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Efficient production of peracetic acid in aqueous solution with cephalosporin-deacetylating acetyl xylan esterase from *Bacillus subtilis*

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ABSTRACT

Peracetic acid (PAA) is widely used in sterilization, bleaching textile industry, environmental engineering, chemical synthesis, and biomimetic chemistry. A previous study reported that acetyl xylan esterase (AXE) of *Bacillus subtilis* CICC 20034 has high activity toward cephalosporin C and 7-aminocephalosporanic acid. In this study, we found that AXE also exhibited high perhydrolysis activity toward acetate esters and endowed itself with great industrial interest on enzyme-catalyzed preparation of PAA. Recombinant AXE of *B. subtilis* CICC 20034 could be efficiently produced in a low-cost autoinduction medium with an activity of 6.8×10^3 U/mL. The reaction conditions for the optimal synthesis of PAA were as follows: 0.30 mg/mL AXE crude enzyme, 300 mM glycerol triacetate, and 1 M hydrogen peroxide, pH 8.0, and 20 °C, which produced approximately 150 mM of PAA within 5 min. The AXE was then immobilized on an acrylate amino resin; the activity of the immobilized AXE was 383.7 U/g. In the presence of 1 g/mL of immobilized AXE resin, PAA titer of the initial reaction batch was approximately 142.5 mM, and about 95.5 mM of PAA could be produced after 10 cycles.

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1. Introduction

PAA is one of the most important kinds of peroxides and a quick-acting, broad-spectrum, efficient disinfectant, which has been widely used in sterilization [1], bleaching textile industry [2], environmental engineering [3,4], chemical synthesis [5,6], and biomimetic chemistry [7]. PAA is industrially produced by the autoxidation of acetaldehyde or acetic acid with hydrogen peroxide, and sulfuric acid as a catalyst; this process is expensive and risky. PAA is unstable and easily decomposed in the presence of high temperature, organic matter, heavy metal ions, and alkali conditions, thus increasing the cost and risk of transport and storage of PAA. As enzyme-catalyzed reactions are efficient, mild, and environmentally friendly, *in situ* production of PAA using enzymes can overcome the aforementioned drawbacks. There is an increasing demand for enzymes that can be used as catalysts for the industrial production of PAA. Considering the environmental conditions required for sterilization and bleaching, enzyme-catalyzed production of PAA in aqueous conditions would gain prominence in future studies. However, Kirk et al. investigated the ability of hydrolases (lipases, esterases, and proteases) to catalyze perhydrolysis of acyl substrates with hydrogen peroxide to form peroxycarboxylic acid, and reported that the process was less efficient in aqueous systems. They concluded that lipases, esterases, and proteases were, in general, not suitable for catalyzing perhydrolysis of simple esters in an aqueous environment [8].

In general, the enzymatic synthesis of PAA is performed using perhydrolase, a subgroup of serine hydrolases that are very efficient catalyzers of perhydrolysis. Some lipases or esterases, particularly serine hydrolases containing a Ser–His–Asp catalytic triad, have been reported to be capable of catalyzing perhydrolysis to produce peroxycarboxylic acids [9–11]. Currently, AXE (EC 3.1.1.72) of *Aspergillus ficuum* has been reported to catalyze the synthesis of PAA from ethyl acetate with the highest PAA titer volume of 134 mM [12,13]. US Patent No. 8518675B2 [14] has reported several enzymes that belong to the carbohydrate esterase 7 (CE-7, cephalosporin-C deacetylases, and AXEs) family. It also described different bacteria with significant perhydrolysis activity for converting carboxylic acid ester substrates to peracids, which showed that AXE may be a potential enzyme that can be used as a catalyst for the preparation of PAA.

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In this study, a novel cephalosporin-deacetylating AXE from *Bacillus subtilis* CICC 20034 with high activity and stability toward cephalosporin C and 7-aminocephalosporanic acid was reported [15], and the ability of *B. subtilis* CICC 20034 AXE to catalyze the perhydrolysis of acetyl substrates to produce PAA was investigated. The ability of the bacteria to increase the commercial production of PAA in an aqueous environment with optimized reaction conditions was also studied.

