Establishing an enzyme cascade for one-pot production of α-olefins from low-cost triglycerides and oils without exogenous H₂O₂ addition

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Abstract

Background: Biological α-olefins can be used as both biofuels and high value-added chemical precursors to lubricants, polymers, and detergents. The prototypic CYP152 peroxygenase family member OleTJE from Jeotgalicoccus sp. ATCC 8456 catalyzes a single-step decarboxylation of free fatty acids (FFAs) to form α-olefins using H₂O₂ as a cofactor, thus attracting much attention since its discovery. To improve the productivity of α-olefins, significant efforts on protein engineering, electron donor engineering, and metabolic engineering of OleTJE have been made. However, little success has been achieved in obtaining α-olefin high-producer microorganisms due to multiple reasons such as the tight regulation of FFA biosynthesis, the difficulty of manipulating multi-enzyme metabolic network, and the poor catalytic performance of OleTJE.

Results: In this study, a novel enzyme cascade was developed for one-pot production of α-olefins from low-cost triacylglycerols (TAGs) and natural oils without exogenous H₂O₂ addition. This artificial biocatalytic route consists of a lipase (CRL, AOL or Lip2) for TAG hydrolysis to produce glycerol and free fatty acids (FFAs), an alditol oxidase (AldO) for H₂O₂ generation upon glycerol oxidation, and the P450 fatty acid decarboxylase OleTJE for FFA decarboxylation using H₂O₂ generated in situ. The multi-enzyme system was systematically optimized leading to the production of α-olefins with the conversion rates ranging from 37.2 to 68.5%. Furthermore, a reaction using lyophilized CRL/OleTJE/AldO enzymes at an optimized ratio (5 U/6 μM/30 μM) gave a promising α-olefin yield of 0.53 g/L from 1500 μM (~1 g/L) coconut oil.

Conclusions: The one-pot enzyme cascade was successfully established and applied to prepare high value-added α-olefins from low-cost and renewable TAGs/natural oils. This system is independent of exogenous addition of H₂O₂, thus not only circumventing the detrimental effect of H₂O₂ on the stability and activity of involved enzymes, but also lower the overall costs on the TAG-to-olefin transformation. It is anticipated that this biotransformation system will become industrially relevant in the future upon more engineering efforts based on this proof-of-concept work.
Background

Volatile geopolitical factors, depletion of petroleum-based fuels, and serious environmental concerns have been spurring the development of alternative, sustainable, and cost-effective biofuels from renewable feedstocks [1, 2]. Biofuels are considered as the most promising green alternatives to petroleum-based fuels because their combustion could be carbon neutral or even negative (when produced by photosynthetic microalgae) and of near-zero air pollution [3]. Among different biofuel types, aliphatic hydrocarbons such as fatty alkanes and alkenes are regarded as ideal biofuels due to their high energy content, low hygroscopicity, and compatibility with the existing engine and distribution systems [4, 5].

Chemically, a number of thermochemical methods including gasification, pyrolysis, and liquefaction, and various metal-based catalytic reactions can be utilized to convert biomass into hydrocarbon-based fuels [6, 7]. However, these chemical approaches often lead to poor hydrocarbon yields, high energy consumption, and considerable side products due to simultaneous occurrence of several types of undesired reactions at high temperatures. Thus, these problems together with significant environmental concerns on hazardous chemical wastes have prompted continuous searches for green, robust, and economic biocatalytic methods to produce aliphatic hydrocarbons [8, 9].

Compared to fatty alkanes, fatty alkenes especially terminal olefins (i.e., α-olefins) are more valuable products since they can be used as both biofuels and important precursors to lubricants, polymers, and detergents [10]. Nature has evolved various α-olefin-producing enzymes that use either free fatty acids (FFAs) or fatty acyl–acyl carrier proteins (acyl-ACPs) as starting materials [8]. Among them, P450 fatty acid decarboxylases have attracted the most attention in recent years because this P450 enzyme family efficiently catalyzes a single-step decarboxylation of FFAs to form α-olefins by consuming H₂O₂ (as sole oxygen and electron donor) stoichiometrically, with varying amounts of hydroxylated fatty acids as side products.

As the first identified P450 fatty acid decarboxylase, OleTJE from Jeotgalicoccus  sp. ATCC 8456 [11] has been intensively studied to understand its unique catalytic mechanism and to harness its valuable decarboxylation capacity [12–17]. Furthermore, significant efforts on protein engineering, electron donor engineering, pathway engineering, and metabolic engineering have been made for both titer improvement and product profile tuning [14, 18, 19]. Nonetheless, the reported highest total alkene titers of different engineered microorganisms have only reached the level of several 100 mg/L, which are far from the high cost requirement of commercial production of α-olefins. The major reasons accounting for the low α-olefin yields include the tightly regulated FFA biosynthesis from glucose, the difficulty of manipulating multi-enzyme metabolic network, and the poor catalytic behaviors of OleTJE likely owing to poor stability, low activity, substrate accessibility, and the availability of H₂O₂ cofactor in vivo [18, 20, 21].

Thus, the multi-enzyme in vitro transformation of renewable oil-based feedstock into α-olefins has appeared to be a promising alternative strategy. For example, in our previous study, a two-enzyme system consisting of the lipase Tll for hydrolysis of triglycerides (TAGs) to generate FFAs and OleTJE for decarboxylation of the resulting FFAs to produce α-olefins in the presence of exogenously added H₂O₂ was engineered; and the overall TAG-to-olefin yields reached 6.7–46.0% [19]. Matthews et al. recently engineered an OleTJE–AldO fusion enzyme, in which the aldil oxidation AldO from Streptomyces coelicolor [22] was responsible for oxidizing glycerol to glyceraldehyde and glyceric acid sequentially generating H₂O₂ as a co-product to drive the following OleTJE-mediated FFA decarboxylation.

