and was neutralized with silica. The crude product was evaporated and purified by flash column chromatography (CH₂Cl₂–MeOH; 99:1, v/v) to give the title compound 2b as a yellowish solid (0.32 g, 50%); [α]D⁰^20 = −51 (c 0.14, CH₂OH); ¹H NMR (600 MHz, CD₂OD): δ 3.88 (s, 3H, OMe), 4.07 (dd, 1H, J₃,4 4.3 Hz, J₃,5b 6.8 Hz, H-3), 4.28–4.34 (m, 2H, H-2, H-5a, H-5b), 4.43–4.47 (m, 2H, H-4–H-5), 5.75 (d, 1H, J₁,2 1.6 Hz, H-1), 6.38 (d, 1H, J 15.8 Hz, Hc=CH), 6.80 (d, 1H, J 7.7 Hz, H-Ar), 7.06 (dd, 1H, J 1.7 Hz, 8.3 Hz, H-Ar), 7.18 (d, 1H, J 15.5 Hz, H-Ar), 7.42 (d, 1H, J 9.1 Hz, H-Ar), 7.64 (d, 1H, J 15.8 Hz, Hc=CH), 8.18 (dd, 1H, J 2.7 Hz, 9.3 Hz, H-Ar), 8.30 (d, 1H, J 2.7 Hz, H-Ar); ESI-MS: m/z calcd for [C₉H₁₀O₈Na⁺]⁺ 504.07; found 504.01.

2.5. Expression of AnFaeA in Pichia pastoris KM71

The enzyme was heterologously expressed in Pichia pastoris KM71 [20] as an extracellular protein following established method [21] in a miniature format. Briefly, the recombinant plasmid pGAPZaA/FaeA-D93G/S187F [21] encoding a variant of FaeA from A. niger CIB 423.1 was linearized by BspHI digestion and electro-transformed into P. pastoris KM71 (Invitrogen, Carlsbad, CA, USA). The transformants were grown on YPD medium containing 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose supplemented with 1 M sorbitol and 100 µg mL⁻¹ zeocin. After incubation at 30 °C for 72 h, single colonies were picked with sterile toothpicks and inoculated into 150 µL YPD medium on sterile 96-well microplates, and incubated for 48 h at 30 °C with gyratory shaking at 280 rpm on an INFORIS Multitron incubator shaker (INFORIS HT, Switzerland). The plate was subjected to centrifugation at 1800 g for 10 min. The supernatant of each well was transferred into another 96-well plate to assay the enzymatic activity.

AnFaeA-D93G/S187F was purified as described previously using a Bio-scale Mini UNOsphere Q column [21]. Protein estimations were done with the BCA Protein Assay Kit (Beyotime, Shanghai, China) with bovine serum albumin as a standard.

2.6. Measurement of enzymatic activity

To evaluate the feasibility of the four mimic substrates for the high-throughput screening system of FAEs in a 96-well microplate format, the reaction mixture was prepared by mixing 160 µL of 100 mM sodium phosphate buffer (pH 6.4) containing 2.5% Triton X-100 with 10 µL of 20 mM substrate in DMSO [10], followed by the addition of 20 µL supernatant and sufficient amount of α-L-arabinofuranosidase (α-AF) (Megazyme, Wicklow, Ireland) only in case of CNPFa and UFA. The reactions were performed in triplicate at 40 °C for 10 min with shaking at 100 rpm. The minimal amount of α-L-arabinofuranosidase needed per reaction was confirmed under the reaction condition above with different amount of α-L-arabinofuranosidase. The formation of 2-chloro-4-nitrophenol or 4-nitrophenol was measured at 425 nm, and the formation of umbellifereone was determined fluorometrically with λex of 360 nm and λem of 460 nm on a Varioskan Flash microplate reader (Thermo scientific, USA). The signal-to-noise ratio (S/N) of each reaction system was defined as the ratio of signal absorbance to the background noise absorbance. The background noise absorbance was acquired for the same reaction system without enzyme. The specific activity of AnFaeA-D93G/S187F toward each substrate was measured with 0.16 µM purified enzyme and 1 mM substrate.

The kinetic parameters of purified AnFaeA-D93G/S187F toward new substrates 1b and 2a were determined under standard conditions with various substrate concentrations. All measurements were performed in triplicates. Data were fitted to the Michaelis–Menten equation using GraphPad Prism v5.0 (GraphPad Software, San Diego, CA, USA) to generate estimates of apparent Michaelis constant (Km) and catalytic turnover frequency (Kat) values.