2. Materials and methods

2.1. Cloning AXE gene (Cah) into the expression vector pET28a

The *Cah* gene from *B. subtilis* encoding AXE [15] was amplified with two primers designed to generate NcoI and XhoI sites (forward, 5′-CATGCCATGGGCATGCAATTATACGACT-3′ and reverse, 5′-CCCTCGAGGCCTTTC AGATGCGCTT-3′). The amplified gene was digested with NcoI and XhoI and inserted into the expression vector pET28a (Novagen) to generate a pET28a-*Cah* plasmid. Then, the recombinant plasmid was transformed into *Escherichia coli* BL21 (DE3).

2.2. Expression and production of recombinant AXE

In order to reduce the cost of enzyme production, autoinduction medium was used to replace the expensive conventional IPTG (isopropyl β-D-1-thiogalactopyranoside) induction. Considering the fact that the amount of glycerin, glucose, and lactose present in the medium could affect the cell growth and enzyme production [16], the culture medium was optimized by the orthogonal test in three factors and three levels: 15 g/L peptone, 25 g/L yeast extract, and 10 g/L NaCl; 0.1–0.3% (v/v) glycerin, 0.1–0.3% (w/v) glucose, and 0.2–0.4% (w/v) lactose. Shake flasks were used for optimizing the medium.

In practice, AXE was produced in a 5-L jar fermentor as follows: The seed medium for the recombinant *E. coli* was Luria–Bertani (LB) broth; the recombinant cells were incubated at 37 °C for 12 h in a 250-mL rotary shaker (200 rpm) containing 50 μg/mL of kanamycin. The seed culture (2%, v/v) was then added to 3 L of the optimized fermentation medium. The agitation rate was maintained at 200 rpm, air was sparged at a rate of 1.5 v/v/m, and the cultures were incubated at 30 °C for 16 h. Bacterial growth was then monitored by measuring the A_{600}.

2.3. Enzyme assays

The cells collected from the culture broth were suspended in 100 mM Tris–HCl buffer (pH 7.4) and were disrupted by sonication; supernatants were collected by centrifugation (10,000 × *g* for 30 min). AXE activity was assayed as described earlier [15]. One unit of enzyme activity (U) was defined as the quantity of enzyme required to release 1 μmol of *p*-nitrophenol per minute at 40 °C for 10 min.

2.4. Preparation of AXE crude enzyme

A concentrated cell suspension in a 3-L culture of *E. coli* BL21-pET28a-*Cah* was obtained by continuous centrifugation at 8000 rpm (centrifuge model Avanti J-26XP, Beckman Coulter, Inc., USA). Then, the cells were washed with 100 mM Tris–HCl buffer (pH 7.4) and were concentrated 10 times with the same buffer. Subsequently, the cells were disrupted through a high-pressure homogenizer (model AH100B, ATS Engineering Inc., Germany) at 800 bar and centrifuged for 20 min at 8000 rpm to remove cell debris. The supernatant was collected and stored at 4 °C until use. It was considered as the AXE crude enzyme. The protein concentration of the supernatant was determined by the Bradford method [17].

2.5. Immobilization of AXE

Before immobilization, acrylate amino resin MI-BS1 (Novocata, China) was activated by the following procedure: The resins were washed with 20 mM Tris–HCl buffer (pH 8.0) and were suspended in the same buffer containing glutaraldehyde (1% v/v) with stirring at 250 rpm and 25 °C for 1 h. Then, the remaining glutaraldehyde solution was removed thoroughly, and the resins were washed with distilled water before use and stored at 4 °C. For the immobilization of AXE, crude AXE solution (0.1 g of protein) and 10 g of activated resins were mixed with 50 mL of 100 mM Tris–HCl buffer (pH 7.4) and incubated at 35 °C for 6 h. After reaction, the immobilized AXE was recovered by filtration and stored in 100 mM Tris–HCl buffer (pH 7.4) at 4 °C until use.

2.6. Evaluation of commercial lipases and recombinant AXE for perhydrolysis reaction

The reaction was performed in a 5-mL test tube with a reaction volume of 1 mL containing glycerol triacetate (250 mM), hydrogen peroxide (1 M), and 0.3 mg of commercial enzymes or recombinant AXE in 100 mM Tris–HCl buffer (pH 7.4) at 25 °C for 10 min.

2.7. Determination of kinetic parameters

Comparison of kinetic parameters between free enzymes and immobilized AXE was performed using the monochlorodimedone assay [18] to measure the initial rates of enzymes with substrates (glycerol triacetate, hydrogen peroxide, and ethyl acetate) at 25 °C and pH 6.5. The apparent K_M values were obtained using the Michaelis–Menten equation.