To further improve the efficiency of TAG-to-olefin transformation and lower the overall costs, a number of key factors including the catalytic efficiencies, the mismatch between the fatty acyl chain length specificity of lipase and P450 fatty acid decarboxylase, the cofactor supply, and the low stability of proteins (especially for P450 enzymes) in presence of H₂O₂ must be addressed. Thus, in this work, a novel enzyme cascade was designed and assembled for efficient one-pot production of α-olefins from low-cost triglycerides and natural oils by integrating the activities of lipase, P450 fatty acid decarboxylase, and aldil oxidase. Conceptually, a lipase hydrolyzes one molecule of TAG to release three molecules of FFAs and one molecule of glycerol; an aldil oxidase (AldO) [22] oxidizes glycerol to generate two equivalents of H₂O₂ in situ as the cofactor of OleTJE to drive FFA decarboxylation yielding α-olefins; the short- age of one equivalent of H₂O₂ can be resolved by exogenous addition of glycerol (Fig. 1).

In specific, three well-characterized lipases including CRL from Candida rugose [23], AOL from Aspergillus oryzae [24], and Lip2 from Yarrowia lipolytica [25] were examined for their cooperativity with OleTJE and AldO.
toward different substrates, including pure TAGs, and several kinds of natural oils. Moreover, the enzyme cascade comprising CRL, OleT\(_{JE}\), and AldO was optimized for the preferable coconut oil using lyophilized enzymes.

**Results**

**Lipase selection in terms of the TAG hydrolytic activity**

The P450 fatty acid decarboxylase OleT\(_{JE}\) favors the FFAs with the acyl chain length between \(C_{10}\) and \(C_{18}\) [18, 26]. To identify an appropriate lipase to efficiently supply the favorable FFA substrates for OleT\(_{JE}\), the hydrolytic activities of three well-characterized lipases toward the pure TAGs (500 \(\mu M\)) with the fatty acyl chains of \(C_{10}^{-}C_{18}\) were evaluated, including the two commercial lipases, namely, CRL from *Candida rugose* and AOL from *Aspergillus oryzae*, and the laboratory-prepared Lip2 from *Yarrowia lipolytica* (Additional file 1: Figure S1) [23–25]. As results, CRL released 1429.9 \(\mu M\) capric acid (\(C_{10}\)), 1450.8 \(\mu M\) lauric acid (\(C_{12}\)), 1293.6 \(\mu M\) myristic acid (\(C_{14}\)), 1016.8 \(\mu M\) palmitic acid (\(C_{16}\)), and 109.6 \(\mu M\) stearic acid (\(C_{18}\)) from their corresponding TAGs, corresponding to the conversion rates of 95.3%, 96.7%, 86.2%, 67.8%, and 7.3%, respectively (Fig. 2). AOL exhibited analogous hydrolytic activities and a similar substrate preference profile to CRL with tricaprin (\(C_{10}\)) as the optimal substrate (92.3% conversion rate). However,
the laboratory-prepared Lip2 showed significantly lower activities than both AOL and CRL, with the highest yield of 58.6% against tricaprin (C10). Considering the overall hydrolytic activity and the factor that OleTJE prefers C10–C14 FFAs [26], CRL and AOL were selected for the following enzyme cascade setup.

Effect of $H_2O_2$ concentration on the OleTJE activity

OleTJE, a CYP152 peroxygenase family member, utilizes $H_2O_2$ as a cofactor (or co-substrate) to support the unique oxidative decarboxylation reactions that convert $C_n$ ($n=4–22$) chain length FFAs into $C_{n-1}$ α-olefins and release one molecule of CO2 [26]. In principle, a higher concentration of $H_2O_2$ co-substrate presumably enhances the reaction rate kinetically. However, excessive addition of $H_2O_2$ would oxidatively inactivate enzymes via radical mechanisms in general [27]. Therefore, it is important to balance the two opposite sides of $H_2O_2$ by identifying an optimal working concentration of $H_2O_2$ for OleTJE's activity.

In specific, 0–5000 μM of $H_2O_2$ was added into each individual OleTJE reaction mixture containing 1 μM P450 enzyme and 500 μM lauric acid. As expected, the conversion of lauric acid increased in proportion to the amount of $H_2O_2$ at the low concentration range (0–550 μM); then, the substrate conversion rates declined with the increase of $H_2O_2$ concentrations between 1000 and 5000 μM (Fig. 3). Taken together, direct addition of $H_2O_2$ to a high concentration appeared not to be an effective way to maintain a high activity of OleTJE.

Determination of the optimal ratio between OleTJE and AldO

Although the OleTJE–AldO fusion enzyme developed by Munro and co-workers could enable efficient channeling of $H_2O_2$ between these two enzyme active sites [28], the fusion nature of OleTJE and AldO fixed the molar ratio of these two functional domains to be 1:1, which might not be an optimal ratio. Moreover, the chimeric protein might cost its high expression level.

Thus, in this study, OleTJE and AldO were separately expressed (Additional file 1: Figure S2), by which their ratio could be conveniently adjusted. Using 500 μM lauric acid as substrate and 0.01% v/v (~1000 μM) glycerol loading, a select number of different OleTJE to AldO (OA) ratios were examined. It was revealed that the best OA ratio turned out to be 1:10 (or 1:5), at which the substrate conversion rate and the alkene production ratio were 99.7% (93.6%) and 64.9% (61.5%) (Fig. 4), respectively. The differences between lauric acid ($C_{12}$) conversion and undecene ($C_{11}$) production were due to the unquantified side products, namely, different hydroxylated fatty acids. In consideration of both the overall catalytic efficiency and cost-effectiveness, the OA ratio of 1:5 with the lower amount of AldO was selected for the following experiments.

Determination of the optimal glycerol concentration

As the substrate of AldO, glycerol is presumably a key factor in the OleTJE/AldO reaction system. Thus, the optimal glycerol concentration for OleTJE’s activity in the range of 0.01–10% was determined with the exogenous addition of $H_2O_2$ as a control. For 500 μM lauric acid, 0.01% glycerol loading resulted in approximately 100% increase in both substrate conversion rate and total turnover number (TTN) compared to the OleTJE reaction supported by 500 μM $H_2O_2$ (Table 1). These results clearly demonstrated the advantage of continuous release of $H_2O_2$ over the direct addition of $H_2O_2$ at once.

