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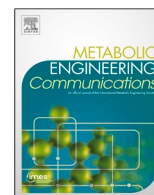

Cell-free metabolic engineering enables selective biotransformation of fatty acids to value-added chemicals

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ABSTRACT

Fatty acid-derived products such as alkanes, fatty aldehydes, and fatty alcohols have many applications in the chemical industry. These products are predominately produced from fossil resources, but their production processes are often not environmentally friendly. While microbes like *Escherichia coli* have been engineered to convert fatty acids to corresponding products, the design and optimization of metabolic pathways in cells for high productivity is challenging due to low mass transfer, heavy metabolic burden, and intermediate/product toxicity. Here, we describe an *E. coli*-based cell-free protein synthesis (CFPS) platform for *in vitro* conversion of long-chain fatty acids to value-added chemicals with product selectivity, which can also avoid the above issues when using microbial production systems. We achieve the selective biotransformation by cell-free expression of different enzymes and the use of different conditions (e.g., light and heating) to drive the biocatalysis toward different final products. Specifically, in response to blue light, cell-free expressed fatty acid photodecarboxylase (CvFAP, a photoenzyme) was able to convert fatty acids to alkanes with approximately 90% conversion. When the expressed enzyme was switched to carboxylic acid reductase (CAR), fatty acids were reduced to corresponding fatty aldehydes, which, however, could be further reduced to fatty alcohols by endogenous reductases in the cell-free system. By using a thermostable CAR and a heating treatment, the endogenous reductases were deactivated and fatty aldehydes could be selectively accumulated (>97% in the product mixture) without over-reduction to alcohols. Overall, our cell-free platform provides a new strategy to convert fatty acids to valuable chemicals with notable properties of operation flexibility, reaction controllability, and product selectivity.

1. Introduction

Environmental issues have attracted more and more attention, which are caused by the development of global industrialization and urbanization. Currently, municipal solid waste disposal has become a critical burden for urban development and kitchen food waste is a major component of municipal waste (Ajay et al., 2021). Each year, about 1.6 billion tons of food waste are generated worldwide, which calls for advanced technologies to recycle and upgrade food waste into valuable products such as energy and materials (Meng et al., 2022). The main compositions of food waste are carbohydrates, proteins, and lipids/oils; their proper disposal and/or recycling play an important role in global sustainable development (Paritosh et al., 2017). Traditional solutions for the municipal waste treatment include landfilling and incineration, easily leading to seriously environmental issues such as air/soil pollution, greenhouse gas emission, and heavy metal leakage (Hassan et al., 2020; Powell et al., 2016; Zhang et al., 2021). The use of food waste disposal units can decrease the amount of food waste; however, this method will aggravate the burden of water consumption and sewerage systems (Iacovidou et al., 2012). Another solution is anaerobic digestion by microorganisms, which not only is environmentally friendly but also can produce bioenergy/biogas (Badgett and Milbrandt, 2021). Yet, bioprocessing equipment and facilities are expensive and complex, which needs elaborate management hindering its wide application. In industry, long-chain hydrocarbon molecules have many applications in fragrances, cosmetics, lubricants, and biofuels, and thus possess great economic benefits (Halfmann et al., 2014; Shi et al., 2018). Since food waste contains a large amount of long-chain fatty acids that can be derived from lipids/oils, using them as feedstocks for valuable product production might be a sustainable approach for the treatment of food waste. For instance, the compositions (%) of lauric acid in palm kernel oil and coconut oil are 47.8 and 46.5, respectively. Palmitic acid

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constitutes 44% of fatty acids in palm oil. In sunflower oil, the composition (%) of stearic acid is 4.5 (Aransiola et al., 2014). While chemical methods have been established for fatty acid conversion, these processes often require harsh reaction conditions and rare-metal catalysts with low efficiency and poor selectivity (Ford et al., 2012; Gomez et al., 2022; Witsuthammakul and Sooknoi, 2016).

In biotechnology, different enzymes have been used to catalyze the bioconversion of fatty acids to value-added products including alkanes, fatty aldehydes, and fatty alcohols with high catalytic efficiency under mild reaction conditions. For example, fatty acid photodecarboxylase (CvFAP) found from the microalga *Chlorella variabilis* NC64A is able to convert fatty acids to alkanes (or alkenes), which can serve as biofuels, through decarboxylation initiated by blue light (Sorigué et al., 2017). When CvFAP is coupled with lipase, triglycerides can also be converted to alkanes via a two-step cascade enzymatic reactions (Huijbers et al., 2018). Another class of enzymes called carboxylic acid reductases (CARs) can catalyze the reduction of fatty acids to corresponding fatty aldehydes (Butler and Kunjapur, 2020; Derrington et al., 2019). Note that CARs need to be post-translationally modified by an auxiliary enzyme phosphopantetheinyl transferase (PPTase) to form active *holo*-CARs (Venkitasubramanian et al., 2007). Previous studies have shown that endogenous enzymes such as alcohol dehydrogenase (ADH) and aldehyde reductase (AHR) in *E. coli* are able to reduce a wide range of aldehydes (e.g., aromatic and aliphatic) to corresponding alcohols (Akhtar et al., 2013; Derrington et al., 2019). As a result, heterologous expression of CARs in *E. coli* generates not only the target products (i.e., aldehydes) but also most often the side products (i.e., alcohols) due to the presence of many endogenous ADH/AHR enzymes. Deletion of specific ADH and/or AHR gene(s) from the host cell might minimize the aldehyde over-reduction. In contrast, if alcohols are final products, overexpression of ADH/AHR enzymes together with CARs is often a reasonable strategy as reported previously (Butler and Kunjapur, 2020; Derrington et al., 2019). However, selective production/accumulation of aldehydes or alcohols using one type of engineered strain is difficult because the requirement of ADH/AHR enzymes is different. It is, therefore, necessary to develop a robust and simple approach for selective transformation of one substrate (e.g., fatty acid) to different target molecules as demanded in a single one-pot reaction.