2.8. Perhydrolysis

Perhydrolysis of acetate esters catalyzed by the recombinant enzyme AXE was performed in a 5-mL test tube containing a reaction volume of 1 mL *via* different reactions. Conditions such as different acetate esters, concentration of the substrate, amount of enzyme, temperature, buffer pH, and reaction time were optimized. The reusability of the immobilized AXE was investigated at the end of each reaction, which was removed from the reaction medium by centrifugation and reused after being thoroughly rinsed with 100 mM Tris–HCl buffer (pH 7.4).

2.9. Determination of PAA by high-performance liquid chromatography

In general, PAA is analyzed with its redox properties by oxidizing methyl *p*-tolyl sulfide (MTS) to the corresponding sulfoxide (MTSO). Thus, PAA was quantitatively measured by estimating the MTSO content with high-performance liquid chromatography (HPLC) analysis, as described by Shaw and Hager [19]. However, after complete consumption of PAA, the excess H_2O_2 present continued to oxidize MTS. In order to avoid a steady increase in the MTSO peak due to MTS oxidation by excess H_2O_2, triphenylphosphine (TPP) was used to initiate the second derivatization step, which was then oxidized to triphenylphosphine oxide (TPPO) by the excess H_2O_2. Thus, PAA could be determined as follows: 0.1 mL of the sample solution was mixed with 0.1 mL of 20 mM MTS in acetonitrile and 0.3 mL of deionized water in the dark, at 25 °C for 10 min. Then, 0.4 mL of acetonitrile and 0.1 mL of a 10 mM solution

Table 1
Amino acid sequence identity between AXEs having perhydrolysis activity.

Amino acid identity between AXEs[a] having perhydrolysis activity								
Source organism (Accession No.)	*B. subtilis* 168[a] (NP_388200)	*B. subtilis* ATCC 31594[a] (BAA01729)	*B. licheniformis* ATCC 14580[a] (YP_077621)	*B. subtilis* ATCC 29233[a] (WP_015715213.1)	*B. pumilus* PS213[a] (CAB76451.2)	*C. thermocellum* ATCC 27405[a] (ZP_00504991)	*T. neapolitanaa* (AAB70869.1)	*A. ficuum* [12] (Q96W96)
Percent identity[b]	84	84	76	84	73	55	40	31

[a] AXEs having perhydrolase activity were provided in US Patent No.8518675B2 [14].
[b] Percent identity is a relationship between polypeptide sequences of the reported AXEs and the AXE of interest from Bacillus subtilis CICC 20034 [15], as determined by the sequence alignments.

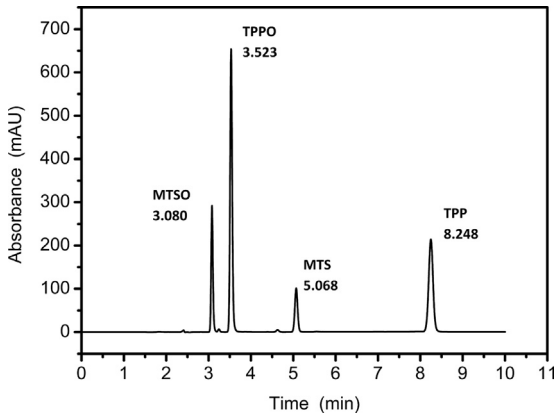

Fig. 1. Chromatogram of standards of MTSO, TPPO, MTS, and TPP.

of TPP in acetonitrile were added to initiate the second derivatization step at 25 °C for 30 min in the dark. The supernatant was taken as the sample and was characterized by HPLC (Thermo Fisher Scientific U-3000, UT) with a Kromasil 100-5C8 (4.6 × 250 mm, ϕ 8 mm) column. The mobile phase was acetonitrile–water, using linear gradient elution, and the concentration of acetonitrile ranged from 80% to 85%. The system was operated at a flow rate of 1 mL/min, monitoring the optical absorption in the effluents at 230 nm, and the column temperature was 40 °C. The retention times of MTS, MTSO, TPP, and TPPO were determined by monitoring the effluent at 230 nm. In this experiment, MTSO eluted first at 3.080 min, followed by TPPO, MTS, and finally TPP (Fig. 1).

