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Construction and expression of two-copy engineered yeast of feruloyl esterase

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ABSTRACT

Background: Aspergillus niger has the ability to secrete feruloyl esterase. However, for economically viable industrial applications, it is necessary to increase their catalytic activities and/or protein yields to satisfy the increasing needs for feruloyl esterases.
Results: The gene AnFaeA that encodes a type A feruloyl esterase was successfully expressed in *Pichia pastoris* by a two-copy engineered yeast. After a screen in shaker flask, a one-copy strain GSKFA3 having the highest feruloyl esterase activity of 2.4 U/mL was obtained. Then, the pPICZαA-AnFaeA plasmid was transformed into GSKFA3 and the transformants were grown on YPDS plates with antibiotic Zeocin. After cultivation, a two-copy strain GSKZαFA20 with the highest feruloyl esterase activity of 15.49 U/mL was obtained. The expressed protein (recombinant AnFaeA) may be a glycoprotein with an apparent molecular weight of 40 kDa. It displayed the maximum activity at pH 6.0 and 50°C, and was stable at a pH range of 4.0–6.5 and at below 45°C. Its activity was not significantly affected by K^+, Ca^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, Na^+ and EDTA, but activated by Fe^{2+}. The Km and Vmax toward 4-nitrophenyl ferulate were 5.5 mM and 69.0 U/mg, respectively.
Conclusions: The two-copy strain GSKZαFA20 showed a 4.4-fold increase in extracellular enzyme activity compared with the one-copy strain GSKFA3. Construction of two-copy strain improved secretion of recombinant AnFaeA in *P. pastoris*.

1. Introduction

Ferulic acid (4-hydroxy-3-methoxycinnamic acid, FA), a phenolic acid, is an extremely abundant phenolic phytochemical found in plant cell walls. It can form diferulic bridges, providing cross-linkages between hemicelluloses, pectin and lignin via ester bonds [1]. Such structures can limit hydrolysis of these substrates by polysaccharide hydrolases and reduce accessibility to polysaccharides (hemicelluloses and cellulose).

Feruloyl esterases, also known as cinnamoyl esterases (EC 3.1.1.73), have been identified as key enzymes that catalyze the hydrolysis of the ester bond between hydroxycinnamic acids and polysaccharides in plant cell walls [2]. Breakdown of the ester linkage makes the plant cell wall much easier to release polysaccharides and ferulic acid [3]. Thus, these enzymes have a very high potential for industrial applications, such as feed, food, pulp and paper industries.

Feruloyl esterases have been purified and characterized from various organisms, such as bacteria and fungi [4]. To date, the industrial applications of feruloyl esterases are still limited by their low catalytic activities. Therefore, it is necessary to increase their catalytic activities and/or protein yields to satisfy the increasing needs for feruloyl esterases. There is considerable evidence that increase of protein yields can be achieved by optimizing the codon usage and expression conditions, as well as constructing double vector system and co-expression system [5,6,7]. Moreover, *Pichia pastoris* transformants with multiple copies of genes could potentially lead to high-level expression of heterologous proteins [8].

In this study, the gene AnFaeA that encodes a type A feruloyl esterase was efficiently expressed in *P. pastoris* by a two-copy engineered yeast. Meanwhile, the expression conditions of the two-copy yeast GSKZαFA20 were optimized. The enzymatic properties were also characterized. To our knowledge, this is the first report on enhancing the expression level of AnFaeA in *P. pastoris* by a two-copy engineered yeast.

2. Materials and methods

2.1. Strains, vectors, and culture media

P. pastoris expression vector pPIC9K (preserved in our laboratory) was used for the construction of the recombinant expression plasmid.

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Escherichia coli host strain DH5α (TIANGEN Biotech, Beijing, China) was used for routine plasmid amplification and *P. pastoris* host strain GS115 (preserved in our laboratory) was used for protein expression. *E. coli* DH5α was cultured at 37°C in the Luria–Bertani medium. *P. pastoris* GS115 and its transformants were cultured and induced as described in the manual of *Pichia* Expression Kit (Catalog Number K1710-01, Invitrogen, USA). The recombinant plasmids pMD19-T-AnFaeA and pPICZαA-AnFaeA were constructed (unpublished data) and preserved in our lab.

