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J. Agric. Food Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jafc.7b05341 • Publication Date (Web): 12 Jan 2018

One-step bioconversion of fatty acids into C8–C9 volatile aroma compounds by a multifunctional lipoxygenase cloned from *Pyropia haitanensis*

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ABSTRACT: The multifunctional lipoxygenase PhLOX cloned from *Pyropia haitanensis* was expressed in *E. coli* with 24.4 $mg \cdot L^{-1}$ yield. PhLOX could catalyze the one-step bioconversion of C18–C22 fatty acids into C8–C9 volatile organic compounds (VOCs), displaying higher catalytic efficiency for eicosenoic and docosenoic acids than for octadecenoic acids. C20:5 was the most suitable substrate among the tested fatty acids. The C8–C9 VOCs were generated in good yields from fatty acids, e.g., 2*E*-nonenal from C20:4, and 2*E*,6*Z*-nonadienal from C20:5. Hydrolyzed oils were also tested as substrates. The reactions mainly generated 2*E*,4*E*-pentadienal, 2*E*-octenal, and 2*E*,4*E*-octadienal from hydrolyzed sunflower seed oil, corn oil, and fish oil, respectively. PhLOX showed good stability after storage at 4 °C for two weeks and broad tolerance to pH and temperature. These desirable properties of PhLOX make it a promising novel biocatalyst for the industrial production of volatile aroma compounds.

KEYWORDS: *Pyropia haitanensis*; lipoxygenase; hydroperoxide lyase; volatile organic compounds; aroma

INTRODUCTION

Among the many flavor compounds commonly used in food industry, certain C6–C9 aldehydes and alcohols are particularly remarkable because they can reintroduce the "fresh green" flavor of fruits and vegetables that is lost during processing.[1,2] These compounds include 2*E*-/3*Z*-hexenal and hexenol, 1-/3-octanol, 2*Z*-octen-1-ol, 1-octen-3-ol, 1-octen-3-one, 2*E*-nonenal, as well as 2*E*,6*Z*-nonadienal. For example, 1-octen-3-ol can give a unique and subtle flavor of fresh mushrooms,[3] and 2*E*-nonenal and 2*E*,6*Z*-nonadienal can provide a fresh flavor that resembles cucumber.[4]

Flavor compounds used to be extracted directly from biomaterials,[5] but the increasing market demand required the industry to exploit biocatalysts to synthesize flavor compounds.[6] Studies have shown that in enzymatic catalysis, volatile organic compounds (VOCs) can be produced from polyunsaturated fatty acids (PUFAs) in two steps. In the first step, the PUFAs are peroxided by lipoxygenase (LOX). Secondly, the hydroperoxides are cleaved by hydroperoxide lyase (HPL) to produce short chain aldehydes. The aldehydes may be additionally reduced to the corresponding alcohols by alcohol dehydrogenase (ADH).[7]

In industry, the biocatalyst for the synthesis of VOCs can be obtained in two ways. Traditionally, a homogenate from plant materials that contains the enzymatic mixture can be used.[8-10] For example, 1-octen-3-ol could be produced from linoleic acid by using the homogenate from the mushroom *Agaricus bisporus* as a biocatalyst, which contained the LOX and the associated HPL.[11] It was also reported that the LOX in the homogenate of the mushroom *Psalliota bispora* could catalytically convert linoleic acid into its 10-hydroperoxide, which was then cleaved at the ω-8 position to produce 1-octen-3-ol by the HPL in the homogenate.[12] Nevertheless, the activities of

enzymes in plant homogenates are affected by many factors, e.g., the varieties and sources of the plant materials, the quality of homogenate, *etc.* Moreover, coexisting enzymes in the homogenates may result in undesirable byproducts or impede the conversion of substrates.

Alternatively, the biosynthesis of VOCs may make use of recombinant LOX and HPL enzymes cloned from different plants.[13-15] For example, the soybean LOX and watermelon HPL genes were cloned and co-overexpressed in the yeast *Saccharomyces cerevisiae*, and it was found that the recombinant cells could convert linolenic acid to 2*E*-hexenal and 3*Z*-hexenal.[16] Although pure recombinant LOX-HPL can increase the catalytic efficiency, the two enzymes need to finish disparate reaction steps at the same time, which can make it difficult to manage the optimal reaction condition, especially since HPL can be easily inactivated *in vitro*.[17] As a result, researchers have been actively exploring novel and efficient biocatalysts to prepare aroma compounds.

We have previously cloned a lipoxygenase gene from the red alga *Pyropia haitanensis*.[18] The obtained enzyme, denoted as PhLOX, was found to be a unique multifunctional lipoxygenase that possessed exceptionally high HPL, LOX, and allene oxide synthase (AOS) activities within only one catalytic domain of the protein. Hence, PhLOX may be a promising enzyme for the one-step biosynthesis of aroma compounds. In this work, we determined the enzymatic activities of PhLOX for different PUFAs and hydrolyzed oils. We also measured the kinetic parameters, the optimal enzymatic conditions, and the enzymatic stability of PhLOX.

MATERIALS AND METHODS

Expression and Purification of PhLOX. The recombinant strain, *E. coli* PhLOX-pET28a-BL21 (DE3), was provided by the Key Laboratory of Applied

Marine Biotechnology of Zhejiang Province. The PhLOX gene was cloned from *P. haitanensis* HML thallus (GenBank Accession No. AFQ59981). Its cloning and recombination has been described in our previous work.[18] Five hundred milliliters of Luria-Bertani medium was incubated with 5 mL of the overnight culture of the recombinant strain and incubated at 37 °C until the cell density had increased to A_{600} 0.6 to 0.8. The expression of PhLOX was then induced by 0.1 mM IPTG at 16 °C for 20 h. The cells were harvested by centrifuging for 10 min at 8,000 rpm (10,800 g) and 4 °C, re-suspended by adding 20 mM Tris-HCl buffer A (200 mL, pH = 8.0, 200 mM NaCl, 0.1% Tween 20, 5% glycerin), lysed by ultrasonication, and centrifuged for 10 min at 12,000 rpm (17,226 g) and 4 °C. The supernatant of cell lysis was purified by affinity chromatography using Ni-Agarose 6× His-Tagged Protein Purification Kit (CWBIO, China). Protein concentration was measured with the DC™ Protein Assay Kit (BioRad, USA). Protein purity was evaluated by 12% SDS-PAGE analysis.

Measurements of PhLOX Activity and Kinetic Parameters. The fatty acid substrates, which included linoleic acid (LA), α-linolenic acid (α-ALA), arachidonic acid (ARA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), were purchased from the Cayman Chemical, USA. In a typical test, 1 μM PhLOX (about 0.1 $mg \cdot mL^{-1}$) was incubated with 100 μM substrate in 20 mM Tris-HCl buffer B (1 mL, pH = 8.0) containing 200 mM NaCl at 20 °C for different times (6 s, 12 s, 0.5, 1, 5, 10, 15, 30, 60 min). In the control group, PhLOX was inactivated by boiling for 5 min. The reaction was quenched by acidification (pH < 2) with 4 M HCl, added with 1 mL ethyl acetate and shaken at 4 °C for 20 min. The mixture was then centrifuged for 10 min at 12,000 rpm (13,400 g) and 4 °C. The organic phase was washed with 1 mL water, dried with a stream of nitrogen, metered volume in 0.5 mL methanol, and then analyzed by HPLC-MS to determine the composition of fatty acids. All assays

were run in triplicate.

The activity of PhLOX was determined by monitoring the consumption of fatty acid substrates, and one unit of PhLOX activity corresponded to 1 μM fatty acid consumed in one minute by 1 mg PhLOX. External standards were used to quantify the substrates consumed by PhLOX, and the calibration curves were built with the five fatty acids mentioned above.

Kinetic parameters were determined at varying substrate concentrations (50, 100, 150, 200, 300, 400, 500, 650 and 800 μM). According to the reaction curve of each fatty acid, the reaction period for LA, α-ALA, ARA, EPA, and DHA was 3 min, 3 min, 6 s, 6 s, and 12 s, respectively. Data were fitted to the Michaelis–Menten model to determine the values of the Michaelis constant K_M and the maximum rate V_m, and k_{cat} was calculated from Equation (1):

$$k_{cat} = V_m/[E] \qquad (1)$$

where [E] is the concentration of the enzyme (in micromolar).

Citric acid/sodium citrate buffer (20 mM, pH = 2.5–5.0), sodium phosphate buffer (20 mM, pH = 6.0–7.0), Tris-HCl buffer (20 mM, pH = 8.0–10.0) and sodium bicarbonate/sodium hydroxide buffer (20 mM, pH = 11.0) were tested as the buffer medium to determine the optimal pH for the reaction. The enzymatic activity was also measured at 0, 4, 10, 20, 30, 40, 50, 55 and 60 °C to determine the optimal temperature. The reaction period for LA, α-ALA, ARA, EPA, and DHA in incubation with PhLOX or inactive PhLOX (as the control) were 3 min, 3 min, 6 s, 6 s, and 12 s, respectively, too. Additionally, a mixture of five PUFAs in 1 mL inactive PhLOX was made and extracted immediately to detect any non-enzyme related substrate depletion by comparing to the fatty acid contents after incubation in the control.