Recently, cell-free protein synthesis (CFPS) systems have been used for *in vitro* protein production and the construction of biomanufacturing factories (Dudley et al., 2020; Ji et al., 2022; Lim and Kim, 2022; Liu et al., 2020; Rasor et al., 2021; Silverman et al., 2020; Xu et al., 2022). CFPS reaction mixture contains cell lysate, energy, amino acids, and salts, mimicking cell metabolism *in vitro*. By directly adding plasmid(s) to CFPS systems, cell-free production of desired products (e.g., proteins) can be achieved in hours without the use of intact living cells (Silverman et al., 2020). Here, we propose to use the well-developed *E. coli*-based CFPS system for selective biotransformation of food waste (fatty acids) to long-chain hydrocarbons (e.g., alkanes, fatty aldehydes, and fatty alcohols) through controllable cell-free reaction conditions (Fig. 1). Using cell-free system, we show two paradigms of selective bioconversions. One is reaction selectivity based on the added plasmids encoding different enzymes (here are CvFAP and CAR), which means that the same substrate can be converted to different products with different plasmid inputs. The other one is product selectivity in the CAR bioconversion system. By elevating the reaction temperature, the endogenous ADH/AHR enzymes originated from *E. coli* cell lysates can be deactivated so as to produce the intermediate fatty aldehydes catalyzed by CARs. Otherwise, ADH/AHR enzymes in the CFPS system will further reduce fatty aldehydes to fatty alcohols. Looking forward, we anticipate that cell-free biotransformation systems can be used for the rapid synthesis of fatty acid-derived high-value chemicals such as fatty aldehydes/alcohols and alkanes of industrial importance when selective biocatalysis in cells are difficult or not possible.

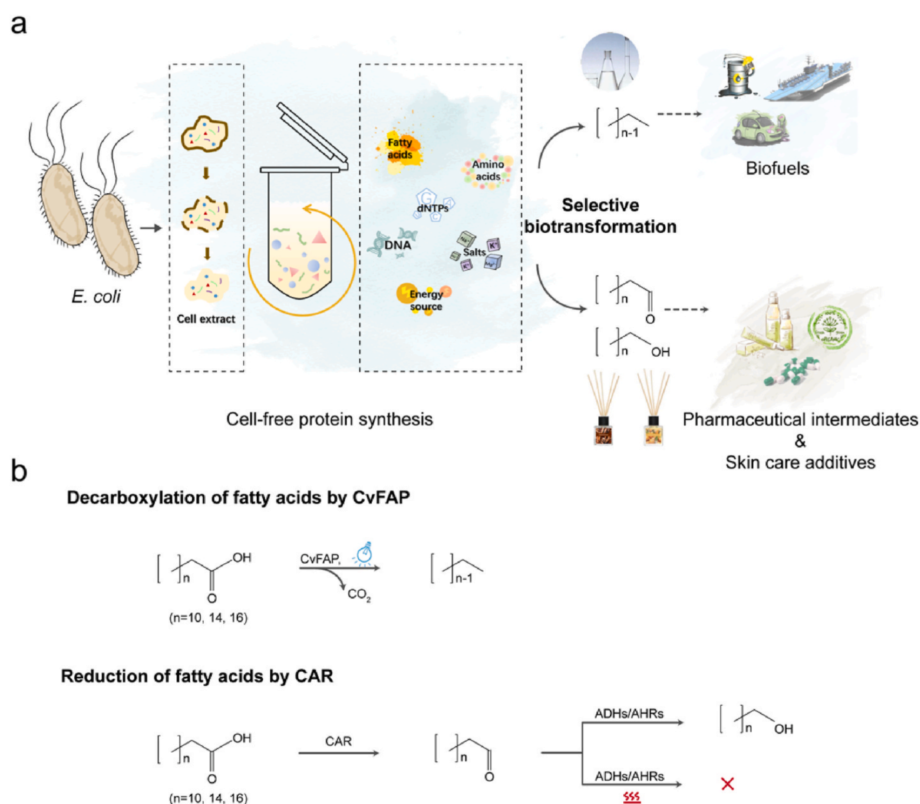

Fig. 1. (**a**) Cell-free selective biotransformation of fatty acids to alkanes and fatty aldehydes/alcohols. Enzymes expressed with cell-free protein synthesis (CFPS) are used to construct *in vitro* metabolic pathways. (**b**) Enzymatic decarboxylation (top) and reduction (bottom) of fatty acids by CvFAP and CAR, respectively. Three fatty acid substrates used in this work are lauric acid (C_{12}), palmitic acid (C_{16}), and stearic acid (C_{18}).

2. Materials and methods

2.1. Chemicals

1-Octanol, lauric acid, lauraldehyde, dodecanol, undecane, palmitic acid, hexadecanal, hexadecanol, pentadecane, stearic acid, octadecanal, octadecanol, and heptadecane were purchased from Aladdin Chemical (Shanghai, China). Antibodies: 6xHis, His-Tag Mouse Monoclonal Antibody (Catalog no. 66005-1-Ig, Proteintech, USA), HRP-Goat Anti-Mouse IgG (H + L) Antibody (Catalog no. SA00001-1, Proteintech, USA). The plasmid miniprep kit was purchased from Sangon Biotech (Shanghai, China).