2.2. Construction of expression vector pPIC9K-AnFaeA

The cDNA encoding mature AnFaeA was amplified using the specific primers (AN-PPICEco-F: 5′-CCGGAATTCGCCTCCACGCAAGGCATC-3′ and AN-PPICNot-R: 5′-ATAAGAATGCGGCCGCCTACCAAGTACAAGCTC-3′) and the plasmid pMD19-T-AnFaeA as a template. Primer AN-PPICEco-F contained an EcoRI site and primer AN-PPICNot-R introduced a NotI site (underlined). The amplified PCR products were double-digested with EcoRI and NotI, gel-purified, and ligated into the pPICZαA vector double-digested with same restriction enzymes. The ligation product was transformed into *E. coli* DH5α. The positive clone was confirmed by colony PCR and DNA sequencing. The recombinant expression vector was designated as pPIC9K-AnFaeA.

2.3. Transformation of AnFaeA gene into P. pastoris GS115 and screening of transformants

After linearization with PmeI, the recombinant plasmid pPIC9K-AnFaeA was transformed into *P. pastoris* GS115 by electroporation as described previously [9]. Positive transformants were screened for their ability to grow on MD plates (1.34% YNB, 4 × 10–5% biotin, 2% dextrose, and 1.5% agar). Expression of AnFaeA in *P. pastoris* was performed according to the EasySelect *Pichia* Expression Kit (Invitrogen, USA; Catalog No. V175-20). From those transformants tested, one strain having the highest feruloyl esterase activity, labeled as GSKFA3, was selected and used for the subsequent transformation.

2.4. Construction of recombinant strain GSKZαFA20 and its expression in P. pastoris

The selected GSKFA3 strain was used as the host strain for transformation of pPICZαA-AnFaeA linearized with SacI. The transformants were grown on YPDS plates containing 100 μg/mL Zeocin and incubated at 28°C for 2–3 d until visible colonies appeared. Through flask expression test, one strain having the highest feruloyl esterase activity, labeled as GSKZαFA20, was obtained and used for subsequent studies.

2.5. Optimization of the expression conditions

Two major factors (methanol concentration and induction time) for AnFaeA expression by GSKZαFA20 strain were optimized as described by Chen et al. [7]. The purity of recombinant AnFaeA (reAnFaeA) was determined using the Bio-Rad ChemiDoc XRS imaging system equipped with Quantity One software (Bio-Rad, Hercules, CA, USA).

2.6. Glycosylation analysis of reAnFaeA

To determine if reAnFaeA is a glycoprotein, the protein was first detected using a glycoprotein staining kit (Thermo Scientific Pierce) according to the manufacturer's instructions. Then the enzyme was digested by using endoglycosidase H (Endo H) (New England Biolabs, Beverly, MA, USA) according to the manufacturer's protocol. The treated protein was analyzed on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining.

2.7. Western blot analysis

The expressed protein was resolved on 12% SDS-PAGE. Proteins were transferred onto a nitrocellulose membrane using the Mini trans-Blot® cell (Bio-Rad), which was then performed by sequential incubation with appropriate primary antibody (1:1000, prepared in our laboratory, rats were backside immunized with natural total protein from *A. niger*) followed by a horseradish peroxidase (HRP)–conjugated goat anti-rat IgG (1:6000, Santa Cruz, CA, USA). Recognition was performed with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

2.8. Enzyme assay

Feruloyl esterase activity was measured against 4-nitrophenyl ferulate (pNPF) as described previously with some modifications [10]. pNPF was synthesized with established method [11]. Release of the p-nitrophenol at pH 5.5 was monitored at 410 nm. One unit (U) of enzyme activity was defined as the amount of enzyme liberating 1 μmol of p-nitrophenol per min under the standard assay conditions.

