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Article

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Identification of a novel esterase from marine environmental genomic DNA libraries and its application in production of free all-*trans*-astaxanthin

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Abstract

Astaxanthin is a pigment with various functions. Free astaxanthin is obtained mainly through saponification methods, which could result in many by-products. Enzymatic methods using lipases have been used in few cases, while there are no reports on the use of esterases for the production of free astaxanthin. Herein we present the screening and identification of a novel esterase (Est3-14) from a marine mud metagenomic library. Est3-14 is pH-sensitive and keeps good stability in akaline buffers (residual activity 94%, pH 8.0, 4 ℃ and 36 h). Meanwhile, Est3-14 keeps a good stability in the medium temperature condition (residual activity 56.7%, pH 8.0, 40 °C and 84 h). Est3-14 displayed high hydrolysis activity to prepare free all-*trans*-astaxanthin in biphasic systems. Furthermore, under optimal conditions (0.5 mL ethanol, 6 mL 0.1 M Tris-HCl buffer, pH 8.0, 0.5% (w/v) *H. pluvialis* oil, 40 ℃), the hydrolytic conversion ratio was 99.3% after 36 h.

Key words: Metagenomic Library, Alkaline Esterase, Free Ax, Enzymatic Methods, High Hydrolytic Conversion Ratio

■ INTRODUCTION

Astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione) (Ax) is a terpenoid with various functions, such as antioxidant activity,[1] antitumor activity,[2] immune response enhancement,[3] anti-nerve injury,[4] inhibition of *Helicobacter pylori* infection,[5] and improvement of memory.[6] Thanks to these properties, it has been widely applied to food, cosmetic, aquaculture and poultry.[7, 8]

Ax is widely distributed in aquatic organisms, such as the shrimp, crab shells and microalgae.[9, 10] In particular, the green alga *Haematococcus pluvialis* (*H. pluvialis*) is considered as the most suitable source for natural Ax being the content of this carotenoid about 4% (w/w) in the dry biomass.[11, 12] However, 95% of Ax in *H. pluvialis* is esterified with long chain (C16-C20) fatty acids.[13] Ax has several isomers, such as three geometrical isomers (9*Z*-, all-*E*- and 13*Z*-) and three optical isomers ((3*S*,3'*S*) (all-*trans*), (3*R*,3'*S*) and (3*R*, 3'*R*)). Under natural conditions, all-*trans* configuration is the most stable structure (Figure S1, Supporting Information).[14] The configuration of Ax in *H. pluvialis* is consistent with the Ax in wild salmon, which is all-*trans*.[15, 16] It has been demonstrated that Ax esters may have different chemical functionalities that confer them a different physiological role, which may limit the wide application of Ax from *H. pluvial.*[13, 17] Free all-*trans*-Ax has been served as a standard to compare the different functions of Ax esters.[18] Ax also has wide application in aquaculture field. Choubert et al.[19] found that the inclusion of Ax in the diet of rainbow trout had a great influence on the muscle pigmentation of this fish.

Therefore, the preparation of free all-*trans*-Ax from its esters produced in *H. pluvialis* is a biotechnological field of major interest.[20-23]

Numerous attempts have been made to hydrolyze Ax esters for obtaining free Ax. Saponification is an efficient method to cleave esters, however, alkaline conditions can cause the formation of by-products (astacene) that may have biological functions different from Ax.[24-27] Enzyme-catalyzed hydrolysis is expected to be an environmentally friendly promising alternative to saponification, thanks to the mild hydrolysis conditions, especially if carried out under a nitrogen atmosphere to inhibit the formation of by-products.[13, 23, 28] In particular, lipases (EC 3.1.1.3) and esterases (EC 3.1.1.1) are a family of hydrolases that catalyze the cleavage of ester bonds and are widespread in nature.[29, 30] Up to now, some studies about preparing the free Ax are mainly focused on lipase-mediated catalysis.[13, 23, 28] Zhao et al. found five lipases could hydrolyze Ax esters from *H. pluvialis* oil, which could hydrolyze 63.2% Ax esters.[13] Nagao et al. attempted to obtain purified Ax by two-step process, they got 63.9% Ax of the initial content in the cell extract finally.[28] However, this group never used this enzyme for the recovery of free Ax from a natural source. Lipases are important industrial biocatalysts and have been widely used for the preparation of free Ax, however, the shortcomings of above researches are the long reaction time and the low conversion efficiency. Indeed, a long reaction time is not suitable for storing Ax, which could cause the degradation of Ax. Besides lipases also esterases could be suitable to improve the catalytic efficiency. It is well known that esterases (EC3.1.1.1)

could hydrolyze esters with short-chain fatty acids (less than 10 carbon atoms). Instead, lipases (EC3.1.1.3) prefer hydrolyzing esters with long chain fatty acids (≥10). Nevertheless, only in a few cases lipases have been used for the cleavage of Ax esters.[13] This lack is due to the absence of known esterases active on these substrates.

Screening of novel biocatalysts is the subject of investigation of many scientists. These studies have facilitated the progress of new methods to separate novel genes. Nevertheless, though the biocatalysts obtained from new isolated genes are abundant, most of the microorganisms from where the isolated genes originated are not cultivable. It is estimated that less than 1% of microorganisms in nature have ever been cultured by traditional methods in lab.[31] Thanks to the development of metagenomics, the loss of diverse biocatalysts can be avoided. Isolation of metagenomic DNA from the environmental samples and cloning it into a special vector can be constructed a metagenomic library. The desired genes can be isolated through functional screening from this metagenomic library. Up to now, this approach is one of the most powerful methods to screen novel biocatalysts without the need for culturing.[32]

Within this work, we constructed a fosmid metagenomic library and screened the lipolytic genes on large scale. A novel esterase (Est3-14) was identified and purified for further characterization. The Est3-14 also displayed high hydrolytic activity in aqueous biphasic systems to prepare the free all-*trans*-Ax from Ax esters.

■ MATERIALS AND METHODS

Materials. DNA amplification used the DNA polymerase from Vazyme (Nanjing, China). Restriction enzymes (*Not*I，*Bam*HI, *Hin*dIII, *Sau*3AI and Calf-intestinal alkaline phosphatase (CIP)) were bought from NEB (New England Biolabs) (MA, USA). The *p*-Nitrophenol (*p*NP) and its esters were bought from Sigma (MO, USA). *H. pluvialis* oil (0.5% (w/w) Ax esters) was bought from Yunnan Alphy Biotech (Yunnan, China). Pre-prepared plates of silica (GF254) were supplied by Qingdao Shenghai silica Chemical Co. Ltd. (Qingdao, China). Methanol, Methyl tert-butylether (MTBE) was purchased from EMD Millipore Corporation (Billerica, MA) and other chemicals and organic solvents were analytical reagents.

The construction of fosmid library and screening. The metagenomic DNA was extracted from marine mud. DNA fragments from 15 to 45 kb were gained by partial digestion with the restriction enzyme *Not*I and used to build a fosmid library by protocol of CopyControl pCC1FOS Fosmid Library Production Kit (Epicentre Biotechnologies). Transformants, with recombinant fosmids, were cultured on Luria-Bertani (LB) agar (containing chloramphenicol (Chl)) at 37 ℃ for 20 h. To identify the effectiveness of library, 5 clones were stochastically chosen and digested with the restriction enzyme *Not*I. The method of construction of metagenomic library is similar to that reported by Gao et al.[38]

Construction of sub-cloning genomic library and sequence analysis of lipolytic genes. For screening the colonies exhibiting lipolytic genes, the positive

126 pools from fosmid library were diluted with sterile saline solution and cultivated at
127 37 °C for 48-72 hours on LB agar (containing Chl and emulsified tributyrin). The
128 positive pools from above LB agar were partially digested with restriction enzyme
129 *Sau*3AI to gather 3-9 kb DNA fragments. Prepared DNA fragments were ligated into
130 pBluescript II SK (+) vector. The products of ligation were introduced into *E. coli*
131 DH5α cells. The transformants were cultivated on LB agar plates (containing
132 ampicillin and tributyrin) at 37 °C for 48 h. Then, positive colonies with clear halos
133 were cultured in LB medium and sent to be sequenced.

134 NCBI open reading frame (ORF) finder (https://www.ncbi.nlm.nih.gov/orffinder/)
135 was used to identify the ORFs in the nucleotide sequences. The functions of ORFs
136 were predicted using BLASTP search (https://blast.ncbi.nlm.nih.gov/Blast.cgi).
137 Multiple sequence alignments were analyzed by Clustal X and ESPript 3.0
138 (http://espript.ibcp.fr/ESPript/ESPript/). The neighbor-joining phylogenetic tree
139 among hydrolases was analyzed by MAGA 6.0 software.

140 **Heterologous expression and purification of esterase Est3-14.** According to
141 the one positive clone with predicted lipolytic gene (*est3-14*), the gene was ligated to
142 pET-28a (+) vector and introduced into the expression host *E. coli* BL21 (DE3).
143 Correct recombinant strain was cultivated at 37 °C until the value of OD_{600} reached
144 0.6-0.8. Then, after adding isopropyl-β-D-1-thiogalactopyranoside (IPTG), it was
145 further cultivated at 20 °C for 20 h. Centrifugation was used to collect cells and the
146 cells were washed with sterile NaCl solution. The collected cells were ruptured by

147 sonication in 0.1 M, Tris-HCl buffer, pH 8.0 (Buffer A). After centrifugation (10,000
148 × g) for 30 min at 4 °C, the supernatant of Est3-14 was loaded onto a Ni-NTA column
149 (1 mL, Qiagen, Hilden, Germany). The target protein was purified by elution with 0.2
150 M imidazole in Buffer A and dialyzed against the same buffer to remove imidazole.[33]
151 Finally, the expression level and the purity of Est3-14 was measured by SDS-PAGE.
152 The esterase activity was determined by using *p*-nitro phenyl butyrate (*p*NPB) (0.02
153 M dissolved in buffer of isopropanol and dimethyl sulfoxide) as substrate and
154 following the formation of *p*NP at 405 nm according to the method reported by
155 Margesin with minor modification.[34] One unit of esterase activity was defined as the
156 amount of esterase needed to form 1 μmol *p*NP per minute. Soluble protein content
157 was estimated by the method of Coomassie brilliant blue.