At a higher concentration of lauric acid (1000 μM), the AldO-based in situ $H_2O_2$-generating system showed more significant improvements under all tested glycerol concentrations relative to 1000 μM $H_2O_2$ (Table 1). In the presence of 0.01–10% glycerol, the TTNs ranged...
When the glycerol concentration was higher, the decarboxylation activity was better. Surprisingly, at the same 0.01% glycerol loading, the reaction with 1000 μM substrate gave a much lower TTN than that with 500 μM substrate. This result suggested that the activity of AldO might be inhibited by the high concentration of hydrophobic products or substrates at a relatively low glycerol concentration.

Evidently, 1 μM OleTJE and 5 μM AldO were not sufficient for complete decarboxylation of 1000 μM lauric acid even in the presence of 10% glycerol. To decarboxylate 1500 μM lauric acid that can be maximally released from 500 μM trilaurin, higher concentrations of OleTJE and AldO were required to be used with an appropriate amount of glycerol. Thus, the reaction system of 3 μM OleTJE, 15 μM AldO, and 1.5% glycerol was proposed and tested, by which we assumed that a triplicated TTN could be achieved by higher enzyme concentrations.

Establishing an enzyme cascade for the transformation from TAGs to α-olefins without exogenous H2O2 addition

Previously, an enzyme cascade was established in this laboratory comprising a lipase and a P450 fatty acid decarboxylase, which is capable of converting TAGs into α-olefins with exogenous addition of H2O2 [19]. To make this system independent of detrimental exogenous H2O2, AldO was introduced into this enzyme cascade to generate H2O2 in situ from glycerol, the by-product of lipase. In specific, 3 μM OleTJE, 15 μM AldO, 5 U lipase, 1.5% glycerol, and 500 μM pure TAGs were mixed together and incubated at 30 °C for 6 h. As results, 594.7 μM 1-nonene (C9), 735.5 μM 1-undecene (C11), 871.4 μM 1-tridecene (C13), 673.3 μM 1-pentadecene (C15), and 56.4 μM 1-heptadecene (C17) were produced from respective TAGs by the CRL/OleTJE/AldO system (Fig. 5a), corresponding to 39.6%, 49.1%, 58.1%, 44.9%, and 3.8% of the theoretical maximum TAG-to-olefin yields (TAG:FFA:α-olefin = 1:3:3). The reactivity pattern of the AOL/OleTJE/AldO was similar to that of the CRL/OleTJE/AldO system (Fig. 5), while the yields of 1-tridecene (61.5%) and 1-heptadecene (10.6%) reached a higher level.

Production of α-olefins from natural oils

Next, the activities of the CRL/OleTJE/AldO and AOL/OleTJE/AldO systems were examined toward a number of natural oils including coconut oil and palm oil featuring saturated fats, peanut oil and olive oil containing monounsaturated fats, and soybean oil representing

### Table 1: OleTJE catalytic activities in two different H2O2-supplying systems

| Substrate               | H2O2 supply | Conversion/% | TTNa    |
|-------------------------|-------------|--------------|---------|
| 500 μM lauric acid      | 500 μM H2O2 | 46.5         | 235 ± 4 |
|                         | AldO + 0.01% glycerol | 93.6 | 467 ± 22** |
| 1000 μM lauric acid     | 1000 μM H2O2 | 18.1         | 181 ± 2 |
|                         | AldO + 0.01% glycerol | 27.3 | 273 ± 4* |
|                         | AldO + 0.05% glycerol | 35.4 | 354 ± 10* |
|                         | AldO + 0.1% glycerol | 38.6 | 386 ± 31** |
|                         | AldO + 0.5% glycerol | 49.3 | 493 ± 24* |
| AldO + 1% glycerol      | 50.1         | 501 ± 39*    |
| AldO + 5% glycerol      | 64.9         | 649 ± 23*    |
| AldO + 10% glycerol     | 69.5         | 695 ± 40*    |

* Reaction conditions: 1 μM OleTJE supported by H2O2 or the 5 μM AldO + glycerol system, at 30 °C for 6 h. Total turnover number (TTN): mean ± standard deviation; standard deviations were derived from at least two independent experiments. Statistical analysis of the adjacent TTN data was performed using a Student’s t test (one-tailed; *P < 0.05, **P < 0.01, ns: P > 0.05, no significant; two-sample unequal variance)

![Fig. 5](image-url) Production of α-alkenes from TAGs by the lipase/OleTJE cascade supported by AldO/glycerol: **(a) CRL/OleTJE/AldO and (b) AOL/OleTJE/AldO**
polysaturated fats. Compared to pure TAGs, these natural oils are more readily available and industrially relevant [29].

In the CRL hydrolytic system, 1304.9, 1108.1, 708.1, 998.8, and 1301.9 μM of total FFAs were produced from 500 μM of coconut oil, palm oil, soybean oil, peanut oil, or olive oil, corresponding to 86.9%, 73.9%, 47.2%, 66.6%, and 86.8% of TAG-to-FFA conversions, respectively. Regarding the released FFA profiles of different natural oils (Additional file 1: Table S2), lauric acid, palmitic acid, and linoleic acid were the main hydrolytic products of coconut oil, palm oil, and soybean oil, respectively, accounting for 47.5%, 41.3%, and 54.7% of total FFAs. For both peanut oil and olive oil, oleic acid was the major released FFA species, reaching 45.6% and 71.9% of total FFAs, respectively. Moreover, the hydrolytic activities (Fig. 6a, b) and FFA profiles of the AOL hydrolytic system (Additional file 1: Table S3) toward the same group of natural oils were highly similar to those of the CRL hydrolytic system. Notably, coconut oil turned out to be the preferred substrate in both hydrolytic systems (Fig. 6a, b).