HPLC-MS analysis. The HPLC-MS assay was performed on a Finnigan

Surveyor with a TSQ Quantum Access system (Thermo Fisher Scientific Inc., Pittsburgh, PA, USA) using a Hypersil GOLD C18 column (2.1 mm × 100 mm, 3 μm, Thermo Fisher Scientific Inc.) under the following conditions: injection volume, 10 μL; column temperature, 30 °C; flow rate, 0.2 $mL \cdot min^{-1}$. The binary eluent consisted of (A) acetonitrile and (B) 0.2% acetic acid. The gradient elution was programmed to increase from 30% A to 100% A over 40 min.

Mass spectrometry was performed on a triple quadrupole mass spectrometer equipped with electrospray ionization (ESI) and operated in negative ionization mode. Samples were scanned over m/z = 100–600. The ionization conditions were: sheath gas pressure (N_2) at a flow rate of 25 $L \cdot min^{-1}$, auxiliary gas pressure (N_2) at a flow rate of 5 $L \cdot min^{-1}$, spray voltage at 2.5 kV, vaporizer temperature at 300 °C, and capillary temperature at 350 °C. Argon was introduced into the trap at an estimated pressure of 6×10^{-6} mbar to improve trapping efficiency and to act as the collision gas. The collision gas pressure was 1.5 mTorr.

Preparation and Component Analysis of Hydrolyzed Oils. Sunflower seed oil, corn oil, and fish oil were purchased from a local supermarket. These oils (0.5 g) were hydrolyzed in MeOH/H_2O (4:1 v/v, 2 mL) containing 6% KOH under N_2 atmosphere at 60 °C for 2 h. The fatty acid products were collected by firstly cooling the reaction mixture to room temperature, then acidification (pH < 2) with 4 M HCl, and finally extraction with hexane/chloroform (4:1 v/v, 1 mL) for three times. The collected hydrolyzed oils were dried with a stream of nitrogen, dissolved in 1 mL methanol and stored at −20 °C.

To analyze the components of hydrolyzed oils after incubation with PhLOX, a mixture containing NaCl (200 mM), PhLOX (1 μM), and hydrolyzed oil (2 μL) in 20 mM Tris-HCl buffer B (2 mL, pH = 8.0) was maintained at 20 °C for 1 h. A boiled

PhLOX and otherwise identical mixture was set up as the control group, and in both cases C19:0 (15 μg) was added as an internal standard (IS). After incubation, the reaction mixture was quenched by acidification (pH < 2) with 4 M HCl, added with 2×1 mL ethyl acetate and shaken at 4 °C for 20 min. The extracts were collected and dried with a stream of N_2, then heated in 14% $BF_3 \cdot CH_3OH$ (500 μL) at 60 °C for 1 h, and finally extracted successively with hexane/chloroform (4:1 v/v, 0.2 mL, twice) and hexane (0.2 mL, once). The combined extracts were mixed with Milli-Q water (1 mL) and centrifuged for 15 min at 12,000 rpm (13,400 g) and 4 °C. The upper layer was separated and dehydrated to give the incubated sample. The sample was then dried with a stream of N_2, redissolved in hexane (0.2 mL), and analyzed on a QP2010 GC-MS (Shimadzu, Kyoto, Japan). Additionally, 2 μL hydrolyzed oil was added into 2 mL boiled PhLOX, and then extracted and derived immediately by following the process above to analyze the fatty acid composition of hydrolyzed oil before incubation.

The GC-MS assay was carried out using a SPB-50 fused silica capillary column (30 m × 0.25 mm × 0.25 μm; Supelco Inc., Bellefonte, PA, USA) under the following conditions: injector temperature, 250 °C; carrier gas, He; constant flow, flow rate, 0.81 $mL \cdot min^{-1}$; pre-column pressure, 73.0 kPa; injection volume, 1 μL; split ratio, 50:1. The oven temperature was programmed as follows: hold at 150 °C for 3.5 min after injection, raise to 200 °C at 20 $°C \cdot min^{-1}$ and hold for 5 min, and raise to 280 °C at 5 $°C \cdot min^{-1}$ and hold for 30 min. The mass spectrometer was operated in the electron impact mode under the following conditions: electron energy, 70 eV; ion source temperature, 200 °C; interface temperature, 250 °C. The mass spectrometer scanned over m/z = 50–600. The polyunsaturated fatty acid components were expressed in micromolar, and their consumption rate was calculated from Equation

(2):

$$R_{FA}(\%) = ([FA]_0 - [FA]_t)/[FA]_0 \times 100\% \qquad (2)$$

where R_{FA} denotes the consumption rate of different fatty acids in the hydrolyzed oils, $[FA]_0$ is the concentration of the fatty acid after incubation with inactive PhLOX, and $[FA]_t$ is the concentration of the fatty acid after incubation with PhLOX.

Production of Volatile Compounds. The substrates for the production of aroma compounds with PhLOX included the five pure fatty acids (LA, α-ALA, ARA, EPA, and DHA) and the three hydrolyzed oil samples. In a typical reaction, a mixture of NaCl (200 mM), PhLOX (1 μM), and the pure fatty acid (100 μM) or hydrolyzed oil (2 μL) was maintained in 20 mM Tris-HCl buffer B (2 mL final volume, pH = 8.0) at 20 °C for 1 h in a closed tube. The boiled PhLOX was used in the control group. The reaction was stopped by acidification (pH < 2) with 4 M HCl, and vanillin (200 μg) was added as an internal standard (IS).

The products were concentrated on a solid phase micro extraction (SPME) fiber assembly polydimethylsiloxane/carboxen/divinylbenzene (Supelco Inc., Bellefonte, PA, USA). [19, 20] The extraction was performed in headspace mode at 40 °C for 50 min with magnetic stirring. After extraction, the SPME device was introduced into the splitless injector of GC-MS and maintained at 210 °C for 5 min, and the products were analyzed on the QP2010 GC-MS fitted with a VOCOL column (60 m × 0.32 mm, 1.8 μm film thickness) (Supelco Inc., Bellefonte, PA, USA) under the following conditions: carrier gas, He; constant flow, flow rate, 1.99 $mL \cdot min^{-1}$; pre-column pressure, 83.5 kPa. The oven temperature was programmed as follows: hold at 35 °C for 3 min, raise to 40 °C at 3 $°C \cdot min^{-1}$ and hold for 1 min, raise to 100 °C at 5 $°C \cdot min^{-1}$, and finally raise to 210 °C at 10 $°C \cdot min^{-1}$ and hold for 30 min. Another column (Agilent HP-5MS, 30 m × 0.25 mm × 0.25 μm) was also used. The oven

temperature was programmed as follows: hold at 35 °C for 3 min, raise to 50 °C at 3 °C·min^{-1} and hold for 1 min, raise to 180 °C at 6 °C·min^{-1} and hold for 2 min, and finally raise to 270 °C at 15 °C·min^{-1} and hold for 7 min. The mass spectrometer was operated in the electron impact mode under the following conditions: electron energy, 70 eV; ion source temperature, 200 °C; interface temperature, 210 °C. The mass spectrometer scanned over *m/z* = 45–1000.

Commercial volatile aroma compounds were used as standard compounds. They were mixed in 2 mL buffer B and then handled by the same procedure of SPME and GC-MS above. Both retention time and mass spectra of analytes were compared with those of the standard compounds. The mass spectra of analytes were also compared with the standard spectra recorded in the NIST 147, the NIST 27 and the WILEY 7 Spectrometry Libraries. The quantification of analytes was calculated from Equation (3) and expressed in micromolar.

$$C_p = [(m_{IS} \times A_p)/(f \times A_{IS})]/(M_p \times V_r) \qquad (3)$$

where C_p denotes the concentration of the volatile product, m_{IS} is the mass of the internal standard (200 μg), A_p is the peak area of the volatile product, f is the relative weight correction factor calculated by the peak area ratio of the standard compound (200 μg) to the internal standard (200 μg), A_{IS} is the peak area of the internal standard (200 μg), M_p is the molecular weight of the volatile product and V_r is the final volume of the enzymatic reaction (2 mL).

The yield of the volatile products was calculated from Equation (4):

$$Y_p\ (\%) = (C_p/[S]) \times 100\% \qquad (4)$$

where Y_p denotes the yield, C_p is the concentration of the volatile product, and [S] is the initial substrate concentration (in micromolar).

The substrate conversion ratio (R_S) was expressed in the percentage (%) of the

227 consumed substrate in the total substrate.

228 Volatile compounds in three hydrolyzed oils were semi-quantitatively determined
229 by using vanillin as an internal standard and the contents of the volatiles were
230 calculated from the GC peak areas relating to the GC peak area of the internal
231 standard.

232 In addition, the enzymatic stability of PhLOX was evaluated by using stored
233 PhLOX (at 4 °C for two weeks) in the incubation of the five pure fatty acids under
234 standard reaction conditions and observing the declined yield of the volatile products.