2.2. Strains and plasmid construction

E. coli DH5α and BL21 Star (DE3) were used for plasmid propagation and cell extract preparation, respectively. The gene of fatty acid photodecarboxylase (CvFAP) from *Chlorella variabilis* NC64A (Sorigué et al., 2017) was synthesized and cloned into pET28a by GENEWIZ (Suzhou, China), generating pET28a-CvFAP. The genes encoding carboxylic acid reductase (NiCAR) from *Nocardia iowensis* (He et al., 2004) and a highly thermostable CAR (AnCAR) generated by ancestral sequence reconstruction (Thomas et al., 2019) were synthesized by GENEWIZ and then cloned into the plasmid pJL1 (Li et al., 2017), yielding pJL1-NiCAR, pJL1-NiCAR_Q283P, and pJL1-AnCAR, respectively. Note that the gene of NiCAR_Q283P was generated by point mutation of NiCAR at the amino acid position of 283 with enhanced catalytic activity (Schwendenwein et al., 2019). The amino acid sequences of CvFAP, NiCAR, NiCAR_Q283P, and AnCAR are shown in Supporting **Information**. To activate CAR *in vitro*, the PPTase Sfp from *Bacillus subtilis* (Quadri et al., 1998) was used for the post-translational phosphopantetheinylation of CARs. The gene *sfp* was cloned into pET15b generating pET15b-Sfp for Sfp expression.

2.3. Expression and purification of Sfp protein

Stocked cells were inoculated from frozen glycerol and grown in the Luria-Bertani (LB) medium at 37 °C and 200 rpm on a shaker. When OD_{600} reached 0.5–0.6, cells were induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), followed by 12 h cultivation at 22 °C for protein expression. Cell pellets were collected by centrifugation at 6000*g* for 10 min and resuspended in lysis buffer (10 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole, pH 7.5). Then, cells were disrupted by the high-pressure French Press. After centrifugation, the supernatant was loaded to a Ni-NTA column (GE Healthcare). The impure proteins were eluted by wash buffer (10 mM Tris-HCl, 500 mM NaCl, 50 mM imidazole, pH 7.5), and then the target proteins were eluted with elution buffer (10 mM Tris-HCl, 500 mM NaCl, 10% glycerol, 250 mM imidazole, pH 7.5). Purified Sfp (see Fig. S1a) was concentrated to 1 mg/mL using an Amicon Ultra filter (Millipore) and stored for subsequent use at 4 °C in storage buffer (10 mM Tris-HCl, 500 mM NaCl, 10% glycerol, pH 7.5).

2.4. Preparation of crude CvFAP solution

The crude CvFAP solution was prepared according to a previous report (Huijbers et al., 2018). Briefly, expression of CvFAP was induced with 0.5 mM IPTG when OD_{600} reached 0.5–0.6, followed by cultivation at 22 °C for 12 h. Afterward, cells were collected by centrifugation at 6000*g* for 10 min and resuspended in lysis buffer (50 mM Tris-HCl, 500 mM NaCl, pH 7.5). Then, cells were disrupted and centrifuged at 12000 g and 4 °C for 30 min. The resulting supernatant was filtered through a 0.45 μm filter, generating the crude CvFAP solution (see Fig. S1b for the expression of CvFAP). The total protein concentration of the cell lysate was determined by Bradford assay (Quick StartTM Bradford, BioRad). The amount of CvFAP in the cell lysate was estimated from the relative intensity of the bands on the gel (the protein band intensity was analyzed by ImageJ).

2.5. Cell extract preparation

E. coli BL21 Star (DE3) cell grown, collection, lysis, and cell extracts were prepared as described previously (Zhuang et al., 2020).

2.6. Cell-free protein synthesis (CFPS) reactions

One standard CFPS reaction (15 μL) consisted of 12 mM magnesium glutamate, 10 mM ammonium glutamate, 130 mM potassium glutamate, 1.2 mM ATP, 0.85 mM each of GTP, UTP, and CTP, 34 μg/mL folinic acid, 170 μg/mL of *E. coli* tRNA mixture, 2 mM each of 20 standard amino acids, 0.33 mM nicotinamide adenine dinucleotide (NAD), 0.27 mM coenzyme A (CoA), 1.5 mM spermidine, 1 mM putrescine, 4 mM sodium oxalate, 33 mM phosphoenolpyruvate (PEP), 13.3 μg/mL plasmid, and 27% (v/v) of cell extract. CFPS reactions were carried out at 30 °C and cell-free expressed proteins were analyzed by Western-blot.

2.7. CvFAP- and CAR-based biotransformation

2.7.1. Decarboxylation of fatty acids by CvFAP

Decarboxylation of fatty acids was carried out with cell-free expressed CvFAP, and the crude CvFAP solution was used as a positive control for comparison. Cell-free expression of CvFAP was initially performed for 6 h. Then, 120 μL of CFPS mixture containing CvFAP was mixed with 10 mM fatty acid and 30% (v/v) DMSO in Tris-HCl buffer (pH 8.5, 100 mM) to a total volume of 500 μL in a transparent glass vial. Afterward, the vial was sealed and exposed to blue LED light with gentle magnetic stirring at 37 °C for the photo-induced decarboxylation of fatty acids. For the control reaction, all conditions were the same as described above except that the CFPS mixture was replaced with the crude CvFAP solution (containing ca. 6 μM of CvFAP).