2.9. Effects of pH and temperature on enzyme activity and stability

The optimal reaction temperature was determined under the standard assay conditions described above, except for reaction temperatures ranging from 30°C to 90°C. To evaluate its temperature stability, the enzyme was preincubated in 100 mM citrate-phosphate buffer (pH 6.0) for 30 min at temperatures ranging from 30°C–70°C. The residual enzyme activity was then detected at 50°C for 15 min as described above. To evaluate its thermostability, the enzyme was preincubated at different temperatures (45°C, 50°C, 55°C) for different times (10, 20, 30, 40, 50, 60 min). The residual enzyme activity was then detected at 40°C for 30 min as described above.

The pH optimum of reAnFaeA was measured at 50°C in 100 mM Na_2HPO_4-citrate acid buffer (pH 3.0– 7.5) and Tris–HCl buffer (pH 8.0–9.0). The pH stability of reAnFaeA was measured by pre-incubating the enzyme without substrate in 100 mM citrate-phosphate buffer (pH 3.0– 7.5) and Tris–HCl buffer (pH 8.0– 9.0) at 35°C for 1 h. The residual enzyme activity was measured at the optimum pH and optimum temperature.

2.10. Effects of metal ions on the enzyme activity

The enzyme samples were preincubated for 1 h at 35°C with various metal ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+}, and Mn^{2+}) and EDTA at a final concentration of 2.0 mM in 100 mM citrate–phosphate buffer (pH 6.0), and the residual enzyme activity was measured at 50°C for 30 min as described above. The enzyme solution without adding any additive was used as a control.

2.11. Kinetic parameters of the reAnFaeA

Hydrolyzing reaction rate (U/mg) of the reAnFaeA was determined under the standard assay conditions, except for pNPF concentrations ranging from 3 to 30 mM. The reaction rate versus the substrate concentration was plotted to confirm whether the hydrolyzing mode of the reAnFaeA conforms to the Michaelis–Menten equation. The kinetic parameters, Km and Vmax were graphically determined from the Lineweaver–Burk plotting.

3. Results and discussion

3.1. Construction of expression vector pPIC9K-AnFaeA

A 783 bp DNA fragment encoding AnFaeA was PCR amplified and inserted into the plasmid pPIC9K. The resulting recombinant plasmid pPIC9K-AnFaeA was subsequently confirmed by colony PCR and sequencing.

3.2. Construction of recombinant strain GSKZαFA20 and screening of the P. pastoris transformants

The resulting expression plasmid pPIC9K-AnFaeA was used to transform *P. pastoris* GS115 and the transformants were used to screen the highest expression level. After induction by methanol for 120 h, the culture supernatants of 10 strains were used for feruloyl esterase activity assay. As a result, one strain (GSKFA3) with the highest feruloyl esterase activity of 2.4 U/mL was obtained and used as the host strain for the transformation of pPICZαA-AnFaeA. A total of 49 strains resistant to 100 μg/mL of Zeocin were picked out for the flask expression test. The GSKZαFA20 having the highest feruloyl esterase activity of 10.6 U/mL was obtained, which was 4.4-fold higher than that of GSKFA3.