3. Results and discussion

3.1. Design and synthesis of mimic substrates

In order to develop the high-throughput screening system of FAEs, we firstly synthesized the previously reported substrate pNPF (1a) (Fig. 1A). And then, we also designed a novel substrate, CNPF (1b) (Fig. 1A) with an almost pH-independent chromophore, 2-chloro-4-nitrophenol, with a view to obtain higher molar extinction coefficient (ε) at neutral pH. The substrate CNPF was easily synthesized through the same synthetic route of substrate pNPF.

According to the previous report [14], AnFaeA displayed over 10-fold catalytic efficiency toward 2-chloro-4-nitrophenyl 5-O-feruloyl-α-L-arabinofuranoside (CNPF) 2b (Fig. 1B) than methyl ferulate due to the resemblance of CNPFa to natural substrates. Thus, we synthesized this substrate, as well as further designed a novel fluorogenic substrate umbelliferyl-5-O-feruloyl-α-L-arabinofuranoside (UFA) 2a (Fig. 1B) with the expectation of achieving better sensitivity.

The synthesis of CNPFa was previously described by Marmuse et al., starting from 1-O-acetyl-2,3,5-tri-O-benzoyl-L-arabinobiofuranoside [14]. The procedure requires the application of various protecting/deprotecting strategies that was quite time-consuming and suffered from poor yield. In order to shorten the lengthy protection/deprotection sequences, 1,2,3,5-tetra-O-acetyl-α-L-arabinofuranoside 3 were used instead of benzoylated counterpart as starting material, which was synthesized from L-arabinose according to the method for the synthesis
of α-arabinofuranose [19]. Then glycolyl donor 3 was coupled with 2-chloro-4-nitrophenol smoothly promoted with BF$_3$·Et$_2$O. The product 4b was obtained in 63% yield. Deacetylation of 4b with 1 M NaOMe in MeOH afforded the expected compound 5b in 77% yield. Selectively acylation of 5b with 4-O-acetylfurfuryl chloride [22] under mild basic conditions [8] and subsequent De-O-acytlation with pyrrolidine in dichloromethane [12] gave 2-chloro-4-nitrophenyl 5-O-furfuryl-α-L-arabinofuranoside 2b (Fig. 1B) in 50% yield.

Compound UFA 2a (Fig. 1B) is an analogue of reported CNPF, in which the 2-chloro-4-nitrophenyl aglycon was replaced by coumarin. Unexpectedly, we were unable to get the glycosylated compound 4a according to the above coupling method. Interestingly, under the BF$_3$·Et$_2$O/TEA condition, the glycosylation of umbelliferone reacted with 1,2,3,5-tetra-O-acetyl-α-L-arabinofuranoside 3 smoothly, producing the expected α-α'-arabinofuranoside 4a in 52% yield [23]. Similarly, deacetylation of 4a with 0.5 M TEA in MeOH gave compound 5a in 80% yield [24]. Then, the coupling reaction of 5a and 4-O-acetylfurfuryl chloride was carried out with pyridine as base and followed by De-O-acytlation to produce the desired umbiliferyl 5-O-feruloyl-α-L-arabinofuranoside 2a (Fig. 1B) in 55% yield.

3.2. Evaluation of the four mimic substrates

Both NPF and CNPF are unstable under alkaline conditions. Catalyzed by the FAEs, they could directly release the chlorophore after the cleavage of the ester bond. After 10-min reactions under pH 6.4, the chlorogenic substrate, pNPF, resulted in a low signal-to-noise ratio (S/N) of 2.4 (Table 1), while the newly designed substrate, CNPF, with $k_M$ and $k_{cat}$ of 5.49 ± 0.66 mM and 116 ± 9.31 min$^{-1}$, showed an significantly improved S/N value of 7.1 under the same conditions, around 3-fold of that of the substrate pNPF (Table 1).

The release of chlorogenic or flavonoid group of CNPFPA and UFA needs a two-step enzymatic process including an FAE-catalyzed release of an intermediate chromogenic arabinoside and the liberation of the chlorophore/umbelliferone catalyzed by α-AF [14]. Under the reaction condition, at least 37.5 or 25 ng of α-AF was required to fully release the chlorophore or fluorophore, respectively, for a 10-min reaction with 20-µL crude AnFaeA (about 13.6 U) (Fig. 2). The apparent Michaelis constant ($k_M$) and catalytic turnover frequency ($k_{cat}$) of the novel substrate UFA was 1.84 ± 0.19 mM and 63.6 ± 3.8 min$^{-1}$. The hydrolysis rates of CNPF were higher than that of pNPF or CNPF, or UFA in 10-min reactions (Table 1). However, UFA displayed the best S/N value of 70 (Table 1). It was also observed that substrate CNPFPA was unstable under alkaline conditions, while substrate UFA could well tolerate alkaline conditions. In addition, umbelliferone is a pH-dependent fluorophore. Therefore, for substrate UFA, glycine buffer (400 mM, pH 10.8) was added before fluorescence detection to enhance the signal intensity, which led to a S/N value of as high as 540 (Table 1).