158 **Enzyme characterization of esterase Est3-14.** Esterase activity and soluble
159 protein content were estimated as described in the section heterologous expression
160 and purification of esterase Est3-14.

161 Substrate specificity of Est3-14 was tested by the same method as described
162 above but adopting *p*NP esters with variable chain length: *p*NP acetate (C2), *p*NP
163 butyrate (C4), *p*NP caprylate (C6), *p*NP decanoate (C8), *p*NP caprate (C10), *p*NP
164 laurate (C12), *p*NP myristate (C14), and *p*NP palmitate (C16).

165 To identify the optimal pH of hydrolysis, the esterase activity of Est3-14 was
166 determined at pH values from 4.0 to 10.0 in four different buffers. In particular
167 sodium citrate, sodium phosphate, Tris-HCl and Na_2CO_3-$NaHCO_3$ were used (0.1 M)

for the pH range of 4.0-6.0, 6.0-8.0, 8.0-9.0 and 9.0-10.0, respectively.

To identify the pH stability of Est3-14, the purified enzyme was incubated in different buffers with pH ranging from 5.0 to 10.0 for 36 h. The residual activity of Est3-14 was determined by the method mentioned above.

The determination of the optimal temperature was conducted at temperatures from 30 ℃ to 80 ℃ using the *p*NP butyrate as substrate. Meanwhile, the thermal stability of Est3-14 was detected by putting the purified enzyme into different temperature conditions (40 ℃, 45 ℃, 50 ℃, 55 ℃, 60 ℃) for up to 84 h, and then the remaining activity was measured.

Effects of surfactants, metal ions and organic solvents on Est3-14 activity. The influence of surfactants on the esterase activity was determined in Buffer A including 0.5% (w/v) variable surfactants (Triton X-100, Tween 80, Tween 60, SDS, Tween 20). The activity measured without adding surfactants was used as the negative control. The effect of metal ions ($CoCl_2$, KCl, LiCl, $FeSO_4$, $FeCl_3$, $MnCl_2$, $CaCl_2$, $MgCl_2$, $ZnCl_2$ and $NiCl_2$) and chelating agent Na_2-EDTA on Est3-14 activity were analyzed at the concentration of 1 mM and 10 mM in Buffer A.

To estimate the tolerance of Est3-14 to organic solvents (methanol, ethanol, acetonitrile, n-hexane, chloroform, dimethylsulfoxide (DMSO), acetone, n-propanol, isopropanol, isooctane, cyclohexane), the catalytic activity of Est3-14 was determined after placing the enzyme solution at 30 ℃ for 3 h in organic solvent/water mixture (25%, 50% (v/v), respectively).[33] To reduce the influence of the organic solvents

189 during the activity assay, the hydrophobic organic solvents were removed by
190 centrifugation or, in the case of hydrophilic organic solvents, diluted to 5%.

191 The identification of pure organic solvents effect on Est3-14 was tested according
192 to the method described by Li et al.[41] Est3-14 powder was added to organic solvents
193 and placed at 30 °C while shaking for 3 h. Centrifugation was taken to remove
194 organic solvents. The remaining esterase activity was measured under standard
195 conditions.

196 **Hydrolysis of Ax esters from *H. pluvialis* oil.** In order to test the hydrolysis
197 ability of Est3-14 towards Ax esters, the supernatants of *E. coli* cell lysate of Est3-14
198 was lyophilized by vacuum freezing, and the dried crude powder was used to catalyze
199 the hydrolysis reaction. Esterase activity per milligram of dried cell powder was
200 determined as above. The hydrolysis reaction was carried out adding the supernatant
201 of Est3-14 lysate to a biphasic system (0.5 mL ethanol; 6 mL Buffer A; 0.5% (w/v) *H.*
202 *pluvialis* oil) at 40 °C and shaking for 48 h in a thermostatic bath. Free all-*trans*-Ax
203 was extracted from the reaction medium by 1/1 (v/v) isopropanol/dichloromethane
204 mixture for several times until the aqueous phase was colorless. After extraction, the
205 method of TLC was used to analyze whether Est3-14 could hydrolyze Ax esters. The
206 mobile phase was the mixture of n-hexane and acetone and the ratio was 4:1 (v/v).
207 The standard of Ax was used as a positive control.

208 **Identification of free all-*trans*-Ax in the hydrolysis medium.** The reaction
209 samples were analyzed by using a high-performance liquid chromatograph (HPLC)

(Agilent) equipped with C-30 column (YMC, 250 × 4.6 mm; 5 μm). The detector wavelength was set to 476 nm and the temperature of column was kept at 35 °C.[23] The elution conditions were as follows: 0-15 min, isocratic conditions 90% A and 10% B, then gradient to 40% A and 60% B until t = 25 min, 25-35 min, the gradients came to initial concentration. Eluent A consisted of methanol, Eluent B consisted of MTBE. Analysis was conducted in triplicate.

The standard curve of the standard of free all-*trans*-Ax was used to quantify the production of free all-*trans*-Ax. (Figure S5, Supporting Information) The Ax ester hydrolysis rate was equal to decrease rate of Ax esters. The equation was as follows: Conversion=Area of residual Ax esters after hydrolysis/ Area of initial Ax esters in the reaction medium

HPLC-MS/APCI was adopted to analyze free all-trans-Ax in the hydrolysis medium. The gradient elution program used in HPLC-MS/APCI was consistent with the method mentioned in HPLC determination. The MS instrument was Bruker maXis II (Bruker, Germany) equipped with a quadrupole mass spectrometer system Navigator. The instrument was fitted with APCI with the positive ionization mode. The voltage of the fragmentation was set to 35 eV and the flow rate of nitrogen was 5 L/min. The range of scanning was set from 200 to 650 (m/z) with the scan time of 0.5 s.

The reaction curve of preparing free all-*trans*-Ax under different reaction times and enzyme loading. Under the initial reaction conditions, the hydrolysis curve

was drawn by sampling the reaction mixture at different times (12 h, 24 h, 36 h, 48 h, 72 h and 84 h) and enzyme loading (180 U, 300 U, 900 U, 1800 U, 2700 U, 3600 U) to identify the efficiency of hydrolysis.

■ RESULTS AND DISCUSSION

The construction of fosmid library and functional screening. According to the results of identification of the marine mud metagenomic library (average insert size 40 kb, 40,000 clones), it was observed that the constructed fosmid metagenomic library is various. The results of digestion with *Not*I of 5 randomly chosen clones suggested that 90% of clones included different DNA fragments. It is suggested that this metagenomic library is of high efficiency. The functional screening was based on the ability of producing transparent circle on the plates of LB agar (containing tributyrin and Chl). After incubation at 37 °C for 48 h, the positive fosmids (40 kb) (Figure S2, Supporting Information) were extracted and used for the construction of sub-clones.

To identify the effective fragments among the positive fosmids, the fosmids were digested by restriction enzyme *Sau*3AI to gather 3.0-9.0 kb fragments (Figure S3, Supporting Inforamtion). Then, the fragments were ligated to pBluescript II SK (+) vector and introduced into *E. coli*. The sub-clones that showed lipolytic activity were sent to be sequenced. After sequenced by Sangon Biotech (Shanghai, China) and on the basis of the ORF finder and BLASTP search, the inserted DNA fragment was

identified.

The sequence analysis and phylogenetic tree construction of esterase gene *est3-14*. The putative lipolytic gene was named *est3-14* and had sequence identities of 51% with the esterase gene (KRO42937.1) from *Acidimicrobium* sp., which has not been expressed and characterized. The low similarity with other esterase genes shows that *est3-14* is a new esterase gene. The nucleotide sequence of esterase Est3-14 was uploaded to Genebank database with the accession number MF568505.

Phylogenetic analysis of *est3-14* was carried out according to the classification reported by Arpigny and Jaeger.[35] On the basis of this classification and on the amino acid sequences of different esterase genes belonging to the Families I-VIII, esterase Est3-14 belongs to family V (Figure 1a). Furthermore, the multiple sequence alignment (Figure 1b) revealed that Est3-14 has the typical catalytic triad (Ser, 115; Asp, 237; His, 266), confirming, Est3-14 was a novel esterase.

Heterologous expression and purification of Est3-14. The *est*3-14 gene (*Bam*HI/*Hin*dIII restriction site) was ligated to pET-28a (+) and transformed into BL21 (DE3) to express active protein. The Est3-14 was expressed successfully in *E. coli* and the expression level was estimated by SDS-PAGE (Figure S4, Supporting Inforamtion). The esterase activity determination showed that this vector could be used to express active esterase. Based on the existence of the terminal 6×His affinity tag, esterase Est3-14 was purified successfully. The purified Est3-14 was separated as a single protein and consistent with the predicted molecular weight (32.8 kDa), which

was shown in Figure 2.

Enzyme characterization of purified esterase Est3-14. The catalytic activity of Est3-14, measured by using *p*NP esters with different acyl chain lengths, showed that this enzyme preferentially hydrolyzed short chain fatty acids (C<10) and showed the highest activity towards *p*NP butyrate (292 U/mg, at pH 8.0 and 37 °C), and the lowest towards *p*NP palmitate (20.9% of the *p*NP butyrate) (pH 8.0 and 37 °C) (Figure 3a). These results indicated that Est3-14 is a "true" esterase that preferentially hydrolyzed the substrate with short acyl chain length.[36, 37]

The effects of pH on Est3-14 activity were tested using *p*NPB as substrate. Est3-14 was very pH-sensitive and showed relatively higher activities at alkaline conditions, with optimal pH at 9.0 in phosphate buffer and inactivation at pH < 6.0 (<10% maximum activity in optimal pH) (Figure 3b). The pH stability of Est3-14 was analyzed by testing the residual activity at different time intervals after pretreatment (4 °C, up to 36 h) in different pH value buffers (pH 5.0-10.0). Data in Figure 3c showed that at pH 8.0 even after 36 h, the residual activity of Est3-14 only slightly decreased (residual activity was 94%). Instead, after incubation for 6 hours at pH 6.0 the enzyme kept about 62% of the original activity. However, the residual activity of Est3-14 decreased sharply at pH < 6.0 buffer, and it plunged to 23.7% of the original activity at pH 5.0. According to these results, Est3-14 displayed high activity in alkaline buffers. Therefore, we can conclude that Est3-14 is a member of alkaline esterases.