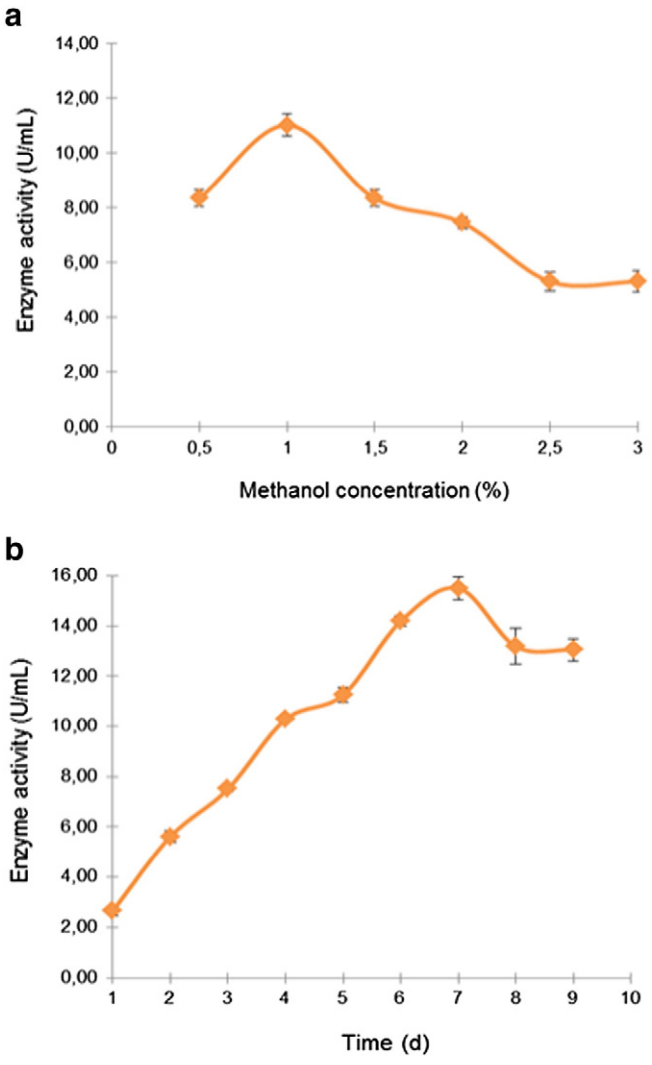

Fig. 1. Effects of induction time and methanol concentration on the expression level of AnFaeA. a: The *P. pastoris* GSKZαFA20 was induced in a BMMY medium containing methanol at different final concentrations (0.5–3.0%). b: The *P. pastoris* GSKZαFA20 was induced in a BMMY medium by adding methanol of 1.0% at 24-h intervals until 216 h at 28°C.

3.3. Optimization of the expression conditions

In this study, the expression conditions, including optimal methanol concentration and induction time for AnFaeA expression by the GSKZαFA20, were optimized. First, the GSKZαFA20 strain was inoculated into BMMY medium and induced by adding different methanol concentrations (0.5 to 3.0% (v/v)) at 24 h intervals at 28°C for 5 d. As shown in Fig. 1a, the maximum feruloyl esterase activity was 11.01 U/mL at a methanol concentration of 1.0%. Second, the GSKZαFA20 strain was inoculated into BMMY medium and induced by adding methanol to a final concentration of 1.0% at 24 h intervals at 28°C for different induction times (0–9 d). As shown in Fig. 1b, the feruloyl esterase activity reached 15.49 U/mL at 7 d of methanol induction and decreased slowly afterwards, which was higher than those of the feruloyl esterases separately from *Aspergillus flavus* (0.5 U/mL) [12], *Aspergillus oryzae* [13] (10.76 U/mL), *Aspergillus usamii* (2.1 U/mL) [14]. The purity of the expressed proteins was 95%, which was higher than that of feruloyl esterase from *A. oryzae* (86%) [12].

3.4. Identification of the reAnFaeA

Equivalent amounts of culture supernatants obtained at different induction times were loaded onto each well and subjected directly to SDS-PAGE. As shown in Fig. 2, a major specific protein band of about 40.0 kDa was observed, and the amount of expressed protein increased with time of induction to approximately 7 d. The molecular weight of expressed protein was consistent with those of feruloyl esterases from *A. niger* CIB 423.1 (40.0 kDa) [12] and *A. niger* CBS 120.49 (40.0 kDa) [15], but larger than those of feruloyl esterases from *A. usamii* (36.0 kDa) [13], *A. oryzae* (37.0 kDa) [14] and *Aspergillus awamori* (35.0 kDa) [16]. The apparent molecular weight of expressed protein was much larger than the calculated molecular mass of 28.6 kDa, which was probably due to glycosylation.

P. pastoris enables some post-translational modifications of recombinant proteins, such as N- and/or O-glycosylation [17]. In this work, there are one putative N-glycosylation site and three O-glycosylation sites in the primary structure of AnFaeA. To verify whether the difference was due to glycosylation, glycoprotein staining and deglycosylation analysis were used. As shown in Fig. 3, the 40.0 kDa band was positively stained, appearing as a magenta band with light pink background, suggesting that the expressed protein was glycosylated. After deglycosylation with Endo H, the expressed protein displayed a single band of about 37.0 kDa (Fig. 4). Western blot analysis showed that the expressed protein was recognized specifically by rat anti-*A. niger* polyclonal antibody (Fig. 5). Meanwhile, PCR