2.7.2. Reduction of fatty acids by CARs

First, CAR enzymes were expressed in CFPS reactions at 30 °C for 6 h. To activate CARs, the PPTase Sfp was added to the cell-free reaction in two ways: (i) coexpression of Sfp with CARs and (ii) addition of purified Sfp. Then, reduction of fatty acids was carried out by adding substrates (fatty acids with appropriate concentrations) and the cofactor of CARs (2 mM NADPH). The reduction reaction with a total volume of 90 μL was performed for another 10 h with shaking at 150 rpm in a 2-mL microcentrifuge tube.

2.8. Selective biotransformation of fatty acids to fatty aldehydes

To selectively convert fatty acids to fatty aldehydes, the thermostable AnCAR was initially expressed in CFPS at 30 °C for 6 h. Note that purified Sfp was added to the reaction to activate AnCAR. Then, the cell-free reaction mixture was heated at 40, 45, 50, 55, or 60 °C for 5 min each in water bath to deactivate endogenous ADH/AHR enzymes. Afterward, the reaction mixture was placed on ice for 3 min, followed by adding 2 mM of NADPH and substrates (the final concentrations of fatty acids: 0.5, 1, and 2 mM). All reactions with a total volume of 90 μL were performed for another 10 h with shaking at 150 rpm in 2-mL microcentrifuge tubes.

2.9. Analytical methods

All final reaction mixtures were extracted with 2 volume of ethyl acetate (containing 1 mM of 1-octanol as an internal standard). Then, 1 μL of each sample was injected and analyzed by gas chromatography-mass spectrometry (GC-MS) (Trace 1300-ISQ, ThermoFisher Scientific) equipped with a HP-5 capillary column (length 15 m, internal diameter 0.25 mm, film thickness 0.25 μm). Helium was used as the carrier gas at a flow rate of 1.0 mL/min. The retention times of all substrates,

products, and the internal standard (1-octanol) are listed in Table S1. Each product concentration was determined according to a linear standard curve generated with a commercial standard. All measurements were performed in triplicate. The conversion (%) was calculated using the following equation:

$$\text{Conversion } (\%) = C_{product} / C_{substrate} \times 100\%$$

where $C_{product}$ is the final product concentration (mM) and $C_{substrate}$ is the initial substrate concentration (mM) added to cell-free reactions. Note that in the CAR-based reactions, the final product concentration is the sum of the mixed products (fatty aldehydes and fatty alcohols).

3. Results and discussion

3.1. Decarboxylation of fatty acids to alkanes

CvFAP (a photoenzyme) was firstly discovered from a microalga in 2017, which can catalyze the decarboxylation of long-chain fatty acids to aliphatic hydrocarbons in response to blue light (Sorigué et al., 2017). After that, its substrate scope and the enzyme activity have been