294 The influence of temperature on Est3-14 catalytic activity was tested in the
295 30-80 °C range, and it was found that it increased quickly with the temperature to
296 60 °C and then declined at 70 °C and 80 °C (Figure 3d). In a recent report, EstK1
297 displayed highest activity at 50 °C (Est3-14, 60 °C),[29] which indicated that Est3-14
298 has a promising application in the high temperature condition. Thermal stability of
299 Est3-14 was identified by measuring the remaining activity at various time intervals
300 after pretreatment of the purified enzyme for 84 h at different temperatures (40 °C,
301 45 °C, 50 °C, 55 °C, 60 °C). Est3-14 preserved over 60% of its original activity after
302 incubation at 40 °C for 78 h. At 55 °C, when the enzyme was pretreated for 24 h, the
303 residual activity of Est3-14 decreased to 50%. However, at 60 °C, the residual activity
304 decreased to 35% of original activity within 6 h (Figure 4e). Based on these data,
305 Est3-14 appeared stable at medium temperature (e.g., 40 °C), which was consistent
306 with esterase EST4 (40 °C) reported by Gao et al.[38] It is a beneficial feature in the
307 perspective of applying this enzyme in the hydrolysis of Ax esters.

308 The various surfactants (0.5%) could cause different effects on esterase activity.
309 As shown in Figure 4f, the activity of Est3-14 was slightly stimulated by Tween 20,
310 Tween 60, Tween 80, Triton X-100. SDS, an anionic surfactant, had a strong
311 inhibitory effect on Est3-14 activity, which is similar to that observed with the
312 esterase Est_p6 reported by Peng et al.[43] and with the esterase Est4 reported by Gao et
313 al.[38]

314 Concerning the influences of the metal ions and Na_2-EDTA on the esterase

315 activity, Est3-14 was slightly stimulated by adding Co^{2+}, K^{+} at the low concentration
316 (1 mM) (Table 1). While when the addition of metal ions and Na_2-EDTA was
317 increased to 10 mM, Ca^{2+}, Mn^{2+}, Mg^{2+}, Co^{2+}, Ni^{2+}, Fe^{3+}, Zn^{2+} and Na_2-EDTA caused a
318 decrease of Est3-14 activity by 20-80%, and the Zn^{2+} displayed highest inhibiting
319 effect to activity of Est 3-14 (decreased by 77%).

320 The effect of organic solvents on Est3-14 was studied at various concentrations of
321 different organic solvents (Table 2) with different Log *P* values from -1.3 to 4.5. Log
322 *P* is an indicator of solvent polarity, and the log *P* value of hydrophobic organic
323 solvents was ≥ 2.[39] Detailed assay method was carried out according to previous
324 report.[40] Methanol, chloroform, acetonitrile and isopropanol had slight inhibitory
325 effects on Est3-14 activity at a concentration of 25% (v/v). However, high organic
326 solvent concentration (50% v/v) decreased the activity sharply by 25.7-81.5%.
327 Furthermore, the purified enzyme was highly denatured by the hydrophilic organic
328 solvents (log $P < 2$). Generally, Est3-14 was more stable in hydrophobic organic
329 solvents, which was similar to lipase SML reported by Li et al.[41] For example, the
330 residual activity of Est3-14 in absolute n-hexane was 88.2% of original activity, which
331 indicates that Est3-14 is stable in organic solvents. Compared with other reports,
332 Est3-14 was much more stable than rEstSL3[44] and Lip_{BA}[45] in organic solvents. It was
333 worth pointing out that Est3-14 displayed high enzyme activity (91.2%) in low
334 concentration of ethanol (25% v/v), which could guarantee the hydrolysis reaction of
335 Ax esters in the biphasic system.

Application of Est3-14 in hydrolysis of Ax esters from *H. pluvialis* oil and the identification of free all-*trans*-Ax. In order to test the ability of Est3-14 of hydrolyzing Ax esters (Scheme 1), 1200 U crude powder was added to start the reaction (0.5 mL ethanol; 6 mL Buffer A; 0.5% (w/v) *H. pluvialis* oil). The TLC result of the hydrolysis of Ax esters was shown in Figure 4. It was observed that Est3-14 can be used to remove the ester bonds of Ax esters to prepare free all-*trans*-Ax. After analyzing by HPLC, the result is consistent with result of TLC. We can see from the Figure 6, the peak of retention time of 7.7 min was the free all-*trans*-Ax. In order to identify the product, we used MS/MS to do further verification. In Figure 5a, the retention time of free all-*trans*-Ax (retention time 7.7 min) in the mixture is consistent with the standard. The MS result of peak (7.7 min) from Figure 5a was expressed in Figure 5c, a strong protonated quasimolecule ion was seen at m/z 597.3934, which corresponded to the $[M+H]^+$ of free all-*trans*-Ax reported by Zhao et al.[13] In the Figure 5d, the peaks at m/z 147.1484 and 173.1588 were characteristic fragments of free all-*trans*-Ax, which were consistent with the standard of free all-*trans*-Ax (Figure 5b). The characteristic fragments of free all-*trans*-Ax corresponded to the results reported by Dong et al.[23] Therefore, the peak (retention time 7.7 min) was eventually identified as all-*trans*-Ax.

The reaction curve of preparing all-*trans*-Ax under different reaction times and enzyme loading. At industrial level, reaction time and enzyme loading play important roles on the final cost of the product. Thus, the time of reaction and the

357 enzyme loading to complete conversion of substrate and the space-yield are crucial
358 parameters to be evaluated.

359 We studied the relationship among reaction time, conversion ratio of Ax esters
360 hydrolysis and space-time-yield. As shown in Figure 6a, with the loading of 1200 U
361 Est3-14 crude powder and the reaction time changing, the tendency of hydrolysis
362 could be detected by HPLC; the conversion of Ax esters reached (88.9%) at 36 h and
363 96.7% at 48 h (Figure 6b); the space-time-yield was shown in Figure 6c, at last we got
364 200 $\mu g \cdot mL^{-1}$ free all-*trans*-Ax.

365 Under the optimal reaction time, we also studied the relationship between
366 enzyme loading and conversion ratio, in Figure 7a, we could find that the content of
367 free all-*trans*-Ax with different enzyme loading and at 1800 U (all-*trans*-Ax: 195
368 $\mu g \cdot mL^{-1}$, the reaction came to balance and the final conversion reached 99.3% (Figure
369 7b).

370 It is important to emphasize that the conversion obtained in the present study is
371 higher than that reported in previous studies. After the reaction, 99.3% Ax esters were
372 hydrolyzed and the production was about 195 $\mu g \cdot mL^{-1}$. According to previous studies,
373 the conversion is higher than them. In a recent study, the hydrolysis of Ax esters was
374 carried out using an alkaline lipase from *Penicillium cyclopium* and the final recovery
375 is about 63.2%.[13] In another report, they used two steps to get the free Ax, first step
376 was used to remove FFA, the second step was used to get free Ax. It is worth pointing
377 that the second hydrolysis step needed 68 h and the total reaction time was 110 h

(final recovery, 63.9%).[28] However, our reaction time is less than 110 h, which is benefit to application. Fermentation also was used to prepare Ax, the conversion of Ax esters was 98.08% at 60 h.[23] Saponification is a traditional method to prepare the free Ax,[24, 42] low temperature (5 °C) was chosen to minimize the degradation of Ax during saponification, and 94.4% Ax esters was transformed into free Ax after saponification for 12 h.[42] However, the low temperature control needs more cost and the hydrolysis of astaxanthin esters by chemical methods from both *H. pluvialis* rarely produced Ax but always astacene and was therefore not considered applicable for industrial use. Therefore, Est3-14 is a promising alternative biocatalyst in preparation of free all-*trans*-Ax.

This is the first report to describe the esterase to prepare the all-*trans*-Ax. In this study, we identified a novel esterase (*est*3-14) gene (51% similarity) from a marine mud metagenomic library. Est3-14 is pH-sensitive and keeps good stability in alkaline buffers, especially in Buffer A (residual activity 94%, 36 h, 4 °C). At the same time, Est3-14 keeps a good stability in the medium temperature condition (residual activity 56.7%, Buffer A, 40 °C, 84 h). Under optimal conditions, the final hydrolysis rate was 99.3% and the yield of free all-*trans*-Ax was 200 $\mu g \cdot mL^{-1}$. According to these results, we found that the hydrolysis rate was high and the by-products of enzymatic method were less than using saponification,[24] which is a pre-requisite for the development of a green chemistry process. The whole reaction system is mild (moderate temperature and less chemical solvents) and the less by-products.[23] Thus, it can be concluded that

399 Est3-14 is an efficient biocatalyst exploitable for the development of a competitive
400 industrial process for obtaining free all-*trans*-Ax. Certainly, the reaction system needs
401 further optimization to improve the volume of production of prepared free
402 all-*trans*-Ax. Furthermore, by the means of enzyme engineering for improving the
403 enzymatic properties, including substrate specificity, new applications for Est3-14 can
404 also be envisaged.
405

406 **■ ABBREVIATIONS**

407 Ax: astaxanthin; LB: Luria-Bertani; CIP: alkaline phosphatase calf intestinal;
408 *p*NP: *p*-nitrophenol; *p*NP acetate (C2): *p*-nitrophenyl acetate; *p*NP butyrate (C4
409 *p*NPB): *p*-nitrophenyl butyrate; *p*NP caprylate (C6): *p*-nitrophenyl caprylate; *p*NP
410 decanoate (C8): *p*NP decanoate, *p*NP caprate (C10): *p*-nitrophenyl caprate; *p*NP
411 laurate (C12): *p*-nitrophenyl laurate; *p*NP myristate (C14): *p*-nitrophenyl myristate;
412 *p*NP palmitate (C16): *p*-nitrophenyl palmitate; ORF: open reading frame; MTBE:
413 methyl tert-butyl ether; HPLC: high-performance liquid chromatography; IPTG:
414 isopropyl-β-D-1-thiogalactopyranoside; TLC: thin layer Chromatography; LB:
415 Luria-Bertani; EDTA: ethylene diamine tetraacetic; SDS: sodium dodecyl sulfate;
416 PAGE: polyacrylamide gel electrophoresis;
417

418 **■ FUNDING**

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Notes

The authors declare that they have no competing interests.

Supporting Information

Figure S1 The structures of Ax. all-*trans*-Ax, 9-cis-Ax, 13-cis-Ax, respectively.