235 **RESULTS**

236 **Expression, Purification, and Enzymatic Characterization of PhLOX.** After
237 SDS-PAGE, the purified PhLOX appeared on the gel as a single band near 100 kDa
238 (Figure 1), which was consistent with the predicted molecular mass of the deduced
239 amino acid sequence of PhLOX with an N-terminal His tag (about 98 KDa). The
240 PhLOX harvested from the total 5 L culture medium amounted to 121.8 mg (*i.e.*, 24.4
241 $mg \cdot L^{-1}$), and the final purity reached about 92% as was calculated from the optical
242 density ratio of the gel.

243 The enzymatic activity of PhLOX was surveyed using the five PUFAs as
244 substrates (*i.e.*, LA, α-ALA, ARA, EPA, and DHA). It was the highest (955.5 U) in
245 the reaction of EPA (Figure 2A). The EPA substrate was consumed by about 24.7 μM
246 in mere 6 s and was exhausted in 1 min (Figure 2B). In the case of DHA, the
247 enzymatic activity of PhLOX reached 807.4 U, and within 5 min 91% of the DHA
248 substrate had been consumed. In the reaction of ARA, the enzymatic activity of
249 PhLOX reached 674.4 U and it took 10–15 min to consume 90% of the ARA
250 substrate. However, the activity of PhLOX was much lower in the reaction of the two
251 C18 PUFAs (*i.e.*, LA and α-ALA), and it took as long as 30 min to consume ≥80% of

the substrate. The results showed that the activity of PhLOX was dramatically higher in the reaction of the C20 and C22 PUFAs than in the reaction of C18 PUFAs (Figure 2B).

Data were fitted to the Michaelis–Menten model to calculate the kinetic parameters of the enzymatic reaction of different substrates. It was found that the V_m of C20 PUFAs was at least 24 times higher than that of C18 PUFAs, and the C22 PUFA (*i.e.*, DHA) also gave a high V_m of 1962.1 μM·min^{-1} (Table 1). In addition, EPA was found to be the most suitable substrate among the five tested PUFAs since it gave a lowest K_M of 286.4 μM. The catalytic efficiency (k_{cat}/K_M) of PhLOX was also significantly higher for C20 and C22 PUFAs than for C18 PUFAs. Again, the catalytic efficiency was the highest when EPA was used as the substrate (k_{cat}/K_M = 14.6 min^{-1}·μM^{-1}) (Table 1).

For all substrates, the optimal condition for the enzymatic reaction with PhLOX was at pH = 8.0 and 20 °C (Figure 3). For the C20 and C22 PUFAs, the activity of PhLOX remained relatively stable over pH = 6.0–10.0 but decreased sharply when the pH value was increased to 11.0 or reduced to 2.5. In contrast, for the C18 PUFAs, the activity of PhLOX was notably affected by the pH value, since only about 20% (for LA) and 9% (for α-ALA) enzyme activity was preserved when the pH value increased from 8.0 to 10.0 (Figure 3B). Similarly, for the C20 and C22 PUFAs, the activity of PhLOX changed relatively little over the temperature range of 10–40 °C. However, for the C18 PUFAs, the activity of PhLOX declined sharply when the temperature deviated from 20 °C (Figure 3A).

Composition of Volatile Products from PUFAs. The volatile products obtained from the five PUFAs by incubation with PhLOX were analyzed. Data showed that after incubation with PhLOX, the conversion rate (R_S) of five PUFAs were all ≥93%

277 and specific volatile organic compounds (VOCs) could be produced from α-ALA,
278 ARA, EPA, and DHA, but not from LA (Figures 4 and S4). Note that the products
279 from ARA included both C8 (1-octen-3-ol, 1-octen-3-one, 2*Z*-octen-1-ol) and C9
280 (2*E*-nonenal) VOCs, whereas EPA and DHA only gave a C9 VOC (2*E*,6*Z*-nonadienal)
281 and a C8 VOC (3*E*,5*Z*-octadien-2-one), respectively. Among the three C20/C22
282 PUFAs, ARA gave the highest total VOC yield (74.7 μM, about 75% yield), which
283 was about 2.5 times and 6.3 times that of EPA and DHA, respectively (Table 2).
284 Among the C8/C9 VOCs generated from ARA, 2*E*-nonenal was the primary product,
285 which amounted to 54.2 μM (about 54% yield). Meanwhile, 2*E*,6*Z*-nonadienal, also a
286 C9 VOC, was generated from EPA and amounted to 30.3 μM (about 30% yield). In
287 addition, various C8 VOCs were generated from ARA and DHA by incubation with
288 PhLOX. For example, 1-octen-3-one (9.7 μM) was produced from ARA and
289 3*E*,5*Z*-octadien-2-one (11.8 μM) was produced from DHA. These C8 VOCs were all
290 obtained in approximately 12% yield. Incubation of α-ALA with PhLOX generated
291 minor amounts of C5/C6 VOCs in <5% yield.

292 Storage of the PhLOX solution at 4 °C for two weeks did not seriously damage
293 the enzymatic activity. Although 2*Z*-pentenal and 2*Z*-octen-1-ol were detected in trace
294 amounts in the reactions with stored PhLOX and showed 0 μM after data conversion,
295 the yield of total VOCs did not decline excessively (Table 2).

296 **Production of VOCs from Hydrolyzed Oils by Incubation with PhLOX.**
297 Three hydrolyzed oils were surveyed as the substrate for the production of VOCs.
298 Data in the column of 'before incubation' showed that hydrolyzed sunflower seed oil
299 and hydrolyzed corn oil were similar in fatty acid composition, *i.e.*, mainly containing
300 C18:2 (902.3 μM and 769.1 μM respectively) followed by C18:1 (>24%), together
301 with minor amount of C18:3 (Table 3). In contrast, hydrolyzed fish oil contained

many more fatty acids, including C16–C22 PUFAs whose degree of unsaturation varied from 2 to 6 (Table 4). The most abundant PUFA was C22:6 (471.9 μM), followed by C20:5 (423.7 μM). There were also minor amounts of C20:4 and C22:5. The contents of other C16–C18 fatty acids were all less than 10 μM. Although it seemed that there was a small decline of PUFAs in these oil samples after incubation with inactive PhLOX as the control, the main fatty acid component was still C18:2 in two hydrolyzed plant oils (>68%), and the main fatty acids were still C22:6 and C20:5 (45.6% and 41.3%, respectively) in hydrolyzed fish oil (Tables 3 and 4). However, when the three hydrolyzed oils were used as substrates for incubation with PhLOX, it was found that only PUFAs were consumed while saturated and monounsaturated fatty acids remained basically unchanged. This was consistent with the predicted enzymatic selection of PhLOX. When hydrolyzed sunflower seed oil was used as the substrate, the minor component C18:3 were completely consumed but the consumption rate of the major component C18:2 only reached about 66% (586.1 μM). Similarly, when hydrolyzed corn oil was used as the substrate, only 49.5% C18:2 were consumed, and a minor amount of C18:3 remained. Among the seven PUFAs in hydrolyzed fish oil, the consumption rate of the major components C20:5 and C22:6 reached 78.8% (322.8 μM) and 87.7% (397.1 μM), respectively, after incubation with PhLOX. The consumption rate of C18:4, C20:4, and C22:5 was all greater than 75%, but the absolute amount of these consumed substrates was not high since they were minor components in the original PUFA profile of hydrolyzed fish oil. It could be seen that among the PUFAs of different degree of unsaturation that coexisted in hydrolyzed fish oil, PhLOX preferentially catalyzed PUFAs of high unsaturation (≥4) and barely targeted the di-unsaturated (C18:2 and C16:2) fatty acids. The consumption rate of C18:2 and C16:2 were very low even though their content in the original PUFA

mixture was already tiny.

GC-MS was used to analyze the new VOCs produced from the three hydrolyzed oils after incubation with PhLOX. For hydrolyzed sunflower seed oil, six new VOCs were tentatively identified after incubation with PhLOX, namely 2*E*,4*E*-pentadienal, *n*-heptanal, 2*E*-heptenal, *n*-octanal, 2*E*-octenal, and 2*E*,4*E*-nonadienal (Figures S1 and S5). The newly generated VOCs were semi-quantified to 517.0 μM. Among the new VOCs, 2*E*,4*E*-pentadienal accounted for 70.5% of the total (364.5 μM), followed by 2*E*-octenal (102.2 μM) (Table 5). Besides, compared with the control group, the content of two existing VOCs increased, most notably *n*-nonanal (by 32.1 μM). In the case of hydrolyzed corn oil, the newly generated VOCs included 2*E*,4*E*-pentadienal, *n*-heptanal, 2*E*-heptenal, *n*-octanal, 2*E*-octenal, and 2*E*,4*E*-nonadienal (Figures S2 and S5). The major new VOCs were 2*E*,4*E*-pentadienal and 2*E*-octenal, which amounted to 69.0% and 23.7% of the total new VOCs, respectively (Table 5). In comparison with the control group, the amount of *n*-nonanal in the products increased most notably (from 16.3 μM to 49.0 μM). In addition to the new and increased production of VOCs above in the two hydrolyzed plant oils, there were still some other VOC compounds tentatively identified such as 3-pentanone, 2-hexanone, hexanoic acid methyl ester, glycerin, undecane, octanoic acid methyl ester, dodecane, decanoic acid methyl ester and dodecanoic acid methyl ester. When hydrolyzed fish oil was used as the substrate, many more volatile products were tentatively identified, including 13 kinds of C5–C10 VOCs (Figures S3 and S5). Among the newly generated VOCs, 2*E*,4*E*-octadienal had a highest yield of 143.6 μM, which accounted for about 48.5% of the total new VOCs (Table 5). Meanwhile, 2*E*,4*Z*-decadienal and 2*E*,6*Z*-nonadienal each also had a high yield of 41.0 μM and 29.3 μM, respectively. In comparison with the control group, the amount of *n*-nonanal in the products increased most notably

(*i.e.*, by 48.6 μM, a 4.5-fold increase). Additionally, some other VOC compounds were tentatively detected, including 3-pentanone, 3-hexanone, 2-hexanone, hexanoic acid methyl ester, glycerin, 1H-imidazole, octanoic acid methyl ester, 4,6-dimethyl-dodecane, 2,4-dimethyl-benzaldehyde, decanoic acid methyl ester and dodecanoic acid methyl ester.