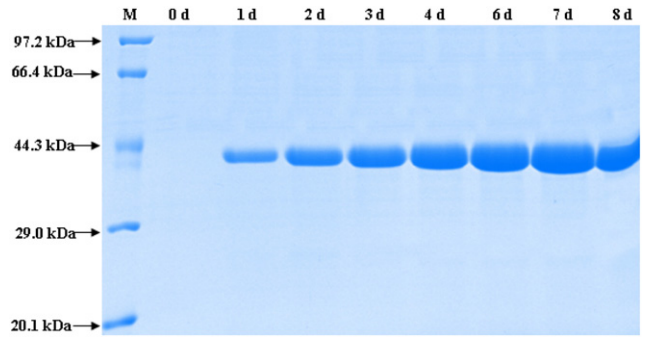

Fig. 2. Time course of AnFaeA expression by *P. pastoris* GSKZαFA20 by SDS-PAGE. Lane M: molecular mass standard protein.

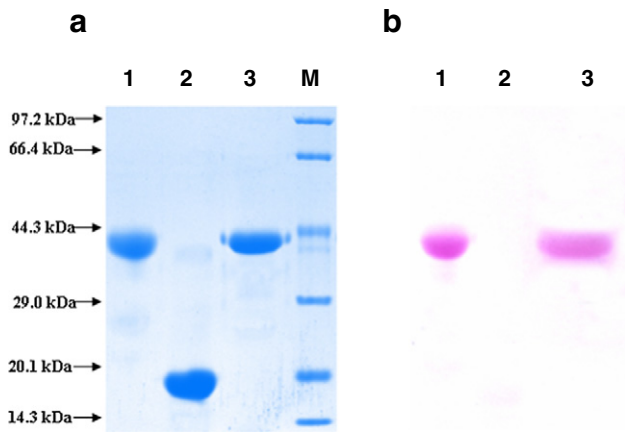

Fig. 3. SDS-PAGE and glycoprotein staining analysis of reAnFaeA expressed by *P. pastoris* GSKZαFA20. a: Coomassie blue staining of polyacrylamide gels. b: Glycoprotein staining of polyacrylamide gels. Lane M: molecular mass standard protein; lane 1: positive control (horseradish peroxidase); lane 2: negative control (soybean trypsin inhibitor); lane 3: reAnFaeA expressed by *P. pastoris* GSKZαFA20.

analysis showed that the AnFaeA gene was stably inserted into the yeast genome (Fig. 6).

3.5. Enzymatic properties of the reAnFaeA

The optimum temperature of reAnFaeA (measured at pH 5.5) was 50°C (Fig. 7a). The enzyme activity retained more than 70% of its original activity at 45°C and 50°C but only retained about 15% of its activity at 55°C for 10 min (Fig. 7b). The pH optimum of reAnFaeA was 6.0 (Fig. 7c). The reAnFaeA was stable at a pH range of 4.0–6.0, retaining more than 60% of its original activity (Fig. 7c), which was similar to that of feruloyl esterase from *A. usamii* [13]. Feruloyl esterase from *A. usamii* retains over 90% of its original activity when preincubated at 45°C for 60 min, and retained 79.2% of its activity at 50°C for 20 min, but entirely lost its activity after 40 min at 55°C [13].

The effects of various metal ions and EDTA on the activity of feruloyl esterase were tested. No significant effect of enzyme activity in the presence of K^+, Ca^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, Na^+, and EDTA was observed (data not shown), whereas it was activated by the addition of Fe^{2+} (2.1-fold). As observed form *A. usamii* and *A. oryzae* feruloyl esterases, the enzyme activity was not significantly affected by Na^+, Ca^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+} and EDTA [13,14], but slightly inhibited by Cu^{2+} [14]. The Michaelis–Menten constant Km and Vmax values toward pNPF at pH 6.0 and 50°C were 5.5 mM and 69.0 U/mg, respectively. The Km of reAnFaeA was much smaller than that (14.4 mM) of recombinant feruloyl esterase from *A. niger* [12].