Figure S2 The functional screening of positive strains from marine mud metagenomic library. M1, M2: with the clear hydrolysis halos on the LB agar with tributyrin (0.5%, v/v).

Figure S3 Partial digestion of positive strains with *Sau*3AI. The digestion time was: lane 1, M1-5 min; lane 2, M1-10 min; lane 3, M2-5 min; lane 4, M2-10 min.

Figure S4 Analysis of the expression level and purification of Est3-14 by SDS-PAGE. lane1, supernatant of pET-28a (+) cell lysate; lane 2, supernatant of *est3-14*-pET-28a (+) cell lysate; lane3, precipitation of *est*3-14-pET-28a (+) cell lysate; lane 4, 0.02 M imidazole with buffer A; lane 5, 0.05 M imidazole with buffer A; lane 6, 0.1 M imidazole with buffer A; lane 7, 0.2 M imidazole with buffer A; lane 8, 0.5 M imidazole with buffer A; Lane M, marker.

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590 **Figure legends**

591 **Figure 1** Bioinformatic analysis of Est3-14. (a) Neighbor-joining phylogenetic tree.
592 Phylogenetic analysis was obtained by MEGA 6.0 software, and Est3-14 is
593 shown as red triangles. (b) Multiple sequence alignments of Est3-14 and other
594 lipolytic enzymes belonging to family V. The typical motif is indicated using
595 blue circles, and the catalytic triad (Ser, Asp, His) is emphasized with pink
596 triangles.

597 **Figure 2** SDS-PAGE analysis of Est3-14. Lanes:0, protein marker;1, supernant of
598 Est3-14 cell lysate; 2, precipitation of Est3-14 cell lysate; 3, purified Est3-14.

599 **Figure 3** Characterization of Est3-14. **a** Substrate specificity of Est3-14 using pNP
600 esters with different acyl chain lengths; **b** Effect of pH on Est3-14 activity, used
601 *p*-NP butyrate as the substrate. The buffers used were citrate (*solid square*),
602 phosphate (*solid circle*), Tris-HCl (*solid triangle*), and Na_2CO_3-$NaHCO_3$ (*solid*
603 *inverted triangle*); **c** Effect of pH on Est3-14 stability, measured at different pHs
604 for 36 h in 0.1 M different pH buffer for various durations; **d** Effect of
605 temperature on Est3-14 activity ranging from 20 °C to 80 °C; **e** Est3-14 was
606 incubated in 0.1 M Tris-HCl buffer, pH 8.0, at 40, 45, 50, 55, or 60 °C for
607 various durations; **f** Effects of surfactants (0.5%) on Est3-14 activity, the reaction
608 without surfactant addition (control) was defined as 100%.

609 **Figure 4** TLC result of the hydrolysis of Ax esters with esterases Est3-14 from
610 metagenomic libraries. Lane 0, free all -*trans*-Ax; Lane 1, the hydrolysis results

611 of Est3-14; Lane 2, the substrate of Ax-esters.

612 **Figure 5** The result of identification of free all-*trans*-Ax from hydrolysis medium. (a)
613 Peak 1: free all-*trans*-Ax; (b) The MS/MS result of free all-*trans*-Ax standard; (c)
614 The MS result of free all-*trans*-Ax; (d) The MS/MS result of free all-*trans*-Ax.

615 **Figure 6** The result of effect of reaction time on hydrolysis of Ax esters. (a) HPLC of
616 hydrolysis of Ax esters at the different reaction time; (b) The conversion of Ax
617 esters under different reaction time; (c) The content of free all-*trans*-Ax under
618 different reaction time.

619 **Figure 7** The result of effect of enzyme loading on hydrolysis of Ax esters. (a)
620 Results of content of free all-*trans*-Ax at the different enzyme loading; (b) The
621 conversion of Ax esters under different enzyme loading.

622 **Scheme 1** Est3-14 catalyzed hydrolysis of Ax esters.

Tables

Tabale 1. Effect of metal ions and Na_2-EDTA on Est3-14 activity

Ion	Relative activity (%)[a]	
	1 mM	**10 mM**
Control	100±2.6	100±4.0
Co^{2+}	107.91±1.1	75.2±1.6
K^{+}	105.6±0.6	96.0±0.4
Li^{+}	96.8±1.3	84.2±0.2
EDTA	94.0±1.5	79.9±1.8
Fe^{3+}	84.3±1.8	44.7±2.4
Mn^{2+}	97.3±0.3	79.6±1.3
Ca^{2+}	95.4±0.2	78.9±2.4
Mg^{2+}	96.1±3.5	81.0±1.0
Zn^{2+}	73.1±3.2	23.2±5.9
Ni^{2+}	98.3±0.1	69.4±2.4

[a] The activity toward *p*NP butyrate measured in the absence of metal ions was defined as 100 %. All measurements were conducted in triplicate.

Table 2. Effects of organic solvents on Est3-14 activity

Organic solvents	log*P*[a]	Residual activity (%)[b] at concentration (%, v/v) of		
		25[a]	50[a]	100[a]
Control	-	100±2.4	100±0.9	100±1.9
DMSO	-1.3	75.9±3.2	69.5±2.4	17.1±1.6
Methanol	-0.76	63.7±2.3	22.5±2.4	20.0±1.4
Ethanol	-0.24	91.2±1.2	18.5±2.7	24.3±2.5
Acetone	-0.23	98.3±2.5	51.2±1.6	33.9±1.6
Acetonitrile	-0.15	73.1±1.9	20.0±0.5	90.2±1.3
Isopropanol	0.1	78.9±1.3	20.1±1.3	52.0±1.8
n-Propanol	0.28	20.7±1.5	19.5±1.7	32.4±1.7
Chloroform	2.0	62.6±1.5	74.3±1.3	26.1±1.2
Cyclohexane	3.2	84.8±1.8	72.9±2.3	41.7±2.4
n-hexane	3.5	54.8±2.3	68.9±4.3	88.2±2.1
Isooctane	4.5	52.3±2.3	64.6±3.7	43.7±0.6

[a] log *P* value is the partition coefficient of an organic solvent between water and *n*-octanol phases

[b] After pretreating Est 3-14 for 3 h in different organic solvents, the remanent activity was measured in Buffer A by using *p*-NP butyrate as the substrate. The 100% activity was defined as the enzyme without adding any organic solvents.

Figures

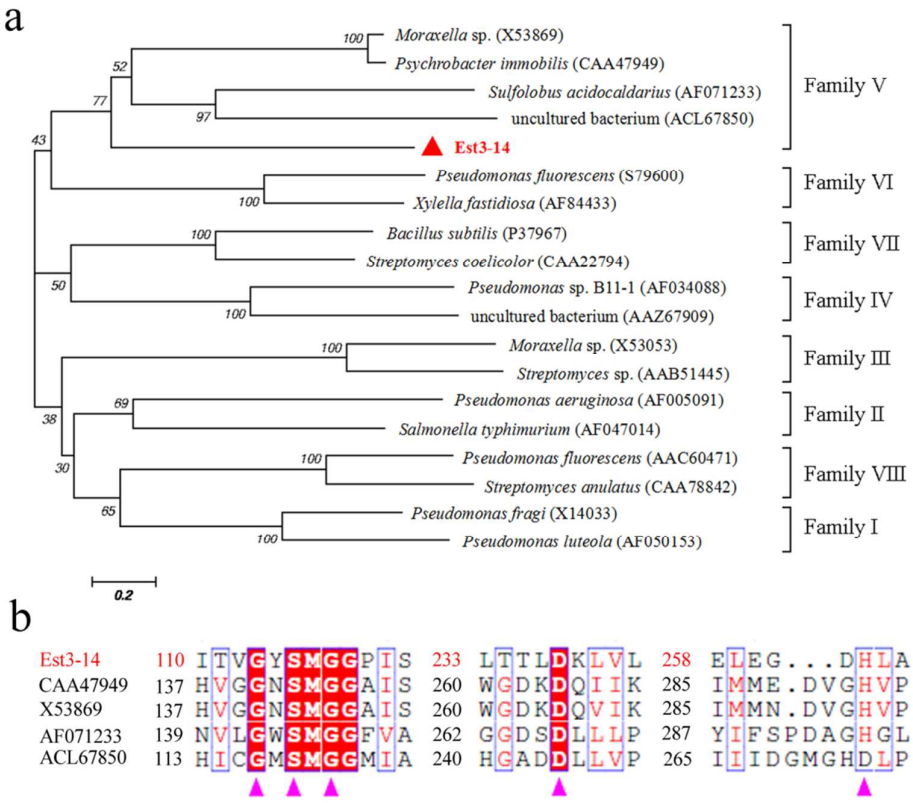


Figure 1 Bioinformatic analysis of Est3-14.