Subsequently, the one-pot enzyme cascade reactions including TAG hydrolysis, glycerol oxidation, and FFA decarboxylation using natural oils as starting materials were carried out. As results, 1027.5 (1002.9), 771.7 (731.3), 558.1 (544.4), 601.2 (543.7), and 730.7 μM (723.6 μM) of total α-olefins (including 1-heptene, 1-nonene, 1-undecene, 1-tridecene, 1-pentadecene, 1-heptadecene, and 1-nonadecene) were produced from coconut oil, palm oil, soybean oil, peanut oil, and olive oil, respectively (Fig. 6c, d, Additional file 1: Tables S4 and S5) by the CRL/AldO/OleTJE (AOL/AldO/OleTJE) systems, corresponding to 68.5% (66.8%), 51.4% (48.8%), 37.2% (36.3%), 40.1% (36.2%), and 48.7% (48.2%) of TAG-to-alkene yields.

Optimization of the coconut oil bioconversion system using the lyophilized CRL/OleTJE/AldO enzymes

Coconut oil among the tested natural oils appeared to be the best feedstock for α-olefin production (Fig. 6c, d), from which 1-undecene derived from lauric acid, the OleTJE-favored substrate, was the predominant product. Thus, the CRL/OleTJE/AldO system using coconut oil as the starting material to produce biological α-olefins was chosen for further optimization. To make the system more practical and cost-effective, lyophilized enzymes were used. Notably, the mixed lyophilized CRL, OleTJE, and AldO demonstrated comparable olefins-producing
activity as the freshly purified proteins at the same levels (Additional file 1: Figure S3).

To examine the application potential of the one-pot reaction system consisting of CRL, OleTJE, AldO, coconut oil, and glycerol, the coconut oil concentration was increased from 500 to 1500 μM (~1 g/L). Under the higher substrate concentration, we re-optimized the amount of each individual component. As a result, 5 U CRL was shown to be able to hydrolyze 1500 μM coconut oil almost to the theoretical maximum yield of 4500 μM, and further increase of lipase amount had no significant beneficial effect on FFA production (Additional file 1: Figure S3). However, when 5 U CRL, 3 μM OleTJE, and 15 μM AldO were reacted with 1500 μM coconut oil and 1.5% glycerol for 6 h, the total alkene yield was 14.3% lower than that of the previous 500 μM substrate system, indicating this reaction system needed to be further optimized.

Since 5 U CRL (among 2.5, 5, and 10 U) gave the highest alkene production (Additional file 1: Figure S4b), we subsequently optimized other contents in the TAG-to-olefin reaction system containing 5 U CRL. As expected, higher concentrations of OleTJE and AldO resulted in improved alkene production under the same glycerol content (Table 2). Moreover, increase of glycerol content significantly improved the total yield of α-olefins. These results suggested that the H₂O₂ releasing upon glycerol oxidation might be the major limit of the overall efficiency of the alkene-producing system. Thus, the highest alkene yield achieved by this enzyme cascade system was 52.6% (0.53 g/L) at a 20% glycerol loading in a 200-μL reaction (Table 2). Of note, in a 10-mL reaction, this system delivered a similar α-olefin yield of 49.1% (0.49 g/L) (Table 2).

**Table 2 α-Olefin production from 1500 μM coconut oil by CRL/OleTJE/AldO at 30 °C for 6 h under different reaction conditions**

| Enzyme concentrations | Glycerol content/% | Total α-olefins/μM | Yield/g/L* |
|-----------------------|-------------------|-------------------|-----------|
| 3 μM OleTJE, 15 μM AldO | 1.5               | 880.5 ± 1.7       | 0.20      |
|                       | 6                 | 1377.9 ± 12.4     | 0.31      |
|                       | 10                | 1535.6 ± 82.2     | 0.34      |
|                       | 20                | 2138.3 ± 121.9    | 0.48      |
| 6 μM OleTJE, 30 μM AldO | 3                 | 1339.8 ± 137.4    | 0.30      |
|                       | 6                 | 1416.6 ± 281.8    | 0.32      |
|                       | 10                | 1820.8 ± 239.5    | 0.41      |
|                       | 20                | 2369.9 ± 65.9     | 0.53      |
|                       | 20                | 2206.9 ± 27.9     | 0.49      |

*200-μL reaction system

**Discussion**

With continuous discovery and characterization of novel hydrocarbon biosynthetic systems, a growing number of α-olefin-producing enzymes have been reported, including P450 fatty acid decarboxylases such as OleTJE and CYP-Sm46 [11, 30], the non-heme iron-dependent decarboxylase UndA [31], and the membrane-bound desaturase-like UndB [32]. Although these α-olefin syntheses can use different chain length FFAs as substrates, when they were coupled to the fatty acid biosynthetic systems of engineered *E. coli* or *Saccharomyces cerevisiae* strains, the total alkene titers turned out to be very low with the highest reported yield (97.6 mg/L) only accounting for about 1% of theoretical maximum yield (3% glucose) [18, 20, 32, 33]. Mechanistically, the complexity and tight regulation of fatty acid metabolic network in vivo could be one of the major reasons for the cost-prohibitive α-olefin yields [9].

To overcome this challenge, we previously engineered an in vitro tandem reaction system, in which the lipase Tll and OleTJE cooperate to transform TAGs to α-olefins with exogenous addition of H₂O₂ [19]. Subsequently, Li et al. developed a genetically encoded synthetic self-assembled Tll/OleTJE complex for bioproduction of fatty alkenes, wherein three non-catalytic modules including cohesion, dockerin, and CBM were used to control enzyme ratio, position, reusability, and stability [34]. However, the α-olefin yields of both approaches were low likely due to the detrimental effect of H₂O₂ for enzymes.