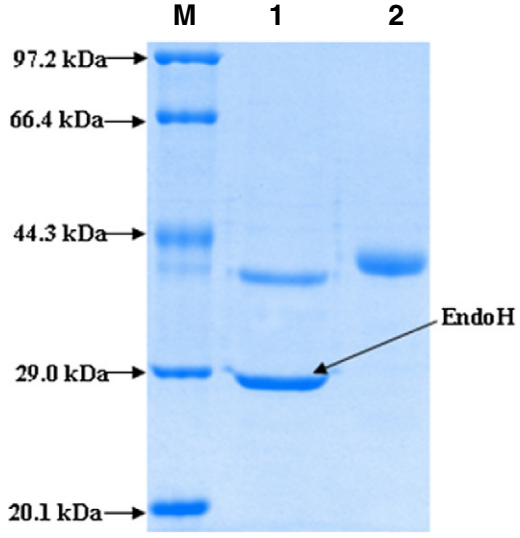

Fig. 4. SDS-PAGE analysis of reAnFaeA after deglycosylation. Lane M: molecular mass standard protein; lane 1: deglycosylated reAnFaeA expressed by *P. pastoris* GSKZαFA20; lane 2: reAnFaeA expressed by *P. pastoris* GSKZαFA20.

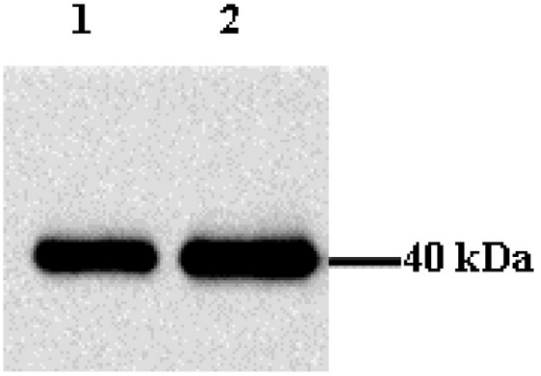

Fig. 5. Western blot analysis of recombinant protein. Lanes 1–2: induced supernatant of *P. pastoris* GSKZαFA20.

In summary, this work led to high-level expression of AnFaeA in *P. pastoris* by two-copy engineered yeast and by optimization of the expression conditions. The GSKZαFA20 having the maximum feruloyl esterase activity of 10.6 U/mL, which was 4.4-fold higher than that for the one-copy strain GSKFA3. When GSKZαFA20 was induced under optimized conditions, the feruloyl esterase activity reached 15.49 U/mL. Construction of two-copy strains may improve secretion of recombinant proteins in *P. pastoris* and the process development

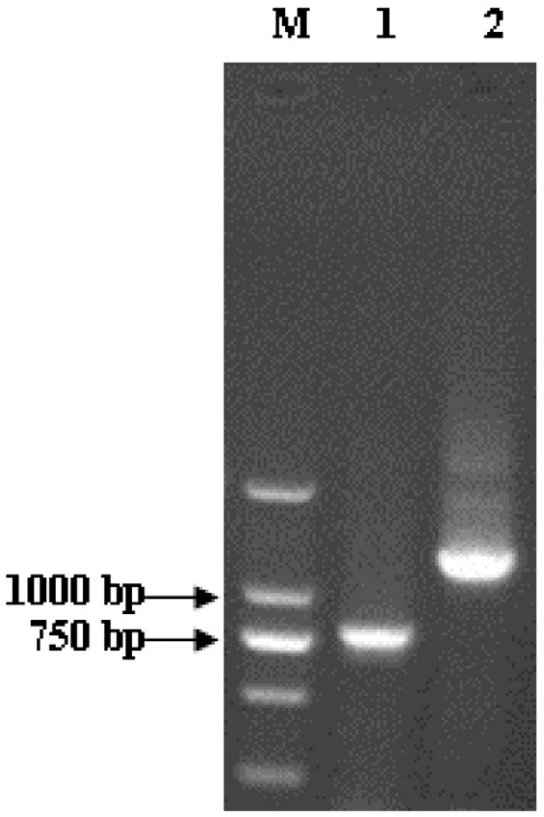

Fig. 6. PCR analysis of *AnFaeA* in genomic DNA of GSKZαFA20 strain. Genomic DNA was extracted from GSKZαFA20 strain. PCR was performed using AN-PPICEco-F/AN-PPICNot-R and 5′AOX1/3′AOX1 primers and genomic DNA as a template. Lame M: DNA marker; Lane 1: AN-PPICEco-F/AN-PPICNot-R; Lane 2: 5′AOX1/3′AOX1.