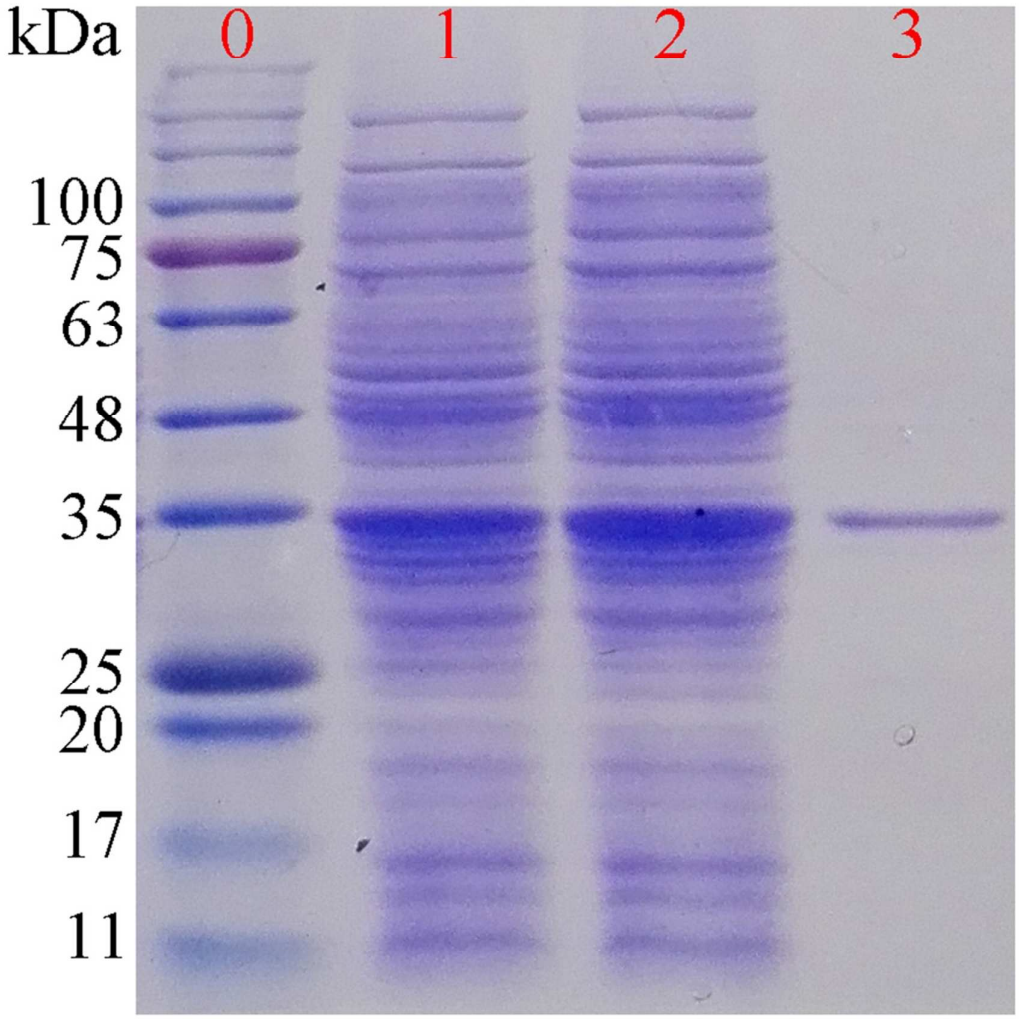


Figure 2 SDS-PAGE analysis of Est3-14.

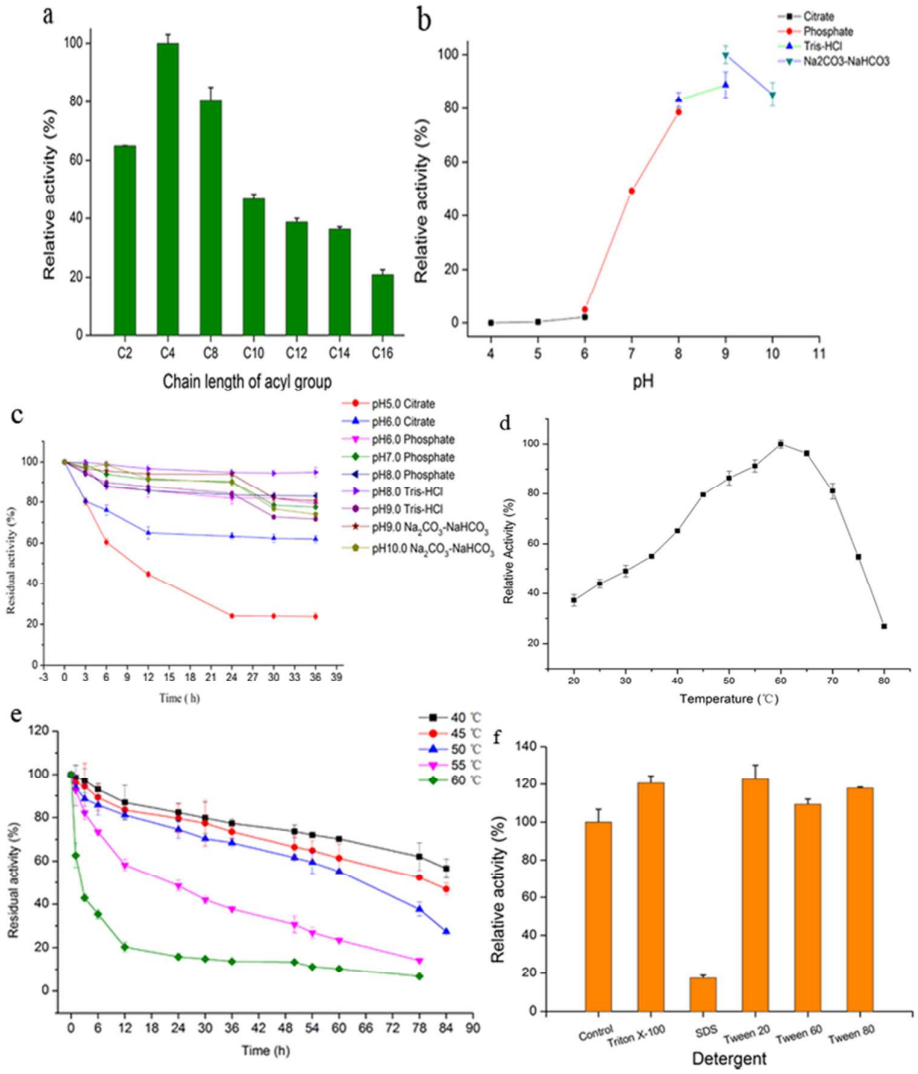


Figure 3 Characterization of Est3-14.

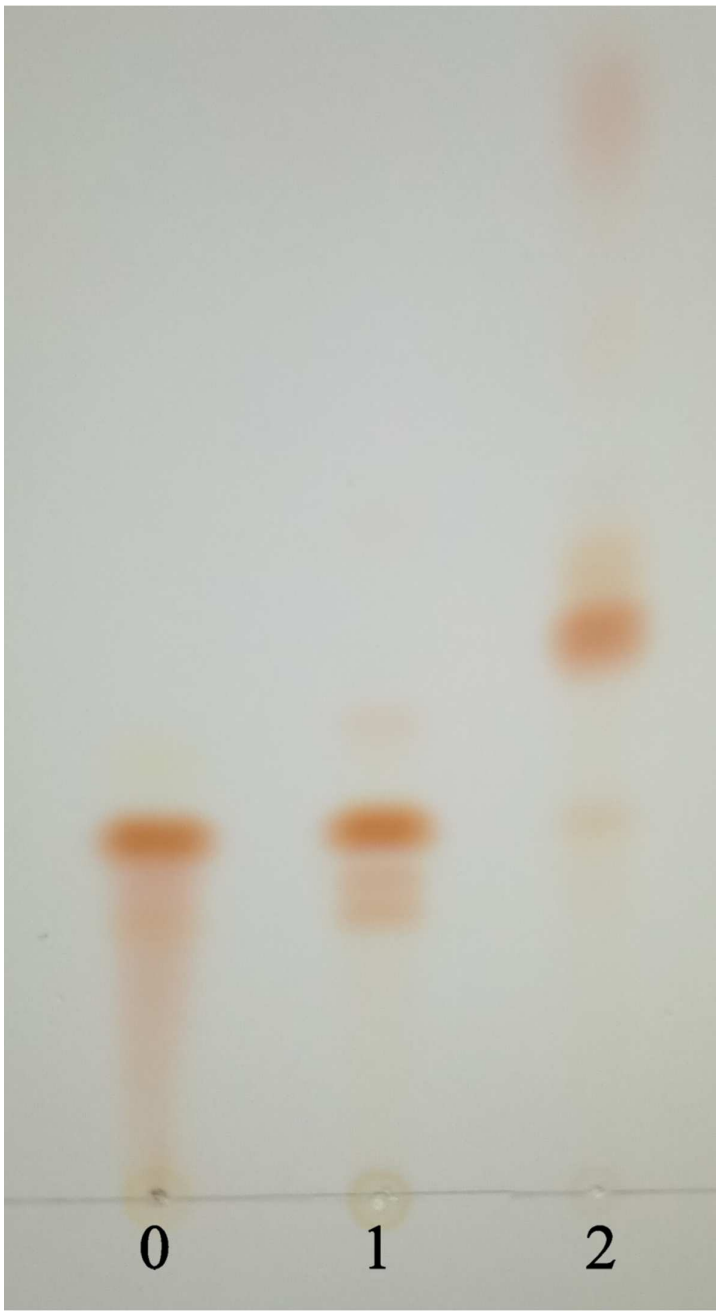


Figure 4 TLC result of the hydrolysis of Ax esters

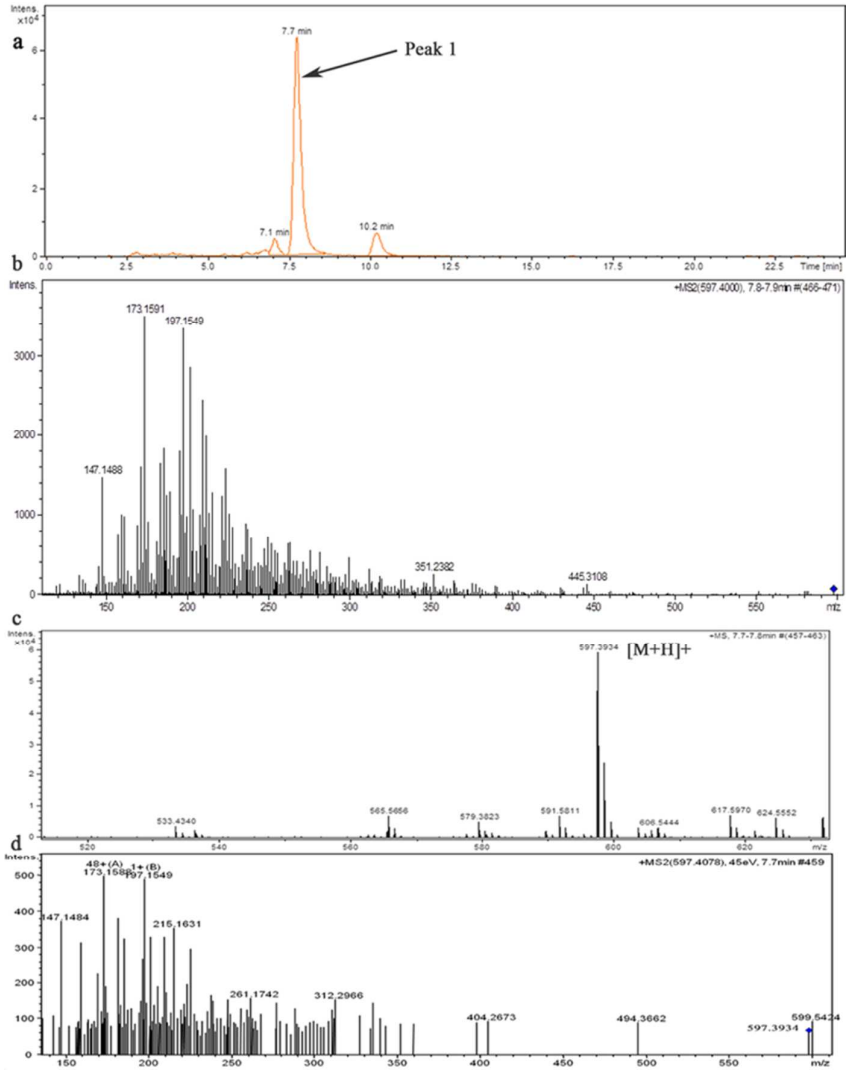


Figure 5 The result of identification of free all-*trans*-Ax from hydrolysis medium.