DISCUSSION

Since the 1980s, scientists have made considerable efforts to produce volatile aroma compounds by enzymatic catalysis. Previous studies mainly exploited the biosynthesis of green leaf volatiles (GLVs) and focused on the associated process optimization.[21,22] Usually, soybean flour is used as the LOX source, and various plant extracts (e.g., green bell pepper, mint leaves, green leaves from sugar beet) are used as the HPL source.[22] However, the HPL activity of plant extracts appears to be unstable and tends to be restricted by the catalytic environment. For example, the HPL activity of plant extracts can be easily inhibited when the concentration of hydroperoxides becomes unfavorable. In addition, the HPL activity is highly dependent on the source plant, and the crude extracts may contain other enzymes that can generate unwanted byproducts.[17] Therefore, the development of effective biocatalysts remains a key task in the biosynthesis of volatile aroma compounds for the industry, e.g., a protein engineering strategy based on directed evolution to enhance the HPL activity.[23] Here, a new and natural biocatalyst, PhLOX, was found to be suitable for the production of some VOCs without harsh catalytic conditions because of the inhibition of HPL activity.

We previously harvested PhLOX from the red alga *P. haitanensis* and reported on its multiple functions on fatty acids, *i.e.*, lipoxygenase (LOX), hydroperoxide lyase (HPL), and allene oxide synthase (AOS).[18] This property of PhLOX is not unique;

some enzymes involved in oxylipin formation have been reported to possess similar, if not overlapping functions. For example, a catalase-like-AOS–LOX fusion protein from coral *Plexaura homomalla* converts arachidonic acid completely to an allene oxide.[24] PpLOX1 from moss *Physcomitrella patens* shows additional HPL activity to produce 2*Z*-octen-1-ol and 1-octen-3-ol from arachidonic acid.[25] However, compared with these fused lipoxygenases, the quite exceptional trait of PhLOX was its LOX-AOS-HPL activities in one catalytic domain which belongs to a conserved non-heme LOX family. And unlike some HPL enzymes harvested from some plants,[26,27] PhLOX does not contain a N-terminal transit peptide. Hence, there was no insertion of the recombinant enzyme in the membrane during expression in *E. coli*, and a larger amount of the enzyme could thus be recovered in the soluble protein fraction. Under the current conditions, the yield of PhLOX from cell lysis reached 24.4 mg protein per 1 L culture, which is much higher than the yield of some recombinant plant HPL, such as recombinant olive HPL*wt* (about 1.3 $mg \cdot L^{-1}$).[15]

Because of its HPL activity, the PhLOX was used as a novel biocatalyst in this study for the one-step conversion of different PUFAs or hydrolyzed oils into VOCs. Among the five tested PUFAs (*i.e.*, LA, α-ALA, ARA, EPA, and DHA), it was found that under identical reaction conditions, the activity of PhLOX was higher when C20 and C22 PUFAs, rather than C18 PUFAs, were used as the substrate. The consumption rate of C20 and C22 PUFAs was also higher than that of C18 PUFAs. According to the Michaelis constant (K_M), the most suitable substrate for PhLOX was EPA. Besides, PhLOX clearly preferred fatty acids with high degree of unsaturation (≥3) as the substrate, since the K_M of LA (459.1 μM) was much higher than that of EPA and DHA (286.4 and 316.3 μM, respectively). The catalytic efficiency of PhLOX was also higher when C20 and C22 PUFAs were used as the substrate, as was

402 indicated by the higher k_{cat}/K_M value of ARA, EPA and DHA than that of LA and
403 α-ALA. These results are in agreement with previous findings. It was reported that the
404 LOXs of Rhodophyta used both octadecenoic acids and eicosenoic acids as
405 substrates.[28] Andreou *et al.* pointed out that the most prominent theme in red algal
406 oxylipin biosynthesis was the metabolism of eicosenoic acids.[29] In our case, we also
407 noted that when C20 and C22 PUFAs were used as substrates, the activity of PhLOX
408 remained stable over a broad range of pH (6.0–10.0) and temperature (10–40 °C).
409 Therefore, it would be desirable for industrial scale synthesis to use biological oils
410 that are rich in C20 and C22 PUFAs when PhLOX is used as the catalyst.

411 Analysis of the VOC products from the PUFAs also showed that PhLOX
412 preferably catalyzed the conversion of PUFAs with high degree of unsaturation (>3)
413 into C8 and C9 VOCs. In the case of C18 PUFA substrates, PhLOX did not convert
414 LA into VOCs and converted α-ALA only into a slight amount of C5 (2*Z*-pentenal)
415 and C6 (3*Z*-hexenal) VOCs. It can be deduced that 2*Z*-pentenal and 3*Z*-hexenal were
416 generated from the cleavage of 13-hydroperoxyoctadecatrienoic acid (13-HpOTE) at
417 ω-C5 and ω-C6, respectively. Although oxidation also happened at ω-C10 and
418 9-HpOTE was detected, PhLOX did not help to generate C9 aldehydes from α-ALA.[18]
419 This differed from some vegetable HPL that are known to convert 9-HpOTE or
420 9-hydroperoxyoctadecadienoic acid (9-HpODE) into C9 aldehydes (e.g.,
421 2*E*,6*Z*-nonadienal and 2*E*-nonenal).[30] Furthermore, C5/C6 VOCs were obtained from
422 α-ALA in a very poor yield of 4.5%. In contrast, the yield of C6/C9 VOCs was within
423 25%–86% when plant extracts were used as the HPL source,[22] or about 93% for
424 hexanal production and 73% for 3*Z*-hexenal production when olive recombinant HPL
425 was used as the catalyst.[15]

426 However, when C20 and C22 PUFAs were used as substrates, PhLOX evidently

427 catalyzed the formation of VOCs. Incubation of ARA with PhLOX produced both C8
428 and C9 VOCs, including 1-octen-3-ol, 1-octen-3-one, 2*Z*-octen-1-ol, and 2*E*-nonenal,
429 and the total yield reached as high as 74.7%. Among these compounds, the three C8
430 VOCs, which contributed significantly to the aroma of mushroom,[31] were formed by
431 the cleavage at ω-C8 of 12-hydroperoxyeicosatetraenoic acid (12-HpETE). Previous
432 reported biosynthesis of 1-octen-3-ol/one mainly proceeded via the aerobic oxidation
433 of linoleic acid using LOX and HPL.[11,32] Hence, the synthesis of 1-octen-3-ol/one
434 from ARA with PhLOX as the biocatalyst could serve as an alternative strategy. In
435 fact, in the PhLOX catalyzed reaction, the predominant volatile product from ARA
436 was the C9 aldehyde 2*E*-nonenal (in 54.2% yield). Forss *et al.* reported that
437 2*E*-nonenal was a major flavor compound of cucumber and could be converted from
438 linoleic acid via 9-HpODE during biosynthesis.[33] However, in the PhLOX catalyzed
439 reaction, 2*E*-nonenal was generated by the cleavage of 12-HpETE at ω-C9. The
440 current PhLOX reaction may provide a feasible pathway for the industrial production
441 of 2*E*-nonenal in high yield.

442 Incubation of EPA and DHA with PhLOX generated 2*E*,6*Z*-nonadienal and
443 3*E*,5*Z*-octadien-2-one as the only volatile product, respectively. Kocsy *et al*. reported
444 that 2*E*,6*Z*-nonadienal gave cucumber flavor.[30] Hu and Pan also reported that
445 2*E*,6*Z*-nonadienal had a fresh fish-like scent, and 3*E*,5*Z*-octadien-2-one had a
446 melon-like flavor.[34] Currently, there are relatively few studies on enzymatic catalysis
447 for the synthesis of 2*E*,6*Z*-nonadienal and 3*E*,5*Z*-octadien-2-one as food aroma
448 compounds. In this work, incubation of EPA and DHA with PhLOX generated
449 12-hydroperoxyeicosapentaenoic acid (12-HpEPE) and
450 14-hydroperoxydocosahexaenoic acid (14-HpDHE), and cleavage at ω-C9 of
451 12-HpEPE and at ω-C8 of 14-HpDHE then gave rise to 2*E*,6*Z*-nonadienal and

452 3*E*,5*Z*-octadien-2-one, respectively. The results here are noteworthy since PhLOX is
453 the first enzyme reported in literature that can use C20/C22 PUFAs as substrates to
454 generate C8/C9 VOCs in one step and on its own.