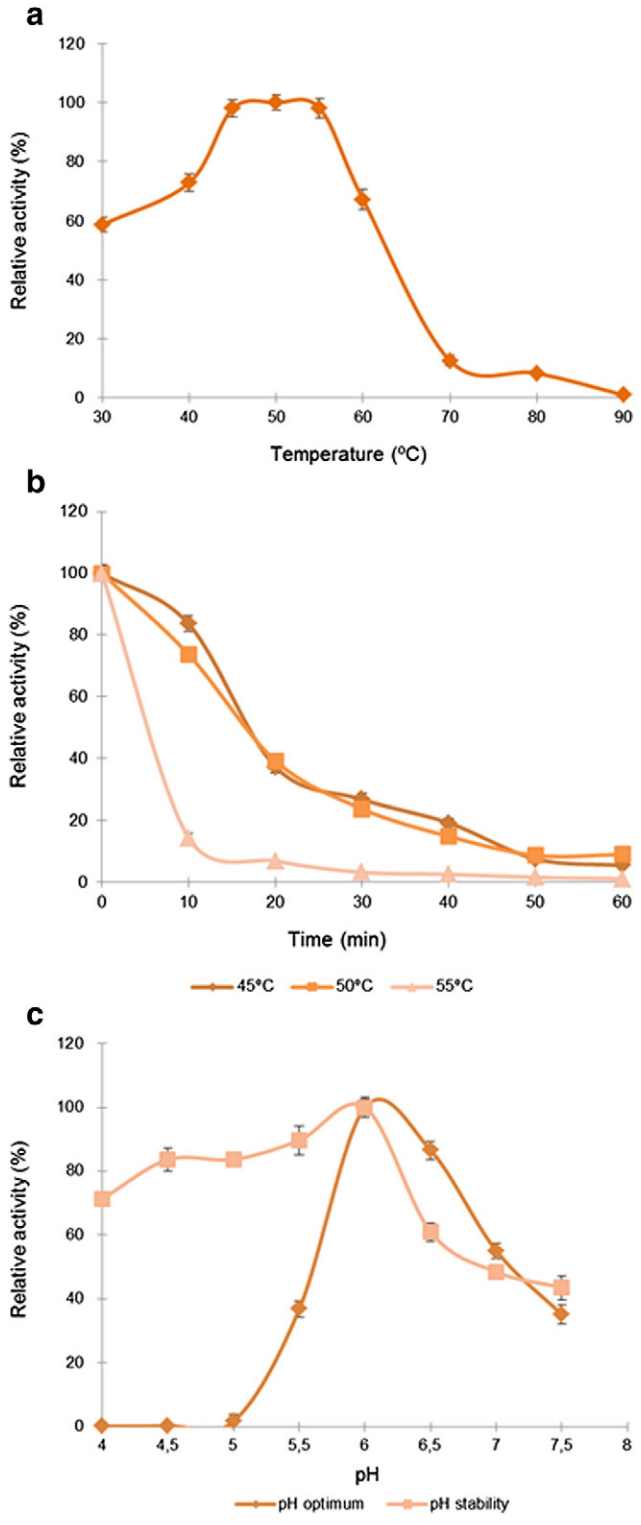

Fig. 7. Effects of temperature and pH on the activity and stability of reAnFaeA expressed by *P. pastoris* GSKZαFA20. a: Effect of temperature on the activity of reAnFaeA expressed by *P. pastoris* GSKZαFA20. b: Effect of temperature on the thermostability of reAnFaeA expressed by *P. pastoris* GSKZαFA20. c: Effect of pH on the activity and stability of reAnFaeA expressed by *P. pastoris* GSKZαFA20. The maximum value was set as 100%. All activity tests were done in triplicate.

technique described here could provide new strategies for other proteins. It is favorable for extending the large-scale production and decreasing production cost.

Conflict of interest

The authors declare no conflict of interest.

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