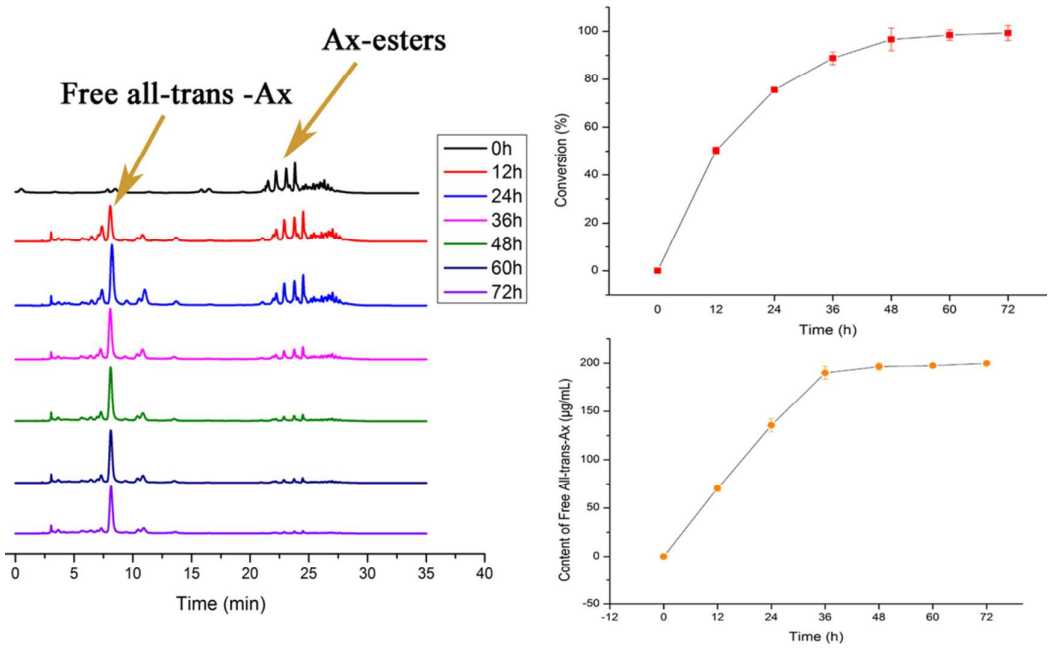


Figure 6 The result of influence of reaction times on hydrolysis of Ax esters.

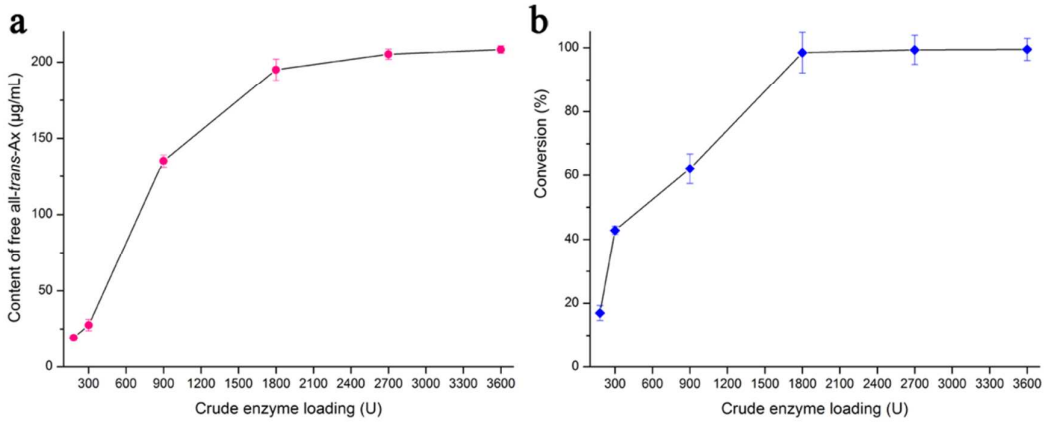
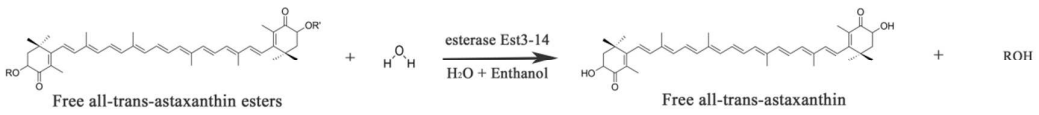
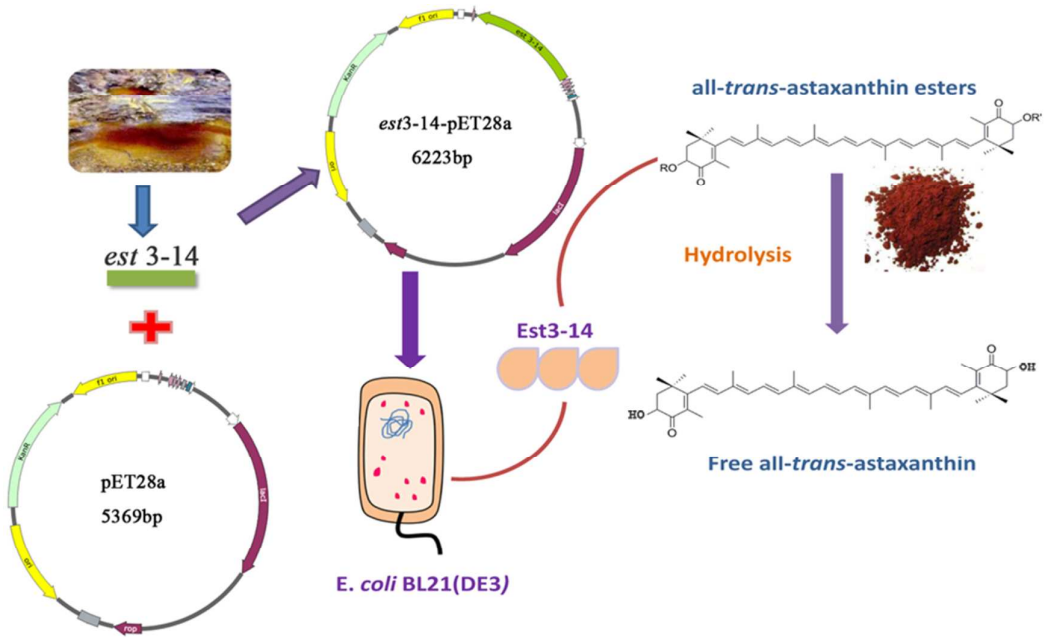


Figure 7 The result of the effect of enzyme loading on hydrolysis of Ax esters.



Scheme 1 Est3-14 catalyzed hydrolysis of Ax esters.


est 3-14
est3-14-pET28a
6223bp
pET28a
5369bp
E. coli BL21(DE3)
Est3-14
all-trans-astaxanthin esters
Hydrolysis
Free all-trans-astaxanthin

TOC Graphic

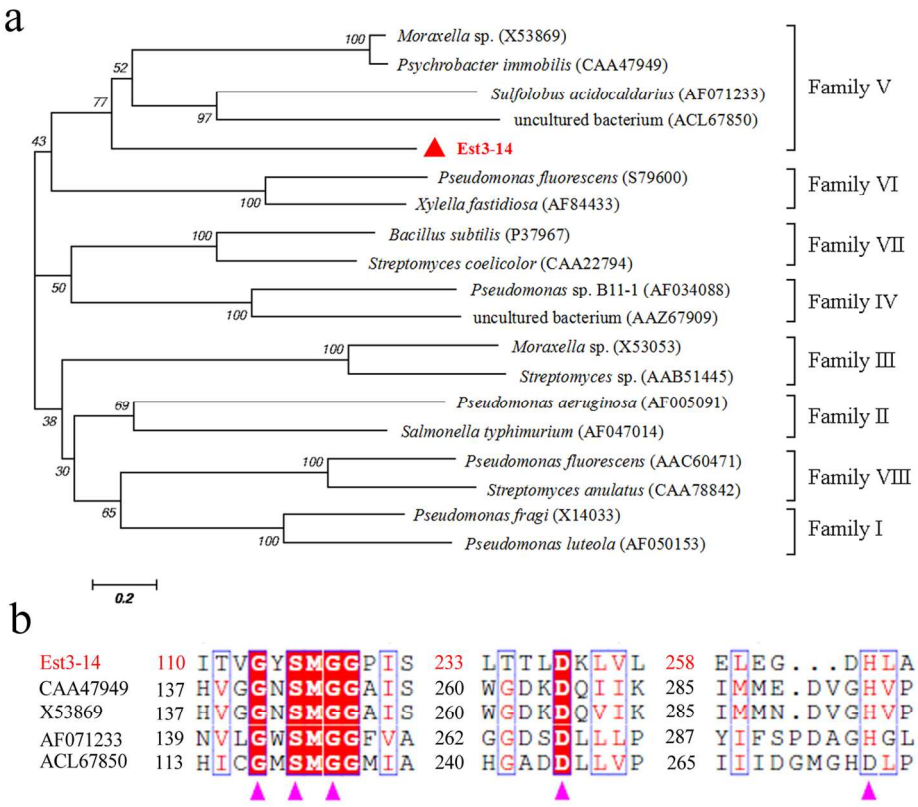


Figure 1 Bioinformatic analysis of Est3-14. (a) Neighbor-joining phylogenetic tree. Phylogenetic analysis was obtained by MEGA 6.0 software, and Est3-14 is shown as red triangles. (b) Multiple sequence alignments of Est3-14 and other lipolytic enzymes belonging to family V. The typical motif is indicated using blue circles, and the catalytic triad (Ser, Asp, His) is emphasized with pink triangles.