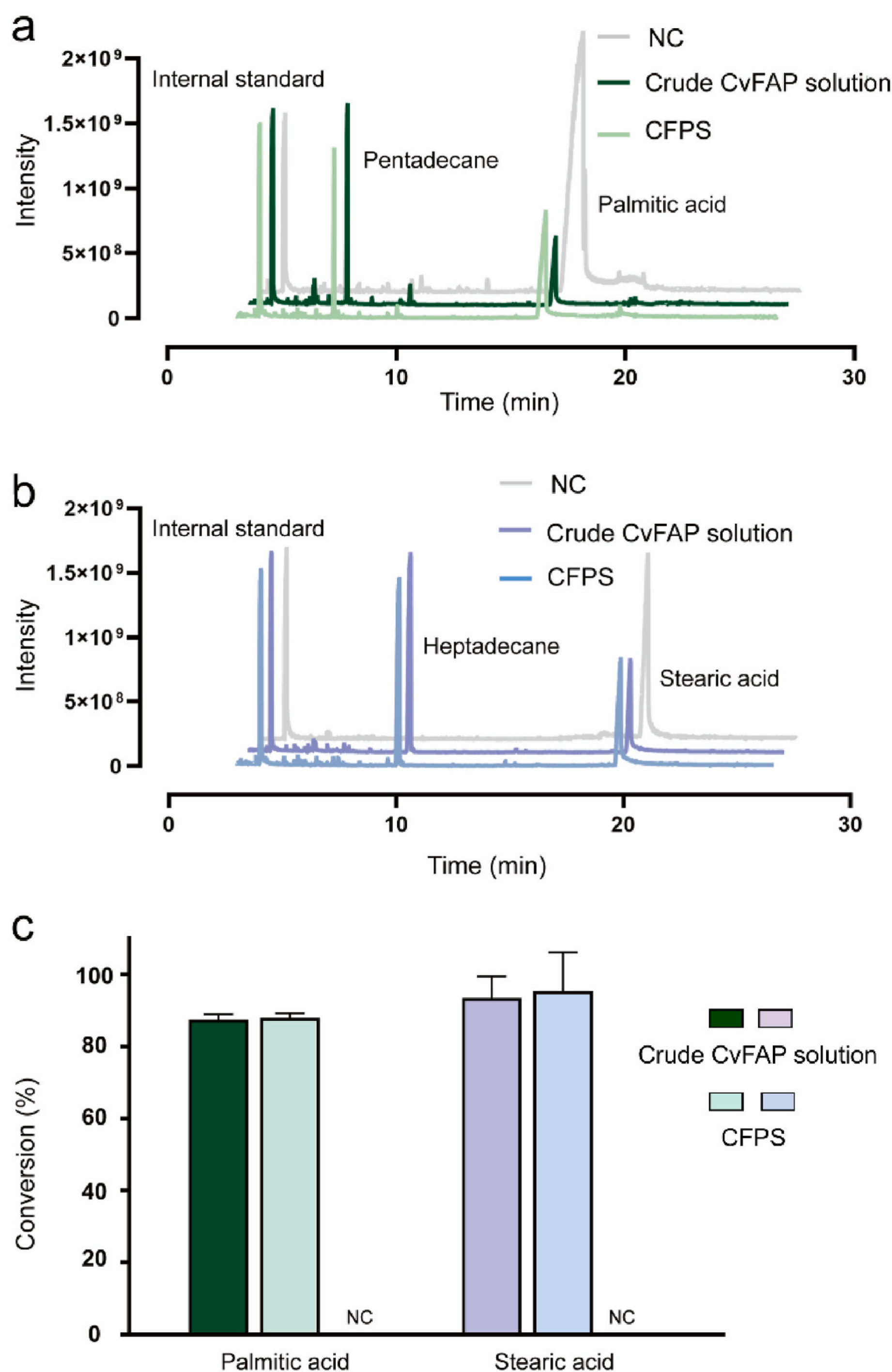

Fig. 2. Decarboxylation of palmitic acid and stearic acid catalyzed by CvFAP. (**a**) GC-MS analysis of palmitic acid and pentadecane. (**b**) GC-MS analysis of stearic acid and heptadecane. (**c**) Substrate conversion (%) with cell-free expressed CvFAP and crude CvFAP enzyme solution. NC, negative control without plasmid in the reaction. Values show means with error bars representing standard deviations (s.d.) of at least three independent experiments.

extensively expanded through enzyme engineering and evolution (Li et al., 2021; Xu et al., 2019; Zeng et al., 2021; Zhang et al., 2019). A previous study reported that the activity of purified CvFAP was lower than that of the crude enzyme solution prepared from the host *E. coli* cells (Huijbers et al., 2018). While the reason remains unclear, it might be due to some unknown, yet necessary enzyme cofactors presented in the crude cell lysate. In this context, crude extract-based CFPS systems could be potential platforms to express CvFAP for efficient enzymatic catalysis without purification. Therefore, here we aim to use an *E. coli*-based CFPS system to express CvFAP that can directly catalyze the decarboxylation of fatty acids *in situ*.

First, cell-free expression of CvFAP was carried out at 30 °C for 6 h. Then, the synthesis of CvFAP was confirmed by Western-blot analysis. The results indicated that CvFAP was successfully expressed with the correct molecular weight (71 kDa), although a small fraction of truncated proteins was formed (Fig. S2a). Having demonstrated the expression of CvFAP in CFPS, we next wanted to test the enzyme activity. For a positive reference, we also expressed CvFAP in *E. coli* (Fig. S1b) and prepared crude CvFAP enzyme solutions for the biocatalysis as reported previously (Huijbers et al., 2018). To this end, we chose three fatty acids with different carbon-chain lengths (i.e., lauric acid, C_{12}; palmitic acid, C_{16}; and stearic acid, C_{18}) as substrates. The catalytic reactions were performed at 37 °C with gentle magnetic stirring under blue light (455–460 nm) illumination for 10 h (Fig. S3). Note that if the reactions were not illuminated with blue light, no products could be detected (Fig. S4). After reaction, the target products were extracted by ethyl acetate (adding 1 mM of 1-octanol as an internal standard) and then all samples were analyzed by GC-MS. The results suggested that all three fatty acids could be converted to their corresponding aliphatic hydrocarbons (alkanes) through decarboxylation by CFPS-expressed CvFAP (Fig. 2a and b, Fig. S5a). We also tested substrate conversions of the three reaction groups over 10 h and observed that the enzymatic reactions basically stopped between 8 and 10 h (Fig. S6). Moreover, we found that our CFPS system could achieve similar final conversions compared to the crude CvFAP enzyme solution-based biocatalysis in each substrate group (Fig. 2c and Fig. S6). The enzyme CvFAP particularly showed a high efficiency in our cell-free system toward palmitic acid (C_{16}, 88% conversion) and stearic acid (C_{18}, 95% conversion) (Fig. 2c). The conversion of lauric acid (C_{12}) was the lowest (13%) among the three tested fatty acids (Fig. S5b). Our finding is in agreement with previous reports that the substrates of C_{16} and C_{18} are more favored by CvFAP than C_{12} (Huijbers et al., 2018; Sorigué et al., 2017). Overall, our results demonstrate that cell-free system is feasible to express the photoenzyme CvFAP with catalytic activity. Since its discovery in 2017, CvFAP has been quickly employed for different enzymatic bioconversions (Ge et al., 2022; Li et al., 2021; Xu et al., 2019; Zeng et al., 2021; Zhang et al., 2019). Therefore, we envision that CFPS will not only can be used for the construction of CvFAP-based metabolic pathway(s) to synthesize various products *in vitro*, but also may serve as a rapid method to engineer, express, and evaluate CvFAP variants if the design-build-test cycles performed in cells are laborious and time-consuming.