3. Results and discussion

3.1. Sequence analysis of AXEs with perhydrolysis activity

AXEs (EC 3.1.1.7) of CE-7 family are serine hydrolases that hydrolyze the ester linkages of the acetyl groups toward both acetylated xylooligosaccharides and cephalosporin C. Several AXEs with a promiscuous activity to catalyze the reversible formation of peracids have been reported. US Patent No. 8518675B2 [14] reported that some AXEs which belong to the CE-7 family catalyze the perhydrolysis reaction of carboxylic esters with H_2O_2 to yield peracids. The results of comparison of the overall percent identity of amino acid sequences between AXEs having perhydrolysis activity are provided in Table 1, which showed that the AXE of interest presented an identity level comparable with those from *B. subtilis* 168 (84%, GenBank no. NP_388200.1), *B. subtilis* ATCC 31954 (84%, GenBank no. BAA01729.1), *Bacillus licheniformis* ATCC 14580 (76%, GenBank no. YP_077621), *B. subtilis* ATCC 29233 (85%, GenBank no. WP_015715213.1), *Bacillus pumilus* PS213 (73%, GenBank no. CAB76451.2), *Clostridium thermocellum* ATCC 27405 (55%, GenBank no. ZP_00504991), and *Thermotoga neapolitana* (40%, GenBank no. AAB70869.1). Recombinant AXE of *A. ficuum* (GenBank no. Q96W96) has also been reported to catalyze the synthesis of PAA from ethyl acetate and hydrogen peroxide [12]; however, the identity of amino acid sequences of both AXEs is only 38%. It is evident from Table 1 that 84% identity could be obtained with the AXEs from *B. subtilis*, which showed that the AXE of interest may be a used as a potential catalyst for the preparation of PAA.

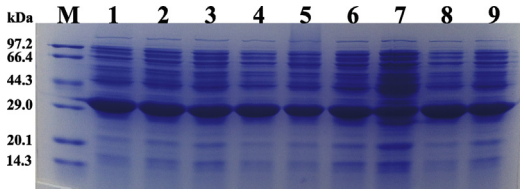

Fig. 2. SDS-PAGE of cell extracts from different cultures. Lane 1, Molecular mass markers; lane 1–9, soluble cell extracts from recombinant *E. coli* BL21-pET-*Cah* in different cultures (the different media components are provided in Table 2). The molecular masses of the markers were 97.2, 66.4, 44.3, 29.0, 20.1, and 14.3.

Table 2
Effects of media components on the production of AXE.

Run No.	Media components			Activity (U/ml)
	Glycerin (%, v/v)	Glucose (%, w/v)	lactose (%, w/v)	
1	0.1	0.1	0.2	1.33×10^3
2	0.1	0.2	0.3	1.30×10^3
3	0.1	0.3	0.4	0.83×10^3
4	0.2	0.1	0.3	1.25×10^3
5	0.2	0.2	0.4	0.96×10^3
6	0.2	0.3	0.2	1.04×10^3
7	0.3	0.1	0.4	0.68×10^3
8	0.3	0.2	0.2	1.36×10^3
9	0.3	0.3	0.3	0.95×10^3

The culture medium was optimized by the orthogonal test in three factors and three levels: 15 g/L peptone, 25 g/L yeast extract, and 10 g/L NaCl; 0.1–0.3% (v/v) glycerin, 0.1–0.3% (w/v) glucose, and 0.2–0.4% (w/v) lactose. The recombinant cells were incubated at 30 °C for 16 h in a 250-mL rotary shaker (200 rpm) containing 50 μg/mL of kanamycin.

3.2. Overexpression of AXE

The recombinant AXE was overexpressed in *E. coli* BL21 (DE3). *E. coli* pET28a-*Cah* cells were grown in different culture media at 30 °C for 16 h, and the total protein of each culture medium was calculated (Fig. 2). Molecular mass of the overexpressed protein band was in agreement with that of AXE (35.6 kDa). The effect of each factor level (medium component) in a given combination was studied in terms of response (activity of AXE and protein yield). Table 2 shows that the experimental combination of 0.3% v/v glycerin, 0.2% w/v glucose, and 0.2% w/v lactose (run no. 8) achieved the highest activity of AXE (1.36×10^3 U/ml), and the corresponding lane (no. 8) shown in Fig. 2 also exhibited a significant high-level expression of AXE protein. Thus, the medium was optimized as follows: 15 g/L peptone, 25 g/L yeast extract, and 10 g/L NaCl; glycerin (0.1%, v/v),

Table 3
Comparison of perhydrolase activity among commercial lipases and AXE.