140x115mm (300 x 300 DPI)

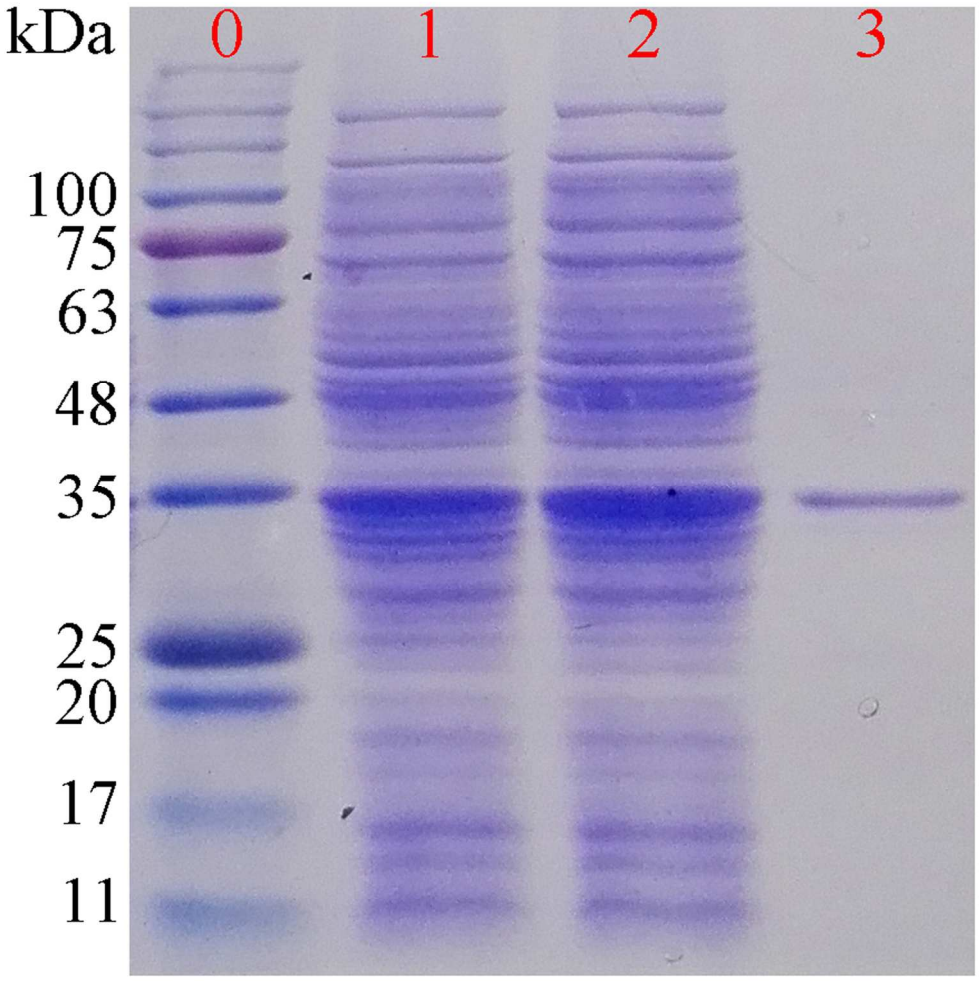


Figure 2 SDS-PAGE analysis of Est3-14. Lanes:0, protein marker;1, supernant of Est3-14 cell lysate; 2, precipitation of Est3-14 cell lysate; 3, purified Est3-14.

150x154mm (300 x 300 DPI)

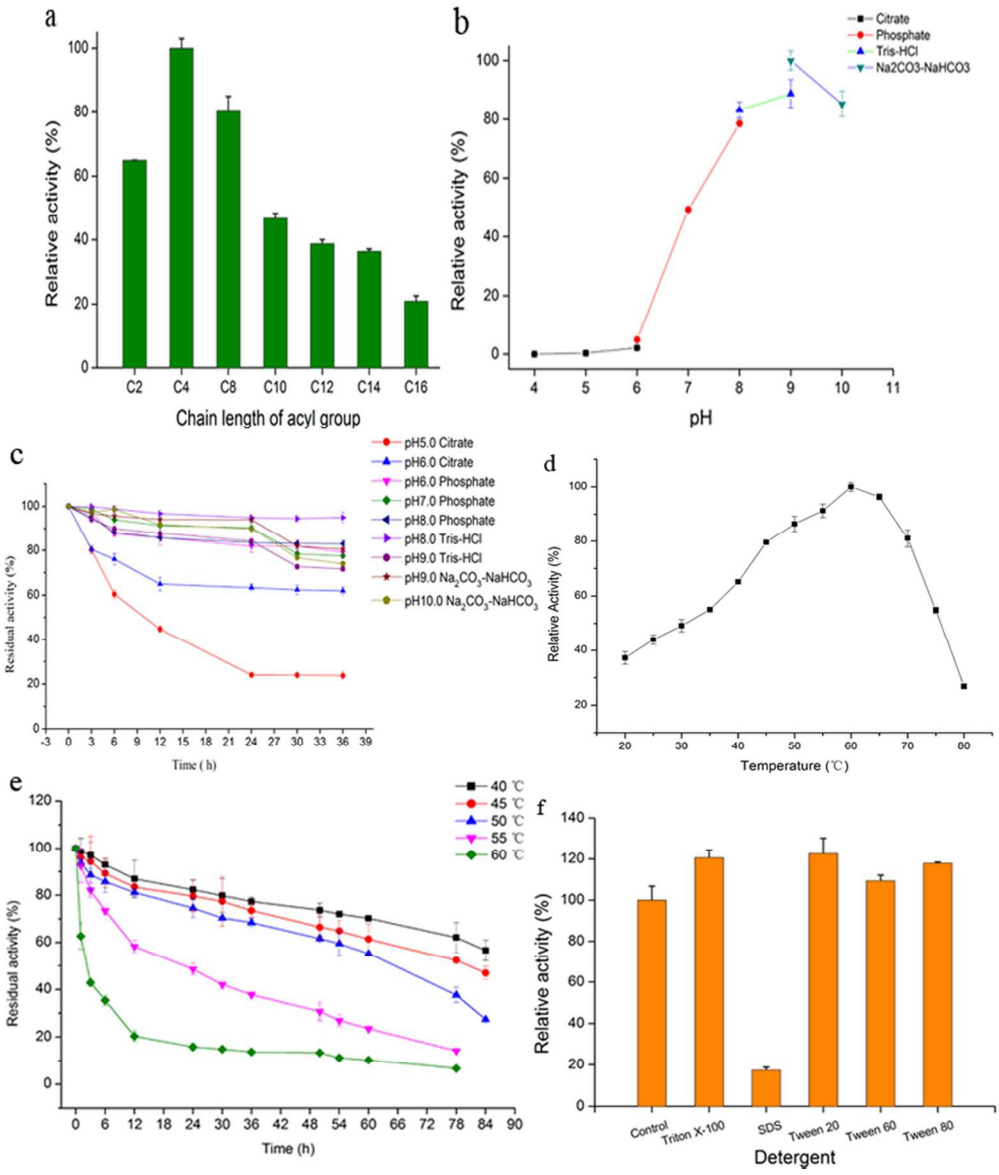


Figure 3 Characterization of Est3-14.

128x150mm (300 x 300 DPI)

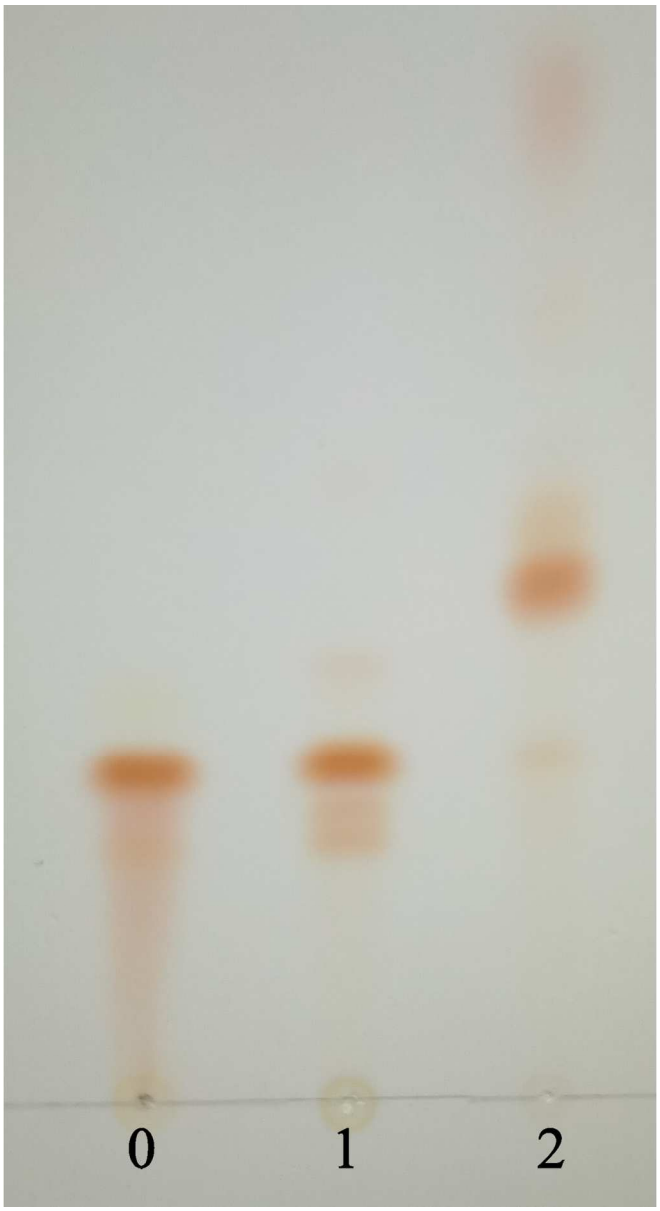


Figure 4 TLC result of the hydrolysis of Ax esters with esterases Est3-14 from metagenomic libraries. Lane 0, free all -trans-Ax; Lane 1, the hydrolysis results of Est3-14; Lane 2, the substrate of Ax-esters.