The results clearly indicated that UFA afforded the highest sensitivity for the assay of AnFaeA expressed in $P$. pastoris on 96-well plate, which could be beneficial for enzymes with low activity.

### Table 1

Hydrolysis of four mimic substrates catalyzed with AnFaeA.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity (µmol min$^{-1}$ mg$^{-1}$)</th>
<th>S/N</th>
<th>α-AF (ng well$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pNPF (1a)</td>
<td>0.52 ± 0.01</td>
<td>2.4</td>
<td>No</td>
</tr>
<tr>
<td>CNPF (1b)</td>
<td>0.62 ± 0.01</td>
<td>7.1</td>
<td>No</td>
</tr>
<tr>
<td>UFA (2a)</td>
<td>0.67 ± 0.01</td>
<td>70 (540$^*$)</td>
<td>&gt;25.0</td>
</tr>
<tr>
<td>CNPFPA (2b)</td>
<td>1.0 ± 0.01</td>
<td>20</td>
<td>&gt;37.5</td>
</tr>
</tbody>
</table>

$^*$ Data in bracket was measured after the addition of equivalent volume of 400 mM glycine buffer (pH 10.8) to quench the reaction.

However, a considerable amount of α-AF was required for the release of the fluorophore, which would add approximately $0.65$ for each well according to the Megazyme catalog (Bray, Ireland), and thus it made it impractical for the assay of large amount of samples. In addition, the synthesis of both CNPFPA and UFA involved a multi-step process, and required expertise in carbohydrate chemistry, which limited their availability. In contrast, the synthesis of CNPF was easily achieved in one step, and no α-AF was required for the assay. A high-throughput screening system based on substrate CNPFPA would be more sensitive than that based on NPF, and at the same time, more convenient than that based on UFA or CNPFPA. The application of CNPFPA afforded an S/N of 7.1, which was sensitive enough for an industrially relevant enzyme. Hence, CNPFPA was chosen as the substrate for the high-throughput screening system.

3.3. Application of CNPF in a high-throughput manner

The reproducibility and uncertainty of the high-throughput screening system using CNPFPA was evaluated with AnFaeA variant expressed in $P$. pastoris. A total of 96 transformants of $P$. pastoris expressing AnFaeA were incubated in the 96-well plate format for a single sequence library and the supernatant of induced culture was subjected to enzymatic assay (Fig. 3). The reaction time was extended to 15 min, which raised the S/N value to 9.8. The mean and standard deviation of the absorbance values from 96 samples were 1.74 and 0.18, respectively (Fig. 3), making the coefficient of variance (CV) of the screening to be 10.3% (CV = [standard deviation/mean] × 100%), which indicates lower variability of the high-throughput screening systems [25,26]. The expected false positives exhibiting higher than 1.5 times the mean value would be close to zero in a typical library of $10^4$–$10^5$ colonies according to statistical calculations [26].

Fig. 2. Release of chlorophore (closed circle) or fluorophore (open circle) with the addition of arabinosidase.

Fig. 3. Evaluation of the feasibility of using 2-chloro-4-nitrophenol ferulate in a high-throughput manner. Enzyme activities are plotted versus plate position (columns) and in descending order (squares).
4. Conclusions

During the last decade, many researchers have been devoted to the design and synthesis of mimic substrates for the rapid assay of FAEs. In this study, we designed and synthesized two new chromo- or fluorogenic substrates, CNPF and UFA, to improve the spectrophotometric and fluorometric assay of FAEs. Compared with their structural analogues, pNPF and CNPFA, respectively, CNPF and UFA displayed significantly enhanced sensitivity. The fluorogenic substrate UFA has better stability at alkaline pH and higher signal-to-noise ratio, and may be suitable for detecting small changes of activity. The chromogenic substrate CNPF could be easily acquired, and well adapted to high-throughput screening. A practical high-throughput screening system with a CV of 10.3% was established for AnFaeA expressed in _P. pastoris_ in the 96-well plate format using the chromogenic substrate CNPF. This system has been successfully applied to our ongoing work on the identification of AnFaeA variants with improved thermostability from random mutagenesis library.

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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.2011.08.011.

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