Lipase	Producer	Lipase source	PAA (mM)
Lipase Type VII	Sigma–Aldrich Chemical Company	*Candida rugosa*	0.98 ± 0.33
LPL	SUNSON Company (China)	*Aspergillus niger*	2.6 ± 0.91
Lipase Type II	Sigma–Aldrich Chemical Company	*Porcine pancreas*	7.1 ± 2.08
CALB	Novozymes	*Candida antarctica*	0
AXE	This study	*Bacillus subtilis* CICC 20034	113.37 ± 3.32

The reaction was performed in a 5-mL test tube with a reaction volume of 1 mL containing glycerol triacetate (250 mM), hydrogen peroxide (1 M), and 0.3 mg of commercial enzymes or recombinant AXE in 100 mM Tris–HCl buffer (pH 7.4) at 25 °C for 10 min. Results are represented as mean ± S.D. of at least three replicates.

glucose (0.2%, w/v), and lactose (0.2%, w/v). After incubation at 30 °C for 16 h, bacterial growth in the fermentation tank reached 11.3 of OD_{600}, and the activity of AXE reached 6.8×10^3 U/mL, which was about 8.5 times higher than that in the LB medium induced by 1 mM IPTG for 2 h. Hydrolysis of 1 mL of the fermentation broth could produce 3.209 mg of AXE crude enzyme. The low-cost autoinduction medium could be efficiently used for AXE production, providing a basis for the industrial use of the enzyme.

3.3. Evaluation of commercial lipases and AXE for perhydrolysis

Many hydrolases (lipases, esterases, and proteases) have been found to catalyze perhydrolysis of acyl substrates with hydrogen peroxide to produce peroxycarboxylic acid; however, most of them have a very low efficiency in aqueous systems. Perhydrolysis activities of four commercial lipases were compared, and it has been found that the prepared AXE acted as a good catalyst for perhydrolysis and produced the highest PAA titer in an aqueous solution in a short time (Table 3). Thus, it may be a good choice for PAA production.

3.4. Immobilization of AXE onto resin

There are many advantages of immobilized enzymes in practical application, including easy separation of biocatalyst from the product, reduced costs of downstream processing, and reuse of biocatalyst [20]. Covalent immobilization of enzymes by glutaraldehyde is the most widely used technique. Glutaraldehyde is a powerful cross-linking agent with two functional aldehyde groups in use for a long time in the design of biocatalysts and a heterofunctional support that permits alteration of the enzyme orientation on the support surface [21]. Therefore, acrylate amino resin was used as a carrier to immobilize AXE. The amino side chains on the acrylate amino resin were preactivated by glutaraldehyde to form covalent bonds with the amino groups of AXE. Approximately 0.42 mg of crude AXE protein was bound to 1 g of resin, and the activity yield of the immobilized AXE was found to be approximately 43% of that of the free AXE (~383.7 U/g). The decrease in the activity of immobilized AXE can be explained by the fact that the enzymes lose their activity due to distortion during some multi-interactions between the enzyme and the support, and that the active center may be blocked during immobilization [22].

3.5. Determination of kinetic parameters of free and immobilized AXEs

Perhydrolysis of hydrogen peroxide and acetate esters was performed. Varying the concentration of H_2O_2 by maintaining a constant glycerol triacetate concentration of 1.14 M yielded the K_M value for H_2O_2 (0–5 mM), whereas varying the concentration of glycerol triacetate or ethyl acetate by maintaining a constant H_2O_2 concentration of 10 mM yielded the K_M value for glycerol triacetate (0–531.4 mM) and ethyl acetate (0–1.33 M) (Table 4). AXE prefers glycerol triacetate than ethyl acetate, because K_{cat}/K_M of glycerol triacetate is 28-fold higher than that of ethyl acetate. The K_M values