99x183mm (300 x 300 DPI)

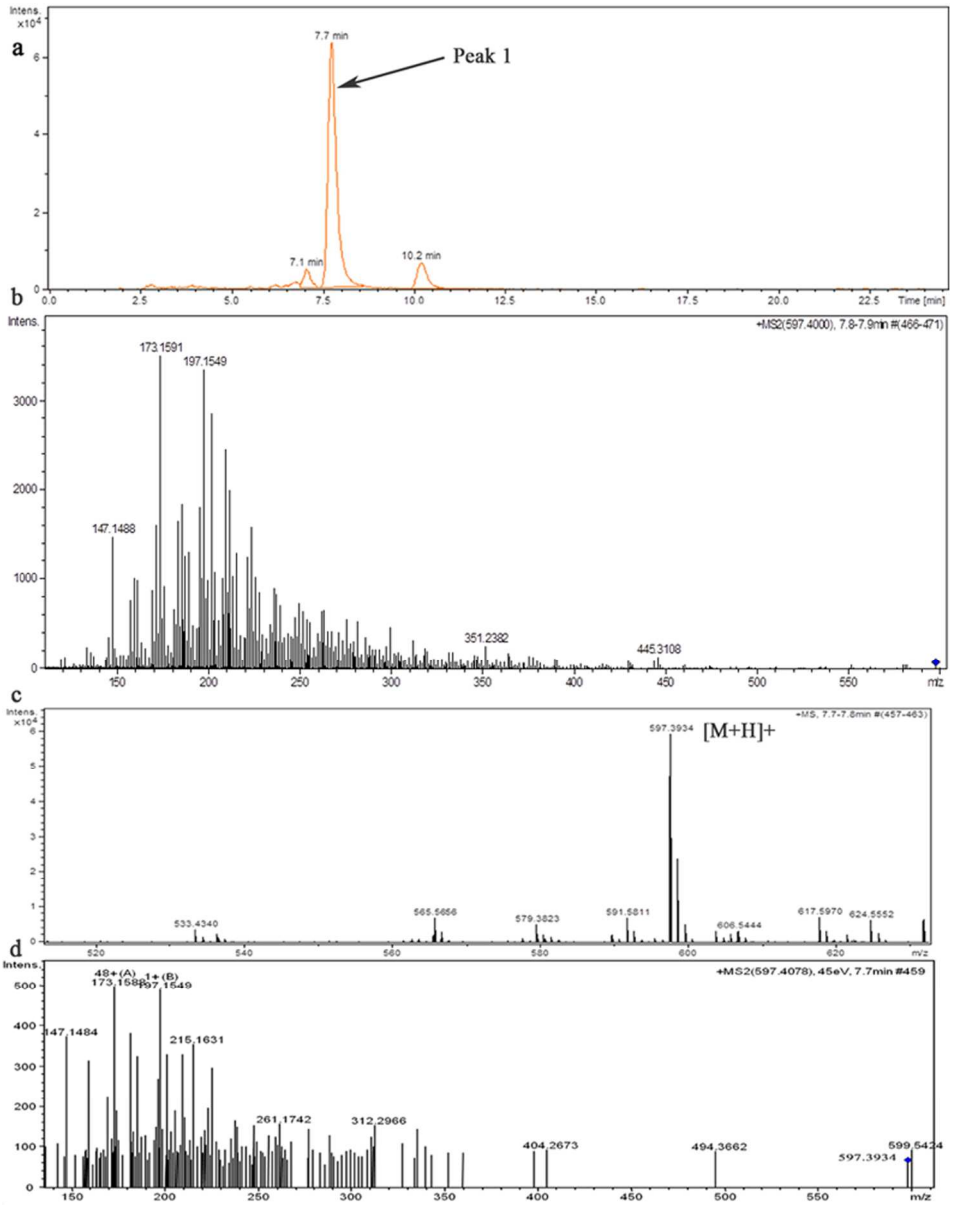


Figure 5 The result of identification of free all-trans-Ax from hydrolysis medium.

118x150mm (300 x 300 DPI)

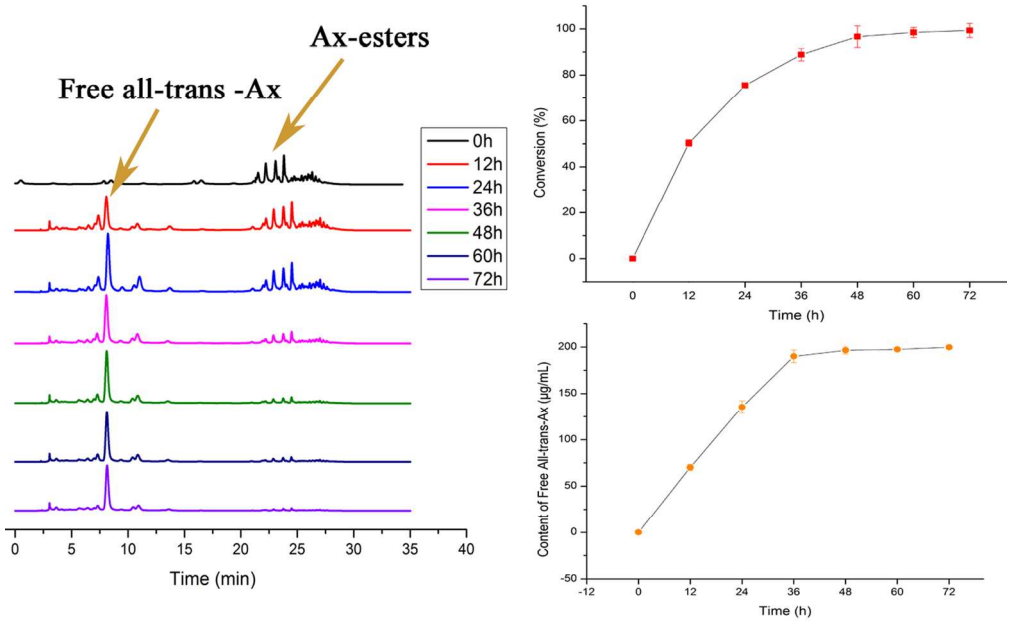


Figure 6 The result of effect of reaction time on hydrolysis of Ax esters.

150x94mm (300 x 300 DPI)

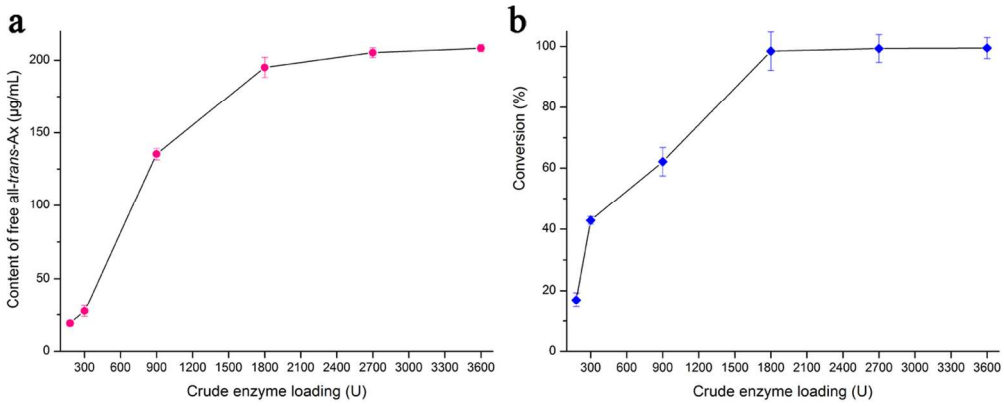
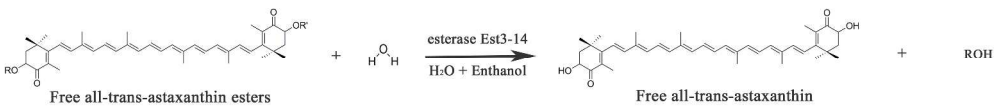


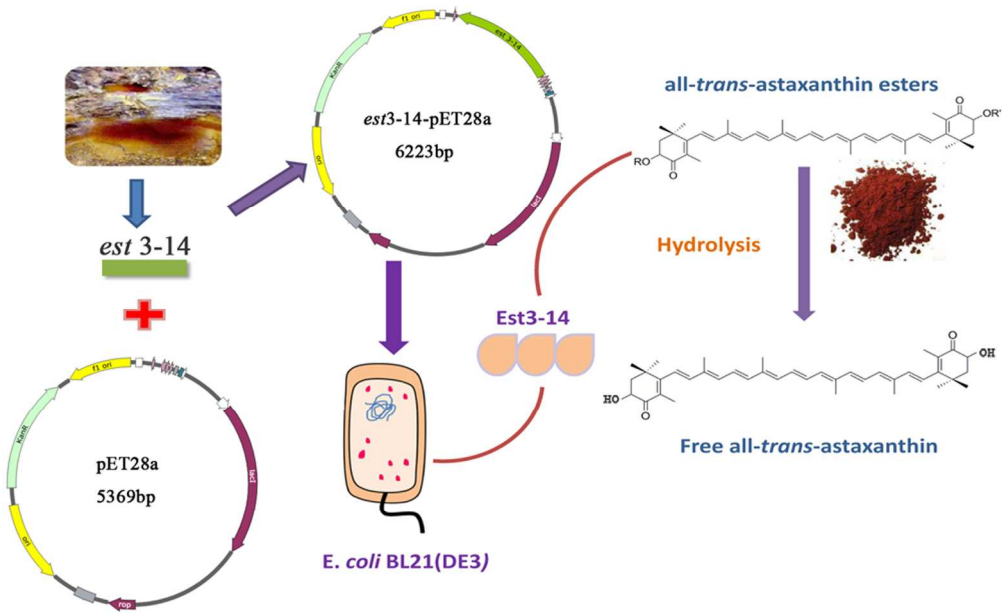
Figure 7 The result of effect of enzyme loading on hydrolysis of Ax esters. (a) Results of content of free all-trans-Ax at the different enzyme loading; (b) The conversion of Ax esters under different enzyme loading.

150x60mm (300 x 300 DPI)



Scheme 1 Est3-14 catalyzed hydrolysis of Ax ester.

391x43mm (300 x 300 DPI)



TOC Graphic

150x90mm (300 x 300 DPI)