Although CYP152 peroxigenases are more H₂O₂-tolerant than most of P450 monoxygenases, a high concentration of H₂O₂ could still result in the loss of catalytic activity of these P450 peroxigenases, which was observed in the process of myristic acid hydroxylation catalyzed by the peroxigenase P450CLA from *Clostridium acetobutylicum* [28, 35]. Despite the high activity (up to 200 min⁻¹), substantial inactivation of P450CLA occurred within 2–4 min when 200 μM H₂O₂ was added at once. Our results also showed that OleTJE was plagued by the H₂O₂ concentrations greater than 1000 μM (Fig. 3). To resolve this problem, a growing number of approaches have been developed to elegantly control the H₂O₂ supply for reduction of the P450 inactivation [18, 36–38]. For example, a light-driven in situ H₂O₂-generating system employed flavin adenine mononucleotide (FMN) as a photocatalyst to reduce O₂ to H₂O with ethylenediaminetetraacetic acid (EDTA) as electron donor, which well supported OleTJE to react with stearic acid. However, the conversion ratios of lauric acid and myristic acid were not satisfactory [38]. Moreover, redox partner engineering by making OleTJE-reductase fusion protein or constructing alternative OleTJE reaction systems using separate redox partners has been proven as an effective approach for reducing the detrimental effect of H₂O₂. 

**References**

[11, 30] CYP-Sm46; [31] UndA; [32] UndB; [18, 20, 32, 33] CYP152 peroxigenases.
method to reconstitute the decarboxylation activity of OleTJE toward C4–C22 FFA s in the presence of NADPH regeneration system (e.g., formate/formate dehydrogenase, glucose/glucose oxidase) [18, 36, 37]. However, the requirement of expensive materials (FMN or NADPH) in both strategies and the complexity in the latter system consisting of four redox proteins would hinder their further application.

In this study, a novel enzyme cascade system was established to convert TAGs and natural oils into α-olefins in vitro (Fig. 1). Using the low-cost and renewable TAG/oil feedstock, the FFAs hydrolyzed off by lipases were well accepted by the downstream P450 fatty acid decarboxylase OleTJE. The required H2O2 cofactor of OleTJE was continuously supplied in situ by the alditol oxidase AldO using the glycerol substrate derived from TAG hydrolysis, through which the H2O2-induced enzyme inactivation was significantly attenuated and the highest yield of α-olefins reached 0.53 g/L from 1500 μM coconut oil.

Although the enzyme cascade system provides a new paradigm for catalytically efficient and cost-effective biotransformation from TAGs/oils to α-olefins, the highest yield is still far from the stringent cost requirement of industrial production of α-olefins. There remain a number of significant problems to be overcome such as enzyme stability and solubility of hydrophobic substrates/products. It is anticipated that more enzyme engineering, process optimization, and other interdisciplinary approaches are required to make this enzyme cascade more industrially relevant.

**Conclusions**

In this study, we established a novel enzyme cascade independent of exogenous addition of H2O2, which is capable of efficiently converting a range of TAGs/natural oils into α-olefins. This one-pot biocatalytic system consisting of CRL (for TAG hydrolysis to provide FFAs and glycerol), AldO (for in situ H2O2 generation upon glycerol oxidation), and OleTJE (for FFA decarboxylation using H2O2 as cofactor) was able to achieve a 68.5% total alkene yield from 500 μM coconut oil. Using the lyophilized enzymes, ~0.5 g/L of α-olefins were produced from the favorable feedstock coconut oil (1500 μM) upon some reaction optimization. Altogether, the three-enzyme cascade provides a new strategy for producing high value-added α-olefins from low-cost and renewable oils, demonstrating promising application potential.

**Methods**

**Materials**

The strains of *Escherichia coli* DH5α and BL21(DE3) and the plasmid pET28(b) were preserved by our laboratory. All antibiotics and chemicals including TAGs, FFAs, and α-olefins were obtained from Tokyo Chemical Industry (TCI) (Shanghai, China), Solarbio (Beijing, China), Sigma Aldrich (St. Louis, MO, USA), and Thermo Scientific (Shanghai, China). Soybean oil, peanut oil, and olive oil were purchased from local market. Coconut oil and palm oil were obtained from Orifera (Malaysia) and Pythonbio (Guangzhou, China), respectively. The 10 × QuickRun™ Fast Running Buffer and FlexiRun™ premixed gel solution for SDS-PAGE analysis were obtained from MDBio (Xinbei, China). Purification of DNA fragments was performed using a MonPure™ Gel & PCR Clean Kit from Monad (Wuhan, China). Ni–NTA resin used for protein purification was purchased from Sangon Biotech (Shanghai, China). PD-10 desalting columns were supplied by GE Healthcare (Piscataway, NJ, USA). Millipore Amicon Ultra centrifugal filters were bought from Millipore (Billericia, MA, USA).

**Molecular cloning and protein purification**

The gene encoding AldO from *Streptomyces coelicolor* A3(2) (GenBank accession number: NC_003888.3) was codon-optimized and synthesized by Qinglan (Yixing, China), and then cloned into the vector pET28b via the NdeI/Xhol restriction sites for expression of the N-terminal His9-tagged recombinant proteins. The sequences of primers used in this study are listed in Additional file 1: Table S1. All cloned sequences were confirmed by DNA sequencing at Sangon Biotech (Shanghai, China), and then used to transform *E. coli* BL21 (DE3) for protein expression. The plasmid pET28b-oleTJE for recombinant OleTJE expression was constructed by this laboratory previously [18].

The *E. coli* BL21 (DE3) cells carrying a certain recombinant expression vector were grown at 37 °C for 12 h with shaking at 220 rpm and then used as seed cultures to inoculate (1:100 ratio) a modified Terrific Broth medium containing a rare salt solution [18]. Cells were grown at 37 °C for 3–4 h until the optical density at 600 nm (OD600) reached 0.8 to 1.0, to which 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added. For P450 expression, 0.5 mM δ-aminolevulinic acid (5-ALA) and 1 mM thiamine were supplemented. Afterward, the cultivation continued for another 24 h at 18 °C for better protein folding [18, 26]. The cells were harvested (6000 × g, 4 °C, 10 min) and stored at −80 °C for later use.