455 Hydrolyzed oils were also tested as substrates and incubated with PhLOX to
456 produce VOCs. During enzymatic incubation, most PUFAs components were
457 transformed by PhLOX, and only a minor fraction was degraded by spontaneous
458 oxidation. In the case of hydrolyzed sunflower seed oil and hydrolyzed corn oil,
459 although ≥50% of C18:2 were metabolized by PhLOX, no corresponding VOCs were
460 produced. Because the degradation of α-ALA by PhLOX produced a low yield (about
461 4.5%) of C5/C6 VOCs (*i.e.*, 2*Z*-pentenal and 3*Z*-hexenal), it is reasonable that no
462 2*Z*-pentenal nor 3*Z*-hexenal were detected when hydrolyzed sunflower seed oil and
463 corn oil were treated with PhLOX since only about 0.4 μM and 9.9 μM C18:3 were
464 consumed. Instead, an isomer of 3*Z*-hexenal, 2*E*-hexenal, was produced. Németh *et al.*
465 reported that both 2*E*-hexenal and 3*Z*-hexenal were formed from hydrolyzed linseed
466 oil by using soybean LOX1 and green bell pepper homogenate.[21] However, it was
467 unexpected that two volatile compounds, 2*E*,4*E*-pentadienal and 2*E*-octenal, were
468 generated in high concentrations and four other VOCs were formed in low
469 concentrations, by comparing with the control group of incubation with inactive
470 PhLOX. It has been reported that some VOC compounds including 1-penten-3-one,
471 hexanal, 1-pentanol, octanal, 1-octen-3-one, 1-octen-3-ol, and 2*E*,4*Z*-heptadienal were
472 crucial to vegetable oil quality.[35,36] For example, Wei *et al.* reported that some
473 characteristic aroma compounds such as 2*E*,4*E*-pentadienal (green, oily),
474 2*E*,4*E*-heptadienal (sweet, hazelnut, woody) and 5-ethyldihydro-2(3H)-furanone
475 (cereal-like) were contained in Xinjiang flaxseed oil (China), although information on
476 flaxseed oil volatiles remains limited.[19] In our experiments, both sunflower seed oil

and corn oil were hydrolyzed and extracted for use as substrates of PhLOX. GC-MS analysis showed that there were still esters and alkanes at high levels in the control group incubated with inactive PhLOX. However, after incubation with PhLOX, more VOCs appeared in both plant oil samples, suggesting that PhLOX could improve and reconstruct the aroma composition of plant oils. In the food industry, both 2*E*,4*E*-pentadienal and 2*E*-octenal are permitted for use as flavor additives in candy, beverage, meat, *etc.*, since 2*E*,4*E*-pentadienal has the flavor of fruit and 2*E*-octenal provides the scent of fat. Currently, these two compounds are mostly obtained through chemical synthesis in industry. The high yield of 2*E*,4*E*-pentadienal and 2*E*-octenal from vegetable oils by incubation with PhLOX indicates that this reaction may be a potentially viable biosynthesis in food industry for these aroma compounds.

When hydrolyzed fish oil was incubated with PhLOX, the enzyme preferentially targeted PUFAs with high degree of unsaturation, since the consumed substrates were mainly C18–C22 PUFAs with a degree of unsaturation no less than 4. Nevertheless, although the consumed substrates were mainly C20:5 and C22:6, the primary new VOC was neither 2*E*,6*Z*-nonadienal nor 3*E*,5*Z*-octadien-2-one, but 2*E*,4*E*-octadienal. When C20/C22-PUFAs were incubated with PhLOX, the yield of 2*E*,6*Z*-nonadienal from EPA and 3*E*,5*Z*-octadien-2-one from DHA were about 30% and 12%, respectively. When hydrolyzed fish oil was incubated with PhLOX, the formed 2*E*,6*Z*-nonadienal and 3*E*,5*Z*-octadien-2-one were about 29 μM and 17 μM, respectively, from 322 μM and 397 μM of C20:5 and C22:6 , with yields of 9.0% and 4.3%. These yields were one third of that of EPA and DHA. In addition, although the yield of 2*E*-nonenal and C8-VOCs (i.e., 1-octen-3-ol, 1-octen-3-one and 2*Z*-octen-1-ol) from ARA by PhLOX was about 54% and 20.5%, respectively, no 2*E*-nonenal but a minor amount of 2*E*-octen-1-ol was detected when fish oil

hydrolysate containing 36 μM C20:4 was treated with PhLOX. It seems that some components in hydrolyzed fish oil interfered with the activity of PhLOX, which was also observed when hydrolyzed vegetable oils were used as substrates for PhLOX. Reports have proved that except the rich unsaturated fatty acid components in plant oils, there were a portion of phytosterols, alcohols, vitamins, polyphenols.[37] It may be appealing to study further the catalyzed metabolism of PhLOX, considering its quite exceptional trait as holding LOX-AOS-HPL activities in one non-heme catalytic domain.[18] Hu and Pan reported that incubating fish oil with the crude extract of a green marine macroalga *Ulva conglobate* could increase desirable unsaturated aldehydes, ketones, and alcohols, such as 2*E*,4*E*-octadienal (oyster flavor), 2*E*,6*Z*-nonadienal (fresh fish flavor), 2*E*-hexenal (apple flavor), 3*E*,5*E*-octadien-2-one (fruit flavor), *etc.*[34] In this work, in addition to giving the major product 2*E*,4*E*-octadienal, the incubation reaction with PhLOX also yielded 2*E*,4*E*-pentadienal, 2*E*-hexenal, 4*Z*-heptenal, 1-hepten-3-ol, 2*E*-octenal, 2*E*,4*Z*-decadienal, *etc*. Hence, using PhLOX as the catalyst for incubation with fish oil may produce fresh seafood flavors and improve the flavor of the oil itself.

In summary, we used a multifunctional enzyme PhLOX to realize the one-step biosynthesis of C8–C9 volatile aroma compounds. The enzyme PhLOX was found to have high efficiency, high stability, and broad tolerance to reaction conditions. It could work with a variety of substrates and thus could serve as a good catalyst to produce C8- and C9-VOCs, especially 1-octen-3-ol/one, 2*E*-nonenal, 2*E*,6*Z*-nonadienal, and 3*E*,5*Z*-octadien-2-one. Hydrolyzed oils (sunflower seed oil, corn oil, and fish oil) could also be incubated with PhLOX to produce 2*E*,4*E*-pentadienal, 2*E*-octenal, and 2*E*,4*E*-octadienal. It is possible that PhLOX may be further combined with lipase in the one-step bioconversion of fatty acids to

produce aroma compounds of more appealing flavor.

ASSOCIATED CONTENT

Supporting Information

GC-MS analysis of the VOCs composition of hydrolyzed oils by incubation with PhLOX.

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Funding

This project was funded by the NSFC project (No. 81370532), the Major Scientific and Technological Project of Zhejiang Province (2016C02055-6B), National Science Foundation of Zhejiang (LY18C190004), Ningbo Programs for Science and Technology Development (2017C110026, 2017C10020), China Agriculture Research System (CAR-50), K.C. Wong Magna Fund of Ningbo University, the 151 talents Project, the Li Dak Sum Marine Biopharmaceutical Development Fund, and the National 111 Project of China.

Notes

The authors declare no competing financial interest.

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668

669 **Figure legends**

670 **Figure 1.** SDS-PAGE analysis of purified PhLOX.

671 **Figure 2.** Analysis of enzymatic activity when different fatty acids were used as
672 substrate for incubation with PhLOX. (A) Enzymatic activity of PhLOX at 1 min for
673 the reaction of five different fatty acid substrates. (B) Consumption of substrate over
674 time.

675 **Figure 3.** Analysis of the impact of (A) temperature and (B) pH on the incubation
676 reaction with PhLOX. The tested substrates included linoleic acid (LA), α-linolenic
677 acid (α-ALA), arachidonic acid (ARA), eicosapentaenoic acid (EPA), and
678 docosahexaenoic acid (DHA). The curves were plotted based on GaussAmp fitness.

679 **Figure 4.** GC-MS analysis of VOCs generated from different fatty acids after
680 incubation with PhLOX.

Table 1. Kinetic parameters of incubating different fatty acids with PhLOX.

Substrate	V_m (μM·min^{-1})	K_M (μM)	k_{cat}/K_M (min^{-1}·μM^{-1})
LA	68.1	459.1	0.1
α-ALA	164.1	395.1	0.4
ARA	3850.6	308.7	12.5
EPA	4192.5	286.4	14.6
DHA	1962.1	316.3	6.2

Table 2. Volatile products of different fatty acids after incubation with PhLOX.