3.2. Reduction of fatty acids by CARs

3.2.1. Demonstrating the activity of cell-free expressed CARs

Having demonstrated the ability of using cell-free expressed CvFAP to perform *in situ* biocatalysis, we next sought to express CAR enzymes in CFPS to convert fatty acids to their reductive products. Typically, CARs are large multi-domain enzymes, consisting of an adenylation (A) domain for substrate activation, a thiolation (T) domain for substrate tethering and transferring, and a reductase (R) domain for substrate reduction (Butler and Kunjapur, 2020; Derrington et al., 2019). Nascent *apo*-CARs are not active once freshly expressed and a post-translational modification is required to form functional *holo*-CARs by an auxiliary enzyme PPTase, which can transfer the phosphopantetheine group from coenzyme A (CoA) to a conserved serine residue in the T domain (Venkitasubramanian et al., 2007). Normally, the promiscuous PPTase Sfp from *B. subtilis* (Quadri et al., 1998) is used for post-modification of various CARs. After activation, *holo*-CARs can catalyze the reduction of carboxylic acids to corresponding aldehyde products.

To reconstitute CAR-based bioconversion *in vitro*, we chose three enzymes: NiCAR (He et al., 2004) and its variant NiCAR_Q283P with enhanced catalytic activity (Schwendenwein et al., 2019), and AnCAR with high thermal stability (Thomas et al., 2019). First, cell-free expression of the three CARs were confirmed by Western-blot analysis. The results showed that three CARs (~129 kDa) were expressed with correct molecular weight bands, their expression levels were comparable, and all enzymes were almost completely soluble (see Western-blot in Fig. S2b). Then, CARs were activated by Sfp to form functional (*holo*) enzymes. Given the flexibility of cell-free reactions, Sfp can be provided with purified Sfp or by coexpression of Sfp with CARs (Fig. 3a). To test the activity of CARs, palmitic acid (0.25 mM) was used as a substrate for the evaluation. After cell-free biotransformation, the samples were analyzed by GC-MS and two kinds of products (hexadecanal and hexadecanol) were observed (see Table S1 for their retention time). The results demonstrated that cell-free expressed CARs could be activated by Sfp to convert fatty acids to aldehyde products; however, aldehydes were further reduced to fatty alcohols by endogenous ADH/AHR enzymes. As shown in Fig. 3b, three CARs were active but with different activities. The highest conversion (>40%) was observed in the group of using NiCAR_Q283P, which has been engineered with a higher activity compared to its parental NiCAR (Schwendenwein et al., 2019). Moreover, we found that purified Sfp worked better than the coexpressed Sfp in all three CAR groups. This is similar to previous reports that purified Sfp can be directly added to cell-free systems to activate nonribosomal peptide synthetases (Goering et al., 2017; Ji et al., 2022). Thus, purified Sfp was chosen to activate CARs during the following investigation and optimization.

3.2.2. Evaluation of substrate concentration on product formation

Since the conversion (%) based on 0.25 mM of substrate (palmitic acid) was low (Fig. 3b), we next wanted to see if the product titer could be increased by adding higher substrate concentrations. Note that a previous optimized CFPS system is used in this work (Jewett and Swartz, 2004; Kwon and Jewett, 2015), cell-free expression of CAR enzymes is not further optimized and their expression levels are comparable (Fig. S2b, CFPS at 30 °C). Thus, we mainly focus on the effect of substrate concentration rather than optimization of enzyme expression/-concentration for the biotransformation. To do this, we selected three fatty acid substrates (i.e., lauric acid, palmitic acid, and stearic acid) and increased their concentrations in cell-free reactions from 0.25 to 2 mM. Meanwhile, three CAR enzymes were tested for comparison. All cell-free reactions were carried out at 30 °C for a total of 16 h, including an initial 6 h for enzyme expression and another 10 h for subsequent biotransformation. Due to the presence of ADH/AHR enzymes in cell-free reactions, partial fatty aldehydes can be further reduced to fatty alcohols. Thus, the concentrations of the mixed products were determined together to calculate the substrate conversion (%). The results of palmitic acid bioconversion with NiCAR, NiCAR_Q283P, and AnCAR are shown in Fig. 4. Clearly, NiCAR_Q283P performed the best and yielded the highest conversion (~47%) at the lowest substrate concentration of 0.25 mM as compared to the other two enzymes (NiCAR and AnCAR). When the substrate concentration was doubled to 0.5 mM, the total titers of the mixed aldehyde and alcohol products (i.e., hexadecanal and hexadecanol) were also increased by nearly 2 times in all three CAR reaction groups. Further increases of substrate concentrations (>1 mM) did not improve the product titers, but reduced the total conversions in each group. This is probably due to the fact that the amount of enzyme expressed in CFPS is not changed and their ability for biocatalysis cannot be further improved regardless of increasing substrate concentration, thus leading to a lower conversion. Similar results were also observed for