Purification of the His-tagged OleTJE was performed by following our previous procedure with some minor modifications [26]. Of note, the elution buffer (pH 7.4, 50 mM NaH3PO4, 500 mM NaCl) used in this study was glycerol free because glycerol is a reactant in the enzyme cascade reaction. Purification of the His-tagged AldO was carried out as described by Liu et al. with some modifications
In brief, the cell pellets stored at −80 °C were taken out to thaw at room temperature, then all the following steps were performed at 4 °C. The cells were re-suspended in 50 mL lysis buffer (pH 8.0, 50 mM NaH$_2$PO$_4$, 300 mM NaCl, 10 mM imidazole) through vortexing and then disrupted by ultra-sonication (SIENTZ JY98-IIIIDN, Ningbo; on 5 s/off 5 s for 30 min). Cell-free lysate was obtained by high-speed centrifugation (Eppendorf Centrifuge 5810R, Germany) at 10,000 × g for 1 h at 4 °C, to which 2 mL Ni–NTA resin slurry was added and mixed gently at 4 °C for 2–3 h. The mixture was loaded onto an empty column and washed with about 200 mL wash buffer (pH 8.0, 50 mM NaH$_2$PO$_4$, 300 mM NaCl, 20 mM imidazole) until no protein was detected in flow-through. The His-tagged proteins bound to Ni–NTA resin were eluted with 5–10 mL elution buffer (pH 8.0, 50 mM NaH$_2$PO$_4$, 500 mM NaCl, 250 mM imidazole). The eluents were concentrated with an Amicon Ultra centrifugal filter (30 kDa cutoff) and then buffer-exchanged into storage buffer (pH 8.0, 50 mM NaH$_2$PO$_4$, 10% glycerol; and 5% EtOH as the co-solvent in 200 μL buffer (pH 7.4, 50 mM NaH$_2$PO$_4$, 500 mM NaCl) was carried out at 30 °C for 6 h. The substrates including TAGs and natural oils were emulsified in water containing 2% (w/v) PVA [41] as emulsifier and dispersing stabilizer to prepare oil-in-water emulsions by ultrasonication for better enzyme–substrate contacts.

For preparation of the lipase Lip2, a single colony of Yarrowia lipolytica strain YLY [39] was inoculated into Yeast Extract Peptone Dextrose (YPD) medium for 12 h at 28 °C, and then used as seed cultures to inoculate (1:40 ratio) a fermentation medium [10 g yeast extract, 20 g tryptone, 10 g sucrose, and 6.7 g yeast nitrogen base (without amino acids) per liter], and shaking cultured at 28 °C, 220 rpm for 72 h. The supernatant containing secreted Lip2 was obtained by removing the cells by centrifugation (4 °C, 6000 × g for 10 min). The supernatant was concentrated with an Amicon Ultra centrifugal filter (30 kDa cutoff) and then buffer-exchanged into a Tris–HCl buffer (50 mM, pH = 8.0). The commercial lipases CRL and AOL were purchased from Sigma Aldrich (St. Louis, MO, USA).

**Enzyme concentration determination**

Analysis of the UV–visible spectroscopic properties for OleTJE was carried out as described previously [26]. The P450 OleTJE protein concentration was calculated based on its reduced CO-bound difference spectrum using the reduced differential extinction coefficient $\epsilon_{450-490}$ of 91,000 M$^{-1}$ cm$^{-1}$ [18]. The concentration of AldO was determined at 452 nm with the reported extinction coefficient of 12,500 M$^{-1}$ cm$^{-1}$ [22]. The hydrolysis activity of lipase (U) was determined by the classical basic titration method using the olive oil emulsion. One hydrolysis activity unit (U) of lipase is defined as the amount of enzyme required to produce 1 μM free fatty acid in 1 min under the specific reaction conditions [40].

**In vitro enzymatic assay**

Since P450 OleTJE was characterized as a moderate halophilic protein requiring salt solution to maintain its stability and hence activity [26], all the reactions of OleTJE were carried out in a buffer containing 500 mM NaCl. Although the high concentration of NaCl might negatively affect the activities of lipase and aldol oxidase, the compromise was made for the best performance of OleTJE.

A typical assay containing 1 μM OleTJE, 500/1000 μM lauric acid; 0–5000 μM H$_2$O$_2$, or 5 μM AldO and 0.01–10% v/v glycerol; and 5% EtOH as the co-solvent in 200 μL reaction buffer (pH 7.4, 50 mM NaH$_2$PO$_4$, 500 mM NaCl) was carried out at 30 °C for 6 h. The substrates including TAGs and natural oils were emulsified in water containing 2% (w/v) PVA [41] as emulsifier and dispersing stabilizer to prepare oil-in-water emulsions by ultrasonication for better enzyme–substrate contacts.

For lipase-catalyzed hydrolysis, 5 U of lipase (CRL/AOL/Lip2), 500 μM TAGs (tricaprin, trilaurin, trimyristin, tripalmitin, or tristearin) or natural oils (coconut oil, palm oil, soybean oil, peanut oil, or olive oil) in 200 μL buffer (pH 7.4, 50 mM NaH$_2$PO$_4$, 500 mM NaCl) were mixed and incubated at 30 °C for 6 h.

For the one-pot reactions, 500 μM TAGs/natural oils, 5 U of lipase (CRL/AOL), 3 μM OleTJE, 15 μM AldO, 1.5% v/v glycerol, and 5% EtOH as the co-solvent in 200 μL buffer (pH 7.4, 50 mM NaH$_2$PO$_4$, 500 mM NaCl) were mixed and incubated at 30 °C for 6 h.

All reactions were quenched by adding 20 μL of 10 M HCl, then heptadecanoic acid (C$_{17}$) was added as internal standard and the mixture was extracted by 150 μL ethyl acetate. The organic phase was analyzed using gas chromatography (GC) as described below. For detection of 1-heptene (C$_7$) product generated from coconut oil in the enzyme cascade reactions, 1.5 mL polytetrafluorethylene (PTFE) septum-sealed glass bottles were used for the 200-μL reactions containing 3 μM OleTJE, 15 μM AldO, and 1.5% glycerol. The reactions were incubated at 30 °C for 6 h with shaking at 200 rpm. Then, the reactions were placed at 4 °C for 12 h to stop reactions prior to heating at 40 °C for 20 min for headspace sampling using a gas-tight Hamilton syringe for GC–MS analysis. Different concentrations of the authentic 1-heptene standard incubated under the same conditions of reactions were analyzed using the same GC–MS method to obtain the standard curve.