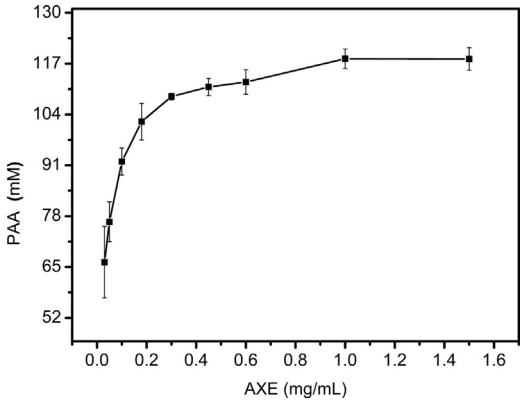

Fig. 3. Effects of enzyme loading on the production of PAA. Reaction conditions: 250 mM glycerol triacetate, 1 M hydrogen peroxide, and 1 mL reaction volume; 25 °C, pH 7.4, 5 min. The error bars represent standard deviation values of three independent experiments ($n = 3$).

of the free and immobilized AXEs for H_2O_2 are almost similar (1.11 and 1.15 mM, respectively), whereas those for glycerol triacetate were different (140.70 and 315.33 mM, respectively). This increase in the K_M values of immobilized AXEs indicated that they have an apparent lower affinity for the substrate. The catalytic ability (K_{cat}/K_M) of the immobilized AXE was decreased for both H_2O_2 and glycerol triacetate. The K_{cat}/K_M of the AXE immobilized on modified graphite oxide was about 270-fold lower than that of the free AXE [23]. This decrease in K_{cat}/K_M may be due to the loss of enzyme flexibility for substrate binding and change in the diffusion restriction caused by the immobilization.

3.6. Optimization of reaction

3.6.1. Effect of substrate

In order to determine the suitable substrate for acetate esters, the reaction was performed in a 5-mL test tube with a reaction volume of 1 mL containing 40 μL acetate esters, 1 M hydrogen peroxide, and 0.3 mg/mL crude AXE at 25 °C for 5 min. Samples withdrawn during the reaction were immediately centrifuged for 2 min at 12,000 rpm. Four different acetate esters used as the substrate to synthesize PAA are presented in Table 5, among which glycerol triacetate was found to be the suitable substrate because of its consistency with the kinetic properties of enzyme. It is worth noting that when ethyl acetate was used as the substrate, the by-product ethanol, which was harmful to AXE, inhibited the production of PAA. Thus, the PAA conversion rate of ethyl acetate for acetate precursor was much lower than that of glycerin acetate.

3.6.2. Effect of catalyst loading

In order to determine the minimum amount of enzyme required for the desired conversion, crude AXE enzymes prepared from *E. coli* pet28a-*Cah* cells were used for the synthesis of PAA. The amount of PAA produced was monitored and the results are presented in Fig. 3. As expected, PAA production increased with the increase in

Table 4
Kinetic properties of free and immobilized AXEs.

Enzyme	Varied substrate	K_M (H_2O_2) [mM]	K_M (acetyl donor) [mM]	K_{cat}/K_M (H_2O_2) [s^{-1} M^{-1}]	K_{cat}/K_M (acetyl donor) [s^{-1} M^{-1}]
Free AXE	Hydrogen peroxide	1.11 ± 0.19		3298	
Free AXE	Glycerol triacetate		140.70 ± 33.94		2534
Free AXE	Ethyl acetate		409.12 ± 48.73		89
Immobilized AXE	Hydrogen peroxide	1.15 ± 0.36		1090	
Immobilized AXE	Glycerol triacetate		315.33 ± 46.28		402

Kinetic constants were measured at 25 °C in citrate buffer (100 mM, pH 6.5) by monitoring the absorbance at 290 nm. The concentration of H_2O_2 was varied from 0 to 5 mM, whereas that of glycerol triacetate was maintained constant at 1.14 M. The concentrations of glycerol triacetate and ethyl acetate were varied from 0 to 531.4 mM and 0 to 1.33 M at a constant H_2O_2 concentration of 10 mM, respectively.

Table 5
Effect of substrates on the production of PAA.

Acetate ester	Substrate (mM)	PAA (mM)	Conversion rate[a] (%)
Ethyl acetate	407.21	35.94 ± 0.58	5.70
Glycerin monoacetate	–	71.42 ± 3.11	–[b]
Glycerin diacetate	–	102.66 ± 0.39	–[b]
Glycerol triacetate	212.33	130.36 ± 1.36	16.37

Results are represented as mean ± S.D. of at least three replicates.
[a] The conversion rate was calculated as the amount of PAA produced from theoretical acetate precursor of substrates.
[b] The substrates glycerin monoacetate and glycerin diacetate were supplied as mixtures; the conversion rates were not available.