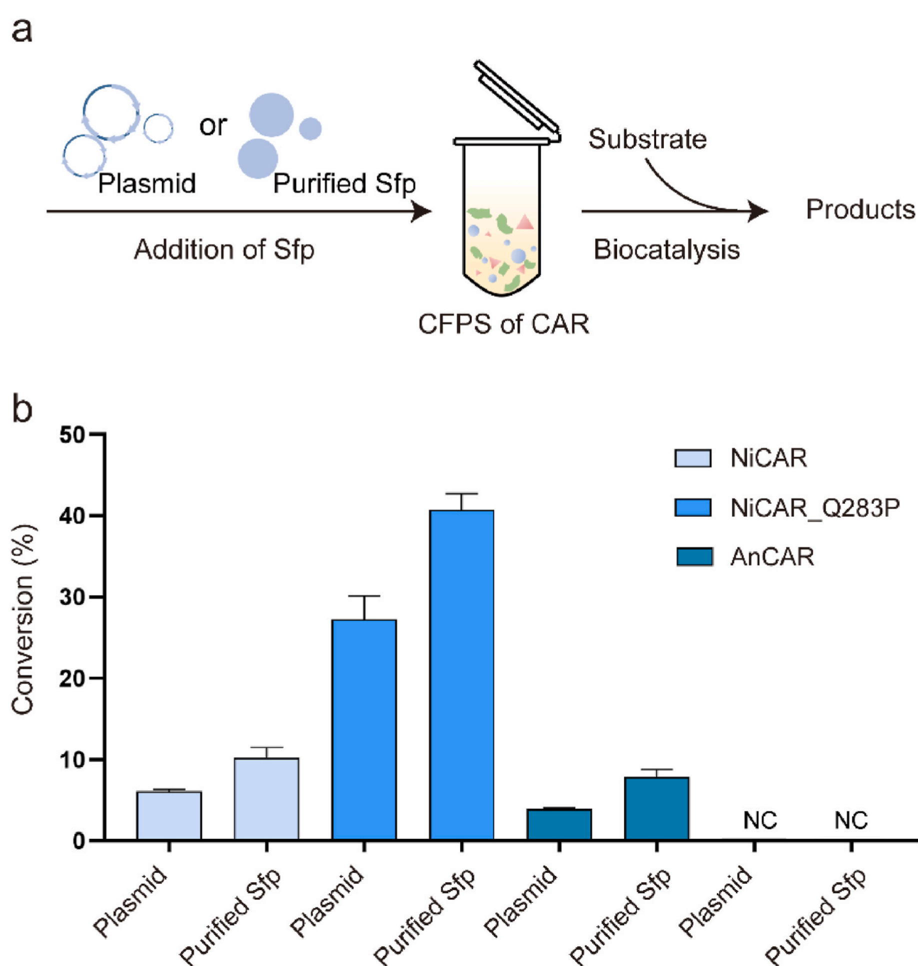

Fig. 3. Reduction of palmitic acid catalyzed by CAR. (**a**) Cell-free expression and activation of CAR for biocatalysis. (**b**) Effect of three CAR enzymes and Sfp (coexpressed or purified Sfp) on the substrate conversion (%). NC, negative control without plasmid in the reaction. Values show means with error bars representing standard deviations (s.d.) of at least three independent experiments.

the other two substrates lauric acid and stearic acid (Figs. S7 and S8). By considering both product titer and substrate conversion, we finally chose the substrate concentration of 0.5 mM for all bioconversion experiments in our following studies.

3.2.3. *Enhancing the substrate conversion*

While cell-free expressed CAR enzymes were active toward three fatty acids (Fig. 4, Figs. S7 and S8), the overall conversion (%) of all tested substrates were relatively low. We next tried to test if an elevated reaction temperature could help improve the bioconversion. To this end, we ran cell-free reactions with different temperatures. The whole reaction process basically consisted of two stages: cell-free expression of CAR for 6 h and biocatalysis for product formation for another 10 h. For these two stages, we used four different temperature combinations, which are (i) 30 °C for both stages, (ii) 37 °C for both stages, (iii) 30 °C for CAR expression + 37 °C for bioconversion, and (iv) 37 °C for CAR expression + 30 °C for bioconversion. Note that in all cases, when the first reaction stage finished, the substrate was then added to the reaction mixture to start the second biocatalysis stage.

The results indicated that different reaction temperature combinations did notably impact substrate conversions among all enzyme/substrate groups (Fig. 5, Figs. S9 and S10). The substrate palmitic acid was taken as an example (Fig. 5). In each enzyme group, the highest conversion (%) was achieved with the same temperature combination, which is 30 °C for CAR expression and 37 °C for bioconversion. Remarkably, the conversion reached 92.7% under this condition (i.e., 30 °C + 37 °C) using the enzyme NiCAR_Q283P, which is > 2 times higher than that of the reaction with 30 °C for both stages (Fig. 5b). However, when both reaction stages were performed at 37 °C, the conversion was sharply reduced to 4.8% (Fig. 5b). Moreover, the substrate conversions from the groups of 37 °C + 30 °C were also as low as the groups of 37 °C + 37 °C (Fig. 5). This is because the optimal temperature of the *E. coli* CFPS system is 30 °C and a high CFPS temperature at 37 °C leads to a low-level expression of CAR enzymes (see Fig. S2b for the comparison of CARs expression at 30 °C and 37 °C). As a result, under the best temperature combination, 30 °C can first support the expression of sufficient CAR and then cell-free expressed enzymes can catalyze the maximum conversion of the substrate at 37 °C, which is likely due to the fact that a higher temperature generally makes the enzymatic reactions going faster. Using the other two substrates lauric acid and stearic acid, we also observed similar trends of the total conversions under the four different reaction temperature combinations (Figs. S9 and S10).