**Analytical methods**

The hydrocarbon and fatty acid samples were analyzed by the methods modified from Guan et al. [42]. The Agilent 7890B gas chromatograph equipped with a capillary
column HP-5 (Agilent Technologies, Santa Clara, CA, USA; 30 m × 0.25 mm × 0.25 μm) or HP-INNOWAX (Agilent Technologies, Inc, Santa Clara, CA, USA; 30 m × 0.25 mm × 0.25 μm) was used for analysis. The flow rate of helium was set to 1 mL min⁻¹. The oven program was set initially at 40 °C for 5 min, then increased to 280 °C by the rate of 10 °C per min and held for 2 min. The injection temperature was set to 280 °C under splitless injection conditions with 1 μL injection volume. The retention times and signal intensity of FAs and α-alkenes were determined and quantified with corresponding authentic standards (FAs: C₈-C₂₀, linoleic acid, linoleic acid; α-alkenes: C₇-C₁₉) and the internal standard [heptadecanoic acid (C₁₇)]. For GC–MS analyses, the gas chromatography was equipped with an Agilent 5975C MSD single quadrupole mass spectrometer operated under electron ionization mode at 70 eV in the m/z scan range of 50 to 500 Da. The GC–MS analysis used the previous protocol adapted from Rude et al. [11] with the Agilent J&W DB-5MS column (30 m × 0.25 mm × 0.25 μm). Peak identity was determined by comparison of the retention time and fragmentation pattern with those of the authentic standards that were available in the National Institute of Standards and Technology, USA mass spectral database.

Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s13068-020-01684-1.

Additional file 1: Figure S1. SDS-PAGE analysis of the secretory lipase Lip2 from Yarrowia lipolytica (lane A) and protein marker (M). Figure S2. SDS-PAGE analysis of the purified native CRL-OleTJE-AldO (lane A), N-His-CRL-OleTJE-OntJ-AldO (lane B), and protein marker (M). Figure S3. Comparison of α-olefin production activities of freshly purified proteins and the lyophilized enzymes in the CRL-OleTJE-AldO tandem reaction system using 500 μM coconut oil as substrate. Error bars represent standard deviations derived from at least two independent experiments. Figure S4. (a) Total FFA released from 1500 μM coconut oil by three different amounts of CRL; (b) the effect of CRL amount on the α-olefin production from 1500 μM coconut oil by the CRL-OleTJE-AldO system (3 μM OleTJE, 15 μM AldO, and 10% glycerol at 30 °C for 6 h). Error bars represent standard deviations derived from at least two independent experiments. Statistical analysis was performed using a Student’s t test (one-tailed; *P < 0.05, **P < 0.01, ***P < 0.001). Table S1. Primers used in this study. Table S2. Released FFA profiles of different natural oils by lipase CRL. Table S3. Released FFA profiles of different natural oils by lipase AOL. Table S4. Distribution of α-olefins produced from natural oils by the tandem hydrolysis–oxidation—decarboxylation reaction system of CRL-OleTJE/AldO. Table S5. Distribution of α-olefins produced from natural oils by the tandem hydrolysis–oxidation—decarboxylation reaction system of AOL/OleTJE/AldO.

Abbreviations
CYP or P450: Cytochrome P450 enzyme; FFA: Free fatty acid; TAGs: Triglycerides; AldO: Alditoll oxidase; CRL: Lipase from Candido rugose; AOL: Lipase from Aspergillus oryzae; Lip2: Lipase from Yarrowia lipolytica; TTN: Total turnover number; PCR: Polymerase chain reaction; PVA: Polyvinyl alcohol; GC–MS: Gas chromatography–mass spectrometry.

Acknowledgements
We thank Prof. Bingnan Han at Zhejiang Sci-Tech University for his kind gift of the Lip2-overproducing Yarrowia lipolytica strain YLY. We are also grateful to Ms. Cong Wang for her assistance in GC–MS data collection.

Authors’ contributions
YJ, CM, and SL conceived and designed the study. YJ, ZL, and SZ performed the experiments and analyzed the results. YJ, YJZ, HK, ZG, CM, and SL wrote the manuscript. All authors read and approved the final manuscript.

Funding
This work was supported by the National Key Research and Development Program of China (2019YFA0905100), the National Science Foundation of China (31872279, 31770844), the DICP&QIEBET program (Grant: DICP&QIEBET UN201706), and the Dalian National Laboratory for Clean Energy (DNL). Chinese Academy of Sciences.

Availability of supporting data
All data generated or analyzed during this study are included in this manuscript (and its Additional files).

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Received: 11 December 2019 Accepted: 21 February 2020
Published online: 16 March 2020