Substrates	Products	Yield (μM)		Y_p (%)*	R_S (%)*
		By fresh PhLOX	By stored PhLOX		
α-ALA	2*Z*-pentenal	1.5 ± 0.2	0	1.5	92.7
	3*Z*-hexenal	3.0 ± 0.1	2.1 ± 0.03	3.0	
ARA	1-octen-3-ol	6.4 ± 0.9	5.7 ± 0.2	6.4	96.9
	1-octen-3-one	9.7 ± 0.4	9.0 ± 1.1	9.7	
	2*Z*-octen-1-ol	4.4 ± 0.8	0	4.4	
	2*E*-nonenal	54.2 ± 6.5	49.7 ± 5.3	54.2	
EPA	2*E*,6*Z*-nonadienal	30.3 ± 0.8	25.8 ± 1.2	30.3	96.2
DHA	3*E*,5*Z*-octadien-2-one	11.9 ± 0.7	9.1 ± 1.2	11.9	100

* datas calculated based on the reaction with fresh PhLOX.

Table 3. Component analysis of hydrolyzed plant oils after incubation reaction. (μM)

Fatty acids	Hydrolyzed sunflower seed oil				Hydrolyzed corn oil			
	Before incubation	Incubation with inactive PhLOX	Incubation with PhLOX	R_{FA} (%)	Before incubation	Incubation with active PhLOX	Incubation with PhLOX	R_{FA} (%)
C14:0	5.1 ± 0.3	4.0 ± 0.1	4.4 ± 0.1		3.6 ± 0.1	2.3 ± 0.1	2.8 ± 0.1	
C16:0	32.0 ± 2.5	29.0 ± 1.1	29.7 ± 0.7		38.5 ± 2.7	36.3 ± 0.9	31.5 ± 1.1	
C16:1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.01		0.7± 0.04	0.4 ± 0.01	0.3 ± 0.01	
C18:0	17.5 ± 0.5	15.6 ± 2.3	13.7 ± 0.3		9.2 ± 0.5	6.2 ± 0.2	5.4 ± 0.2	
C18:1	315.5 ± 24.7	325.7 ± 26.7	346.1 ± 41.7		300.5 ± 24.7	285.6 ± 15.5	286.5 ± 17.8	
C18:2	902.3 ± 58.4	884.2 ± 47.2	298.1 ± 20.2	66.3	769.1 ± 56.9	745.5 ± 32.3	376.2 ± 21.9	49.5
C18:3	1.5 ± 0.2	0.4 ± 0.1	0	100	14.3 ± 1.6	10.3 ± 0.4	0.4 ± 0.02	96.1
C20:0	0.7 ± 0.02	0.4 ± 0.02	0.3 ± 0.01		1.5 ± 0.1	0.5 ± 0.2	0.4 ± 0.01	
C20:1	0.5 ± 0.01	0.2 ± 0.1	0		3.1 ± 0.2	0.3 ± 0.01	0.2 ± 0.01	

Table 4. Component analysis of hydrolyzed fish oil after incubation reaction. (μM)

Fatty acids	Before incubation	After incubation		R_{FA} (%)
		with inactive PhLOX	with PhLOX	
C14:0	7.8 ± 1.0	8.2 ± 1.7	8.8 ± 1.0	
C16:0	10.1 ± 1.2	9.7 ± 0.9	10.8 ± 1.2	
C16:1	8.5 ± 0.9	8.2 ± 0.8	8.8 ± 1.0	
C16:2	1.5 ± 0.1	1.3 ± 0.1	1.4 ± 0.1	0
C18:0	2.0 ± 0.4	2.1 ± 0.2	2.5 ± 0.4	
C18:1	6.9 ± 0.5	7.8 ± 0.8	6.4 ± 1.1	
C18:2	1.1 ± 0.1	0.8 ± 0.1	1.0 ± 0.1	0
C18:4	1.5 ± 0.2	1.3 ± 0.1	0.3 ± 0.02	74.8
C20:0	0.3 ± 0.01	0.1 ± 0.01	0.1 ± 0.01	
C20:1	0.7 ± 0.01	0.4 ± 0.04	0.4 ± 0.1	
C20:4	52.3± 4.9	48.5 ± 5.0	11.8 ± 1.11	75.7
C20:5	423.7 ± 15.7	409.7 ± 20.7	86.9 ± 9.1	78.8
C22:5	43.9 ± 2.9	41.1 ± 3.1	7.5 ± 0.7	81.8
C22:6	471.9 ± 35.4	452.6 ± 30.3	55.5 ± 7.1	87.7

Table 5. Semi-quantitative determinations of volatile products in different hydrolyzed oils incubated with PhLOX. (μM)

	VOCs	Hydrolyzed sunflower seed oil		Hydrolyzed corn oil		Hydrolyzed fish oil	
		Blank	After reaction	Blank	After reaction	Blank	After reaction
New generated	2*E*,4*E*-pentadienal	-	364.5 ± 8.7	-	267.2 ± 15.7	-	18.1 ± 1.6
	2*E*-hexenal	-	(trace amount)	-	(trace amount)	-	8.8 ± 0.2
	n-heptanal	-	14.6 ± 1.4	-	9.7 ± 1.7	-	-
	4*Z*-heptenal	-	-	-	-	-	10.4 ± 1.3
	2*E*-heptenal	-	5.5 ± 0.6	-	4.1 ± 0.8	-	-
	1-hepten-3-ol	-	-	-	-	-	4.4 ± 0.5
	n-octanal	-	9.3 ± 0.3	-	8.9 ± 0.5	-	7.9 ± 0.2
	2*E*-octenal	-	102.2 ± 9.3	-	91.9 ± 5.9	-	12.4 ± 1.6
	2*E*,4*E*-octadienal	-	-	-	-	-	143.6 ± 8.0
	3*E*,5*E*-octadien-2-one	-	-	-	-	-	17.4 ± 1.3
	2*E*-octen-1-ol	-	-	-	-	-	2.8 ± 0.2
	2*E*,6*Z*-nonadienal	-	-	-	-	-	29.3 ± 2.8
	2*E*,4*E*-nonadienal	-	21.0 ± 3.2	-	5.2 ± 0.3	-	-
	2*E*,4*Z*-decadienal	-	-	-	-	-	41.0 ± 1.0
Increased	*n*-nonanal	17.9 ± 4.4	41.0 ± 0.8	16.3 ± 1.2	49.0 ± 5.6	13.8 ± 1.6	62.4 ± 6.1
	n-decanal	7.5 ± 0.8	12.7 ± 0.8	7.9 ± 0.4	11.6 ± 0.8	6.2 ± 0.3	15.5 ± 1.8

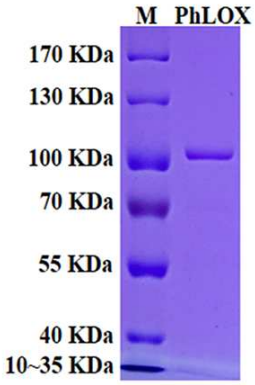


Figure 1. SDS-PAGE analysis of purified PhLOX.

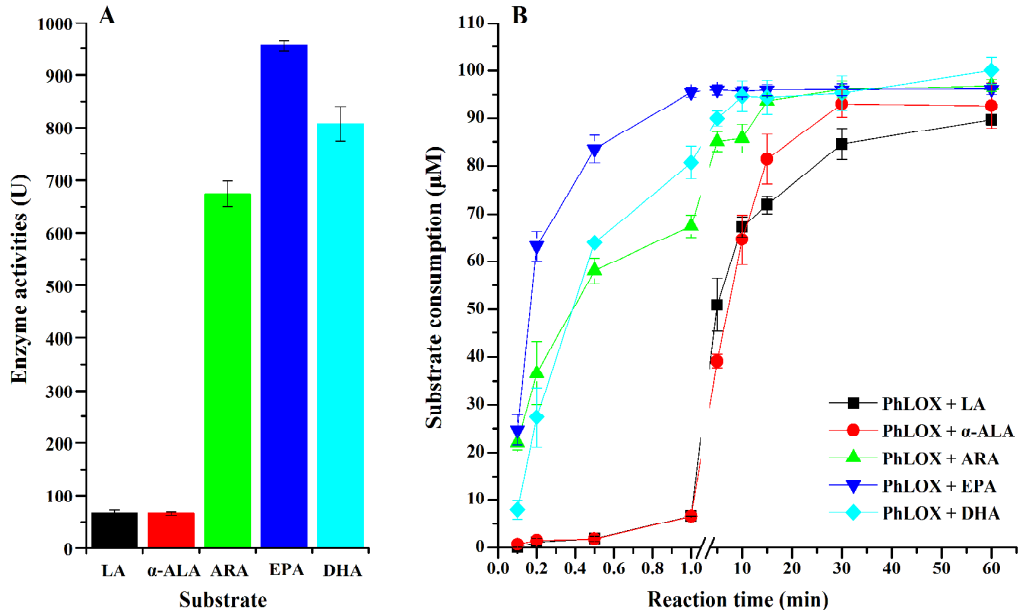


Figure 2. Analysis of enzymatic activity when different fatty acids were used as substrate for incubation with PhLOX. (A) Enzymatic activity of PhLOX at 1 min for the reaction of five different fatty acid substrates. (B) Consumption of substrate over time.