Table 6
Effect of temperature on the production of PAA.

Temperature (°C)	PAA (mM)
20	137.8 ± 2.12
25	129.1 ± 2.16
30	127.9 ± 4.23
37	110.6 ± 3.04

Results are represented as mean ± standard deviation of at least three replicates.

the amount of enzyme used. However, when the amount of enzyme exceeded 0.30 mg/mL, the reaction rate decreased. The highest PAA titer (~108.6 mM) was achieved at 5 min when 0.30 mg/mL of enzyme was used at 25 °C (pH 7.4).

3.6.3. *Effect of concentrations of glycerol triacetate and hydrogen peroxide*

The mechanism of perhydrolysis is described as follows: First, a carboxylic acid reacts with the active-site serine group to form an acyl–enzyme intermediate, which then reacts with H_2O_2 to form a peracid. The switch of nucleophile from water to H_2O_2 could be considered as a change in substrate selectivity for the production of peracids [9]. However, it is also capable of severely damaging the chemical structure of the proteins, thereby inactivating the enzymes [24]. Better conversions were achieved by increasing the concentration of H_2O_2, but extremely high concentration resulted in the irreversible denaturation of the enzyme [10,25]. The effects of substrate concentration on the reaction were investigated by maintaining the concentration of AXE at 0.30 mg/mL (Fig. 4). It was also observed that an excess amount of H_2O_2 (>1 M) could inhibit the conversion. Higher concentrations of glycerol triacetate resulted in an increased reaction rate; however, production of PAA did not increase beyond 300 mM of glycerol triacetate titer.

3.6.4. *Effect of pH and temperature*

The temperature dependence of the reaction was investigated over a temperature range of 20–37 °C. The optimum reaction temperature was found to be 20 °C (Table 6), which means that PAA can be efficiently produced by AXE at room temperature.

The optimum pH of the reaction was evaluated over a pH range of 6.5–9.5 at 20 °C for 5 min. The highest yield of PAA (149.7 mM) was obtained at pH 8.0 (Fig. 5). This in turn proved the ability of AXE to synthesize PAA at room temperature and in a short duration (5 min), thus having a widespread commercial value and prospects for development. The highest PAA titer produced by AXEs from CE-7 family in US Patent No. 8518675B2 was about 70 mM. It was also found that AXE from *B. subtilis* CICC 20034 presented the highest efficiency of perhydrolysis activity for producing PAA.

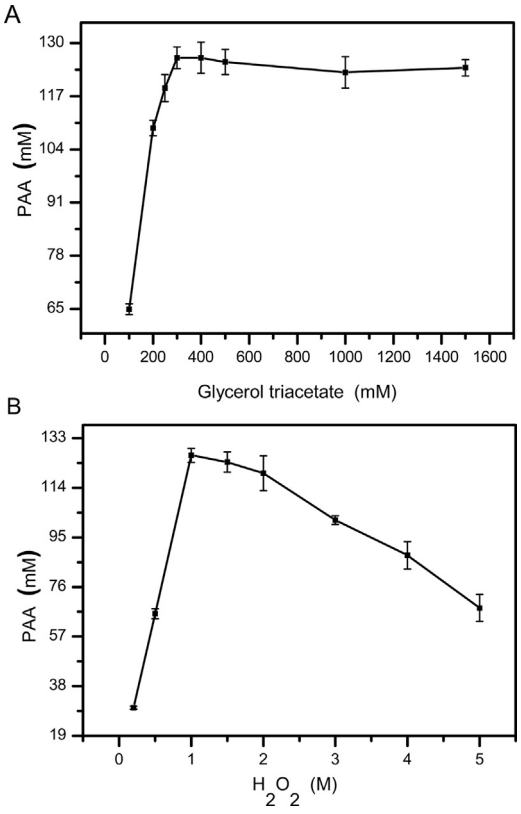

Fig. 4. Effects of different concentrations of glycerol triacetate and hydrogen peroxide on PAA production (A) The concentrations of glycerol triacetate; reaction conditions: 1 M hydrogen peroxide, 0.3 mg/mL AXE crude enzyme, and 1 mL reaction volume; 25 °C, pH 7.4, 5 min. (B) The concentrations of hydrogen peroxide; reaction conditions: 300 mM glycerol triacetate, 0.3 mg/mL AXE crude enzyme, and 1 mL reaction volume; 25 °C, pH 7.4, 5 min. The error bars represent standard deviation values of three independent experiments ($n = 3$).