References
1. Stephanopoulos G. Challenges in engineering microbes for biofuels production. Science. 2007;315:801–4.
2. Shafiee S, Topal E. When will fossil fuel reserves be diminished? Energy Policy. 2009;37:181–9.
3. Zhou YJ, Bujs NA, Sewers V, Nielsen J. Fatty acid-derived biofuels and chemicals production in Saccharomyces cerevisiae. Front Bioeng Biotechnol. 2014;2:32.
4. Herman NA, Zhang W. Enzymes for the conversion of fatty acids into fuels and chemicals. Curr Opin Chem Biol. 2016;35:22–8.
5. Liu K, Li S. Biosynthesis of fatty acid-derived hydrocarbons: perspectives on enzymeology and enzyme engineering. Curr Opin Biotechnol. 2019;62:7–14.
6. Serrano-Ruiz JC, Ramos-Fernández EV, Sepúlveda-Escobar A. From biodiesel and bioethanol to liquid hydrocarbons: new hydrotreatment and advanced microbial technologies. Energy Environ Sci. 2012;5:5638–52.
7. Koçar G, Çiğav N. An overview of biofuels from energy crops: current status and future prospects. Renew Sust Energ Rev. 2013;28:900–16.
8. Schirmer A, Rude MA, Li X, Popova E, Del Cardayre SB. Microbial biosynthesis of alkanes. Science. 2010;329:559–62.
9. Ahktar MK, Turner NJ, Jones PR. Carboxylic acid reductase is a versatile enzyme for the conversion of fatty acids into fuels and chemical commodities. Proc Natl Acad Sci USA. 2013;110:87–92.
17. Du J, Liu L, Guo LZ, Yao XJ, Yang JM. Molecular basis of P450 OleTJE: an
16. Pickl M, Kurakin S, Cantú-Reinhard FG, Schmid P, Pöcheim A, Winkler CK,
12. Belcher J, McLean KJ, Matthews S, Woodward LS, Fisher K, Rigby SE,
11. Rude MA, Baron TS, Brubaker S, Alibhai M, Del Cardayre SB, Schirmer
15. Fang B, Xu H, Liu Y, Qi F, Zhang W, Chen H, Wang C, Wang Y, Yang W, Li S.
13. Grant JL, Hsieh CH, Makris TM. Decarboxylation of fatty acids to
26. Jiang Y, Li Z, Wang C, Zhou YJ, Xu H, Li S. Biochemical characterization of
25. Pignède G, Wang H, Fudalej F, Gaillardin C, Seman M, Nicaud J-M. Charac-
24. Ohnishi K, Yoshida Y, Sekiguchi J. Lipase production of
23. Benjamin S, Pandey A.
22. Heuts DP, van Hellemond EW, Janssen DB, Fraaije MW. Discovery, char-
21. Rui Z, Li X, Zhu X, Liu J, Domigan B, Barr I, Cate JHD, Zhang W. Microbial
20. Chen B, Lee D-Y, Chang MW. Combinatorial metabolic engineering of
19. Yan J, Liu Y, Wang C, Han B, Li S. Assembly of lipase and P450 fatty acid
decarboxylase to terminal alkyls by cytochrome P450 compound I. J Am Chem Soc.
2015;137:4940–3.
18. Amaya JA, Rutland CD, Leschinsky N, Makris TM. A distal loop controls
product release and chemo- and regioselectivity in cytochrome P450
decarboxylases. Biochemistry. 2017;57:544–53.
15. Fang B, Xu H, Liu Y, Qi F, Zhang W, Chen H, Wang C, Wang Y, Yang W, Li S.
Mutagenesis and redox partners analysis of the P450 fatty acid
decarboxylase OleTJE. Sci Rep. 2017;7:44258.
14. Pickl M, Kurakin S, Cantú-Reinhard FG, Schmid P, Pöcheim A, Winkler CK,
Krouhl W, de Visser SP, Faber K. Mechanistic studies of fatty acid activation by
CYP152 peroxygenase reveal unexpected desaturation activity. ACS Catal.
2018;8:9565–77.
12. Du J, Liu L, Guo LZ, Yao XL, Yang JM. Molecular basis of P450 OleTJE: an
investigation of substrate binding mechanism and major pathways. J Compu Mol Des.
2017;31:483–95.
11. Liu Y, Wang C, Yan J, Zhang W, Guan W, Lu X, Li S. Hydrogen peroxide
independent production of α-olefins by OleTJE P450 fatty acid decar-
decarboxylase. Biotechnol Biofuels. 2014;7:28.
13. Yan J, Liu Y, Wang C, Han B, Li S. Assembly of lipase and P450 fatty acid
decarboxylase to constitute a novel biosynthetic pathway for production of
1-alkenes from renewable triglycerides and oils. Biotechnol Biofuels.
2015;8:34.
10. Ray S, Rao PV, Choudhary NV. Poly-o-olefin-based synthetic lubricants: a
short review on various synthetic routes. Lubri Sci. 2012;24:23–44.
9. Rude MA, Baron TS, Brubaker S, Alihba M, Del Cardayre SB, Schirmer A. Terminal olefin (1-alkene) biosynthesis by a novel P450 fatty acid
decarboxylase from Jeotgalicoccus species. Appl Environ Microbiol.
2011;77:1718–27.
8. Belcher J, McLean KJ, Matthews S, Woodward LS, Fisher K, Rigby SE,
Nelson DR, Potts D, Baynham MT, Parker DA, et al. Structure and bio-
chemical properties of the alkene producing cytochrome P450 OleTJE (CYP152L1) from the Jeotgalicoccus sp. 8456. J Biol Chem.
2014;289:6355–50.
7. Grant JL, Hsieh CH, Makris TM. Decarboxylation of fatty acids to
terminal alkyls by cytochrome P450 compound I. J Am Chem Soc.
2015;137:4940–3.
6. Amaya JA, Rutland CD, Leschinsky N, Makris TM. A distal loop controls
product release and chemo- and regioselectivity in cytochrome P450
decarboxylases. Biochemistry. 2017;57:544–53.
5. Fang B, Xu H, Liu Y, Qi F, Zhang W, Chen H, Wang C, Wang Y, Yang W, Li S.
Mutagenesis and redox partners analysis of the P450 fatty acid
decarboxylase OleTJE. Sci Rep. 2017;7:44258.
4. Pickl M, Kurakin S, Cantú-Reinhard FG, Schmid P, Pöcheim A, Winkler CK,
Krouhl W, de Visser SP, Faber K. Mechanistic studies of fatty acid activation by
CYP152 peroxygenase reveal unexpected desaturation activity. ACS Catal.
2018;8:9565–77.
3. Du J, Liu L, Guo LZ, Yao XL, Yang JM. Molecular basis of P450 OleTJE: an
investigation of substrate binding mechanism and major pathways. J Compu Mol Des.
2017;31:483–95.
2. Liu Y, Wang C, Yan J, Zhang W, Guan W, Lu X, Li S. Hydrogen peroxide
independent production of α-olefins by OleTJE P450 fatty acid decar-
decarboxylase. Biotechnol Biofuels. 2014;7:28.
1. Yan J, Liu Y, Wang C, Han B, Li S. Assembly of lipase and P450 fatty acid
decarboxylase to constitute a novel biosynthetic pathway for production of
1-alkenes from renewable triglycerides and oils. Biotechnol Biofuels.
2015;8:34.

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