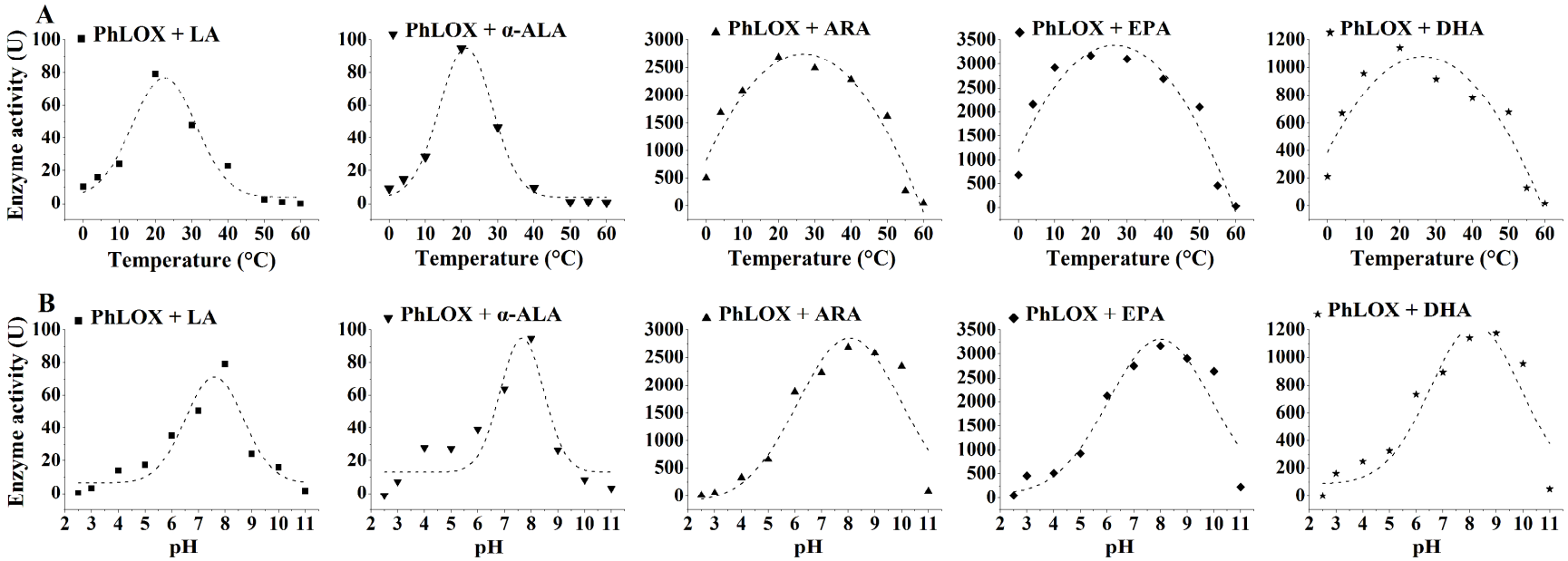


Figure 3. Analysis of the impact of (A) temperature and (B) pH on the incubation reaction with PhLOX. The tested substrates included linoleic acid (LA), α-linolenic acid (α-ALA), arachidonic acid (ARA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). The curves were plotted based on GaussAmp fitness.

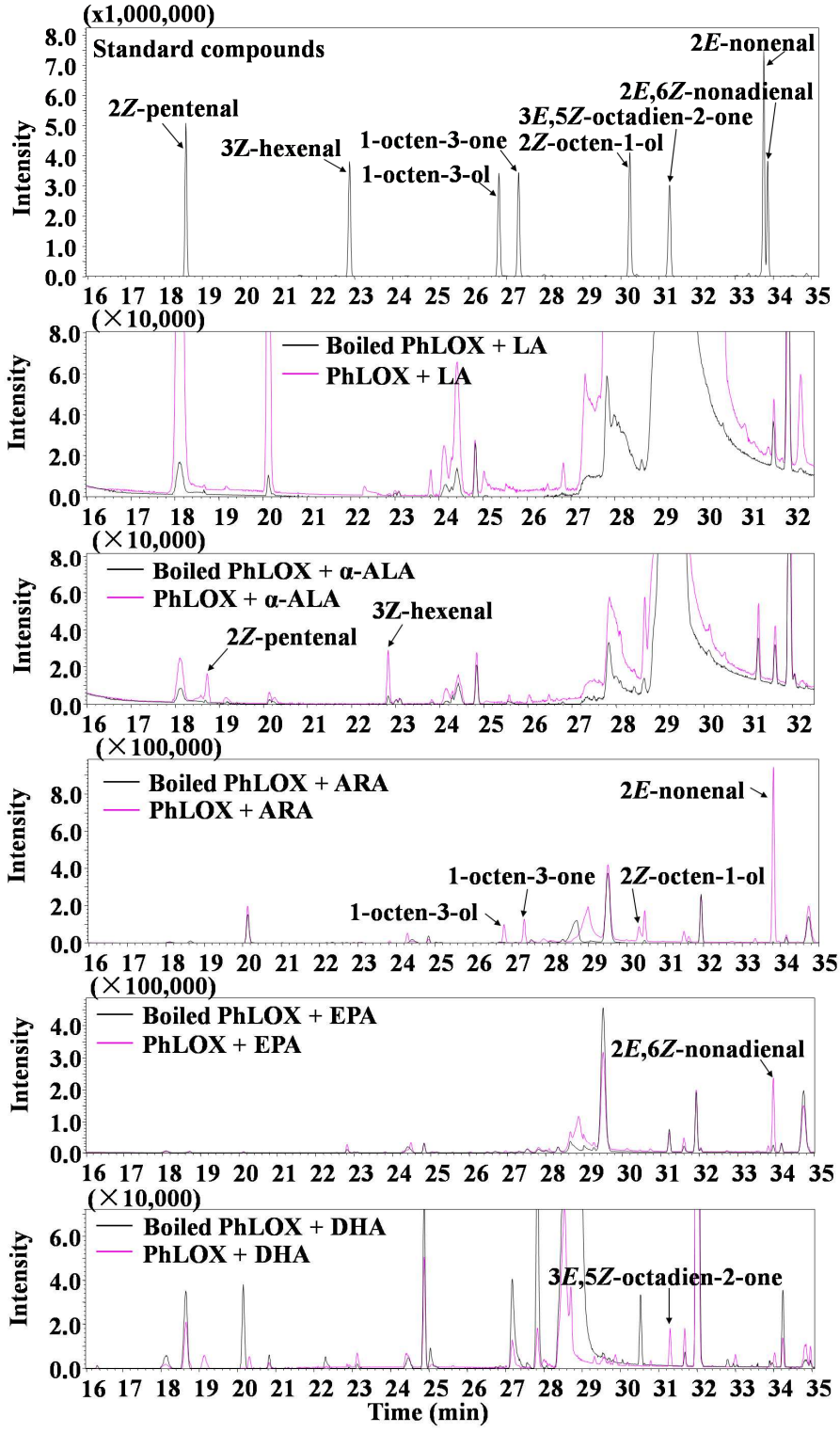
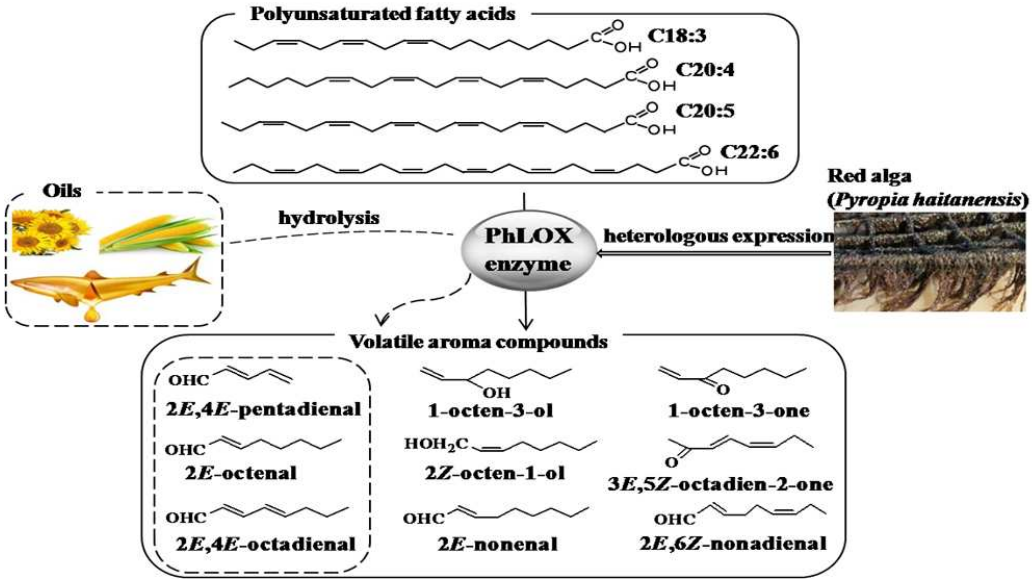
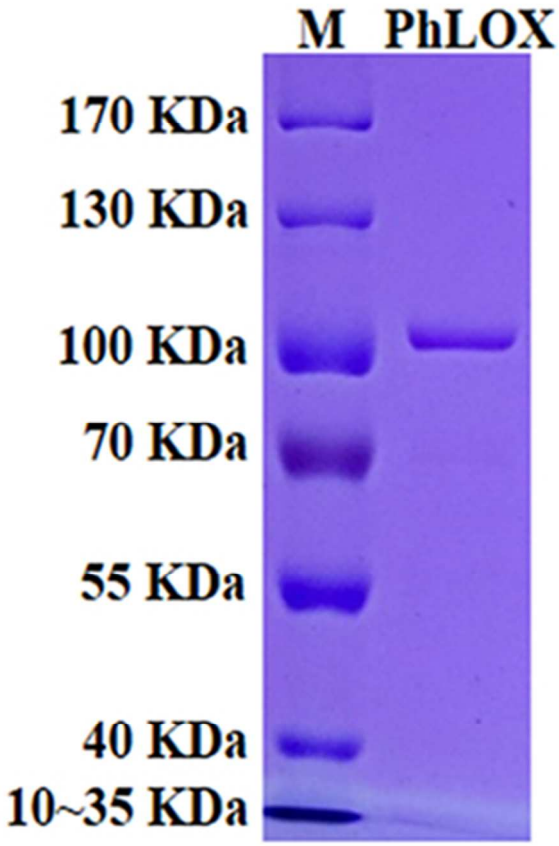


Figure 4. GC-MS analysis of VOCs generated from different fatty acids after incubation with PhLOX.