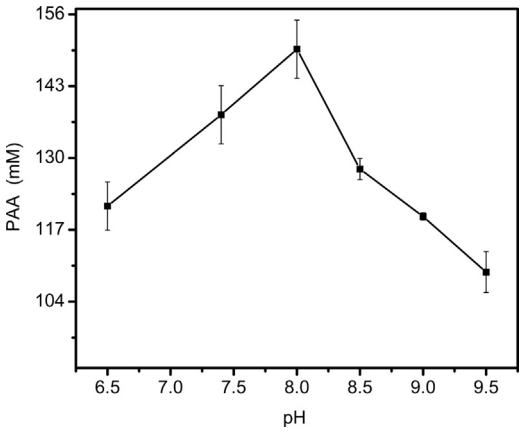

Fig. 5. Optimum pH for the synthesis of PAA. Reaction conditions: 300 mM glycerol triacetate, 1 M hydrogen peroxide, 0.3 mg/mL AXE crude enzyme, 1 mL reaction volume, 20 °C, 5 min. The error bars represent standard deviation values of three independent experiments ($n = 3$).

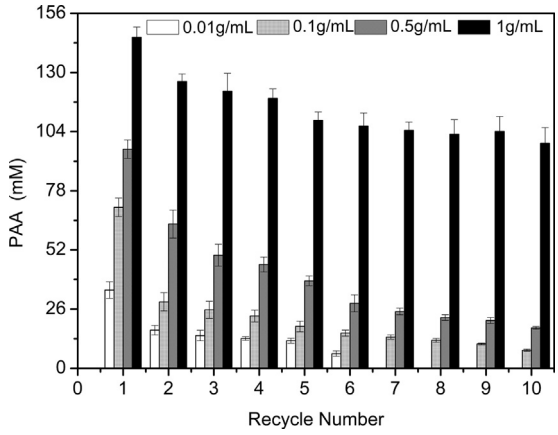

Fig. 6. Reusability of the immobilized AXE. Reaction conditions: 300 mM glycerol triacetate, 1 M hydrogen peroxide, and 10 mL reaction volume; 20 °C, pH 8.0, 5 min. The four different colors of bars indicate different immobilized enzyme loading ranging from 0.01 to 1 g/mL. The error bars represent standard deviation values of three independent experiments ($n = 3$).

3.7. PAA production by immobilized AXE and its reusability

The reusability of the immobilized AXE was investigated in a 50-mL tube filled with 10 mL Tris–HCl buffer (pH 8.0) containing 300 mM glycerol triacetate, 1 M H_2O_2, and immobilized AXE resin (0.01–1 g/mL) at 20 °C for 5 min. It was found that a higher catalyst loading could significantly improve the production and reusability of PAA. The PAA concentration obtained using 0.1 g/mL of immobilized AXE resin at the first cycle was approximately 70 mM, which decreased to 7.88 mM after 10 production cycles, about 11% of the initial level (Fig. 6). However, a high enzyme loading (1 g/mL of immobilized AXE resin) could overcome the deficiency; about 142.5 mM of PAA was produced in the first reaction cycle and about 95.5 mM after 10 cycles, 67% of the initial level. AXE of *A. ficuum* immobilized on modified graphite oxide and chitosan-coated Fe_3O_4 magnetic nanoparticles could produce PAA at 50% and 40% of the initial levels after 10 production cycles, respectively [23,13]. This result confirmed the high-quality performance of the prepared AXE, and thus it could be used as a potential catalyst for PAA synthesis.

4. Conclusion

This study concludes that the AXE from *B. subtilis* CICC 20034 exhibited high perhydrolysis activity toward acetate esters and can be used for efficient PAA synthesis. The recombinant AXE could be efficiently produced in a low-cost autoinduction medium with an activity of 6.8×10^3 U/mL. The highest PAA titer of approximately 150 mM was obtained in an aqueous solution. The immobilized AXE could be reused 10 times with the remaining activity of >67%. These results indicate that the recombinant AXE of *B. subtilis* CICC 20034 exhibited significant perhydrolase activity for the conversion of acetate esters (in the presence of hydrogen peroxide) into PAA in an aqueous condition at concentrations sufficient for use as a disinfectant and/or bleaching agent.

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