TOC Graphic


Polyunsaturated fatty acids
C18:3
C20:4
C20:5
C22:6
Oils
hydrolysis
PhLOX enzyme
heterologous expression
Red alga
(Pyropia haitanensis)
Volatile aroma compounds
2E,4E-pentadienal
1-octen-3-ol
1-octen-3-one
2E-octenal
2Z-octen-1-ol
3E,5Z-octadien-2-one
2E,4E-octadienal
2E-nonenal
2E,6Z-nonadienal



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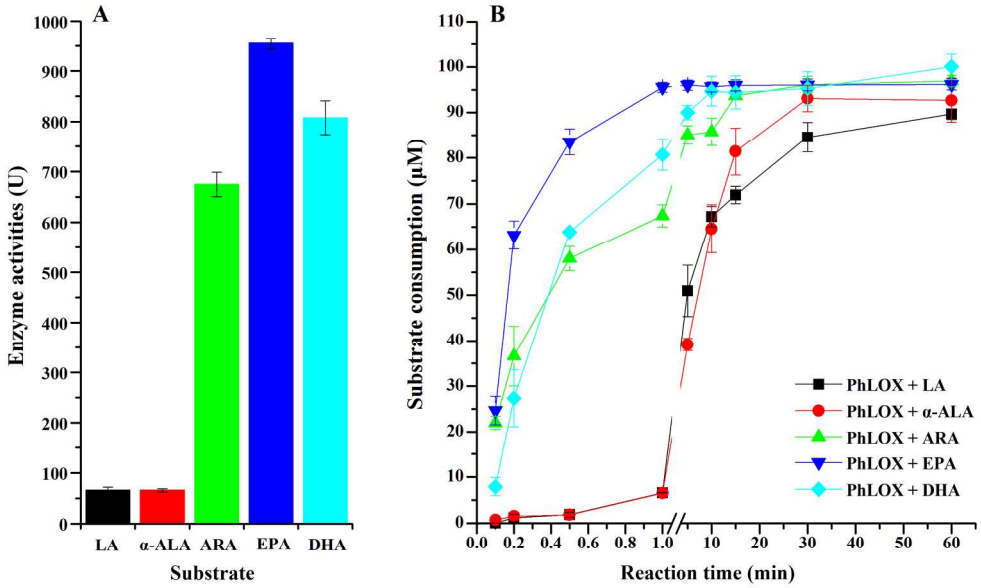

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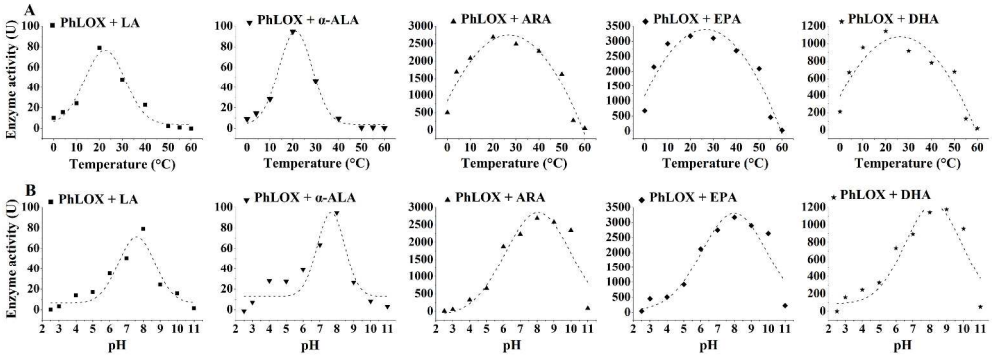

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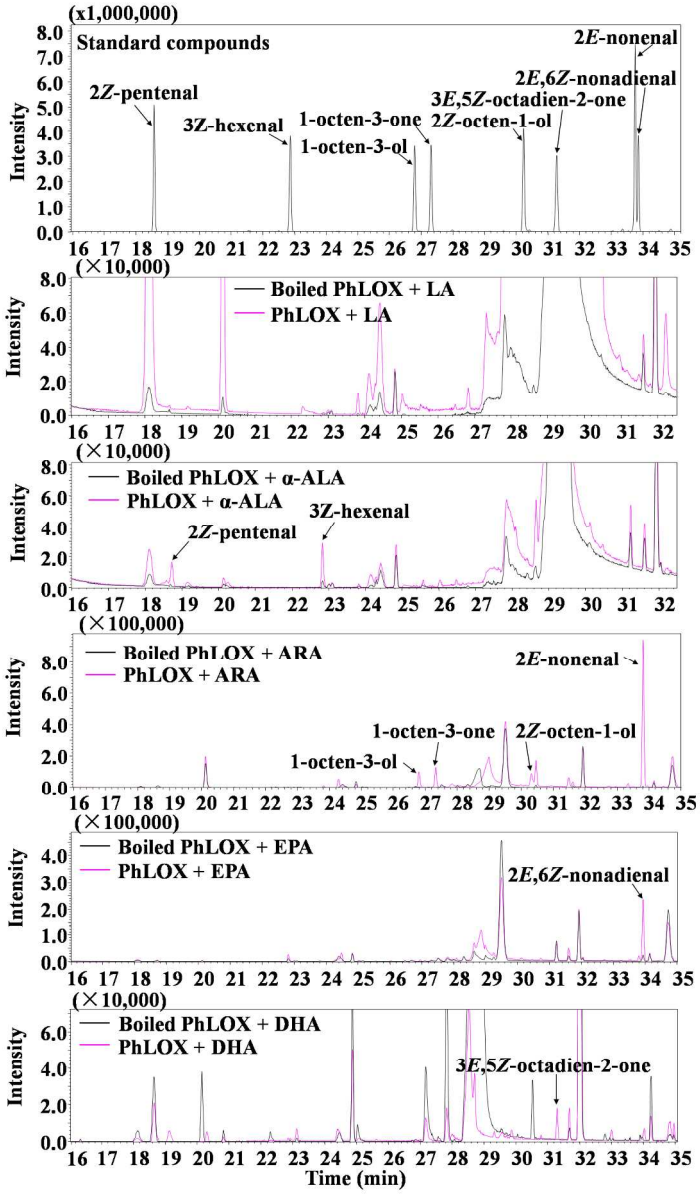

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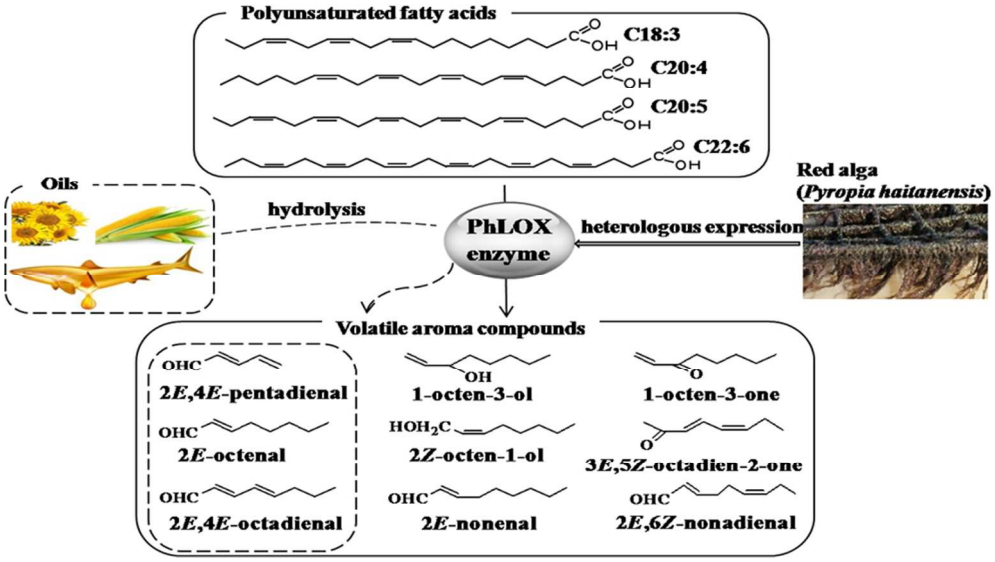

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