3.2.4. *Selective production of fatty aldehyde through thermal regulation*

Like cells, crude extract-based cell-free systems also contain endogenous ADH/AHR enzymes, leading to the over-reduction of fatty acids to alcohols. While it is challenging to control the selective transformation of fatty acids to aldehyde products *in vivo*, we propose that using cell-free systems might be a promising strategy to solve this issue. To address this opportunity, we next attempted to use a thermal regulation approach to deactivate ADHs/AHRs by heating to accumulate aldehydes rather than alcohol by-products in our cell-free reactions (Fig. 6a). To achieve the goal, we selected the thermostable AnCAR as a biocatalyst

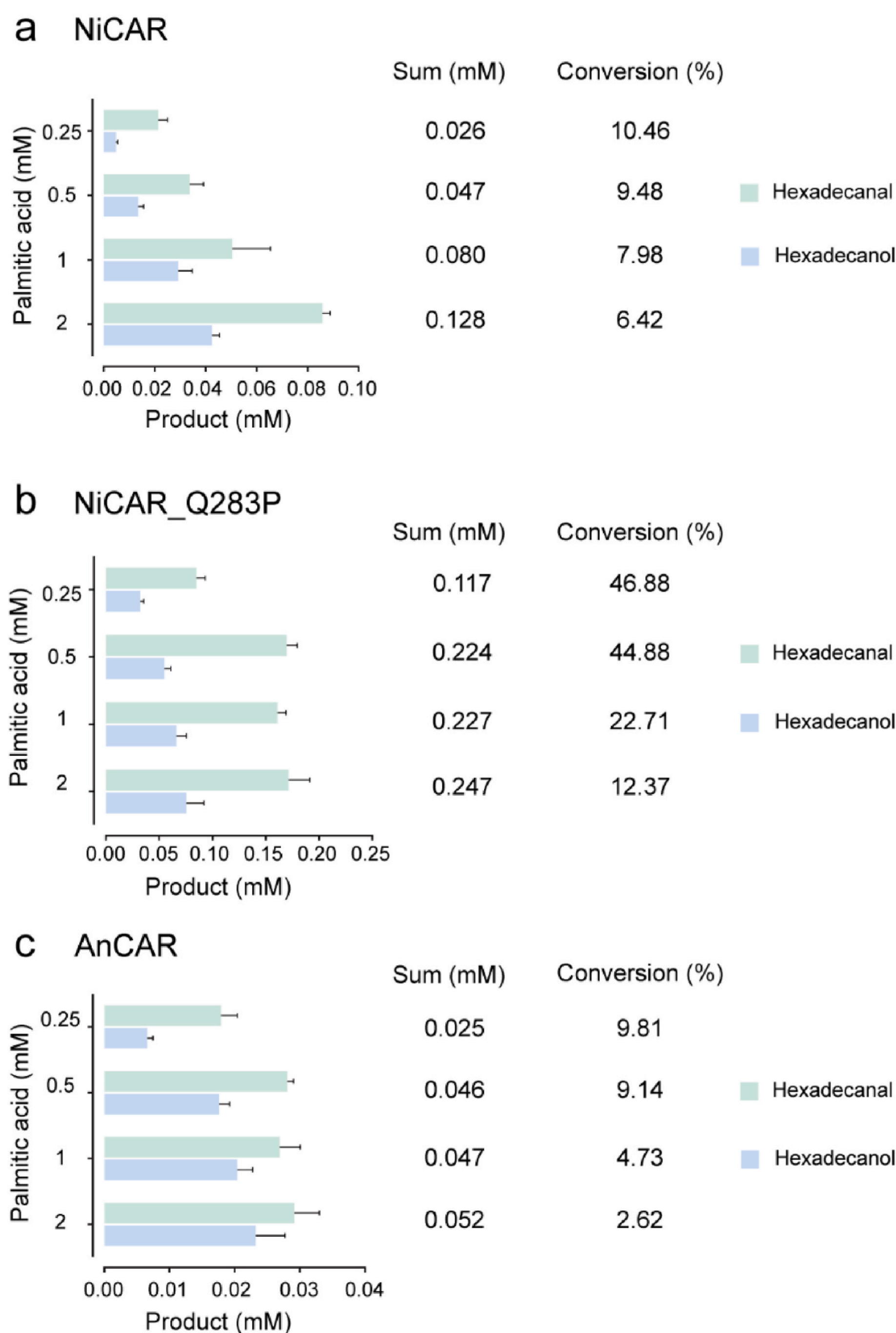

Fig. 4. Effect of palmitic acid concentration on product formation catalyzed by (**a**) NiCAR, (**b**) NiCAR_Q283P, and (**c**) AnCAR, respectively. Final concentrations of the residual palmitic acid in each reaction group are summarized in Table S2. Values show means with error bars representing standard deviations (s.d.) of at least three independent experiments.

(Thomas et al., 2019). After AnCAR was expressed, cell-free reactions were heated and then fatty acid substrates were added to the heat-treated mixtures to synthesize fatty aldehydes catalyzed by AnCAR. Note that the experiments with NiCAR and NiCAR_Q283P were also performed for comparison. To heat cell-free reaction mixtures, we incubated the reaction tubes in water bath for 5 min at temperatures ranging from 40 to 60 °C. Meanwhile, control experiments by heating at 30 °C were performed as well. Afterward, all biocatalysis reactions were carried out at 37 °C for 10 h, followed by detection of fatty aldehydes and corresponding alcohols using GC-MS.

Overall, we found that as the heating temperature increased, the proportion (%) of fatty aldehydes in the product mixture (aldehydes and alcohols) significantly increased up to >90% (Fig. 6b, c, and d). It is clear that the thermostable AnCAR is more tolerant to heating than the other two enzymes (NiCAR and NiCAR_Q283P). When the reaction was heated over 50 °C, NiCAR and NiCAR_Q283P lost most of their activity. After heating at 50 °C, the product titers in the groups of NiCAR and NiCAR_Q283P significantly decreased by 80.5% and 83.3%, respectively, as compared to their control group (i.e., heating at 30 °C for 5 min) (Fig. 6b and c). By contrast, AnCAR could retain approximately half of its original activity after heating above 50 °C (Fig. 6d). In particular, when the reaction was heated at 55 °C, the proportion of the target product hexadecanal in the final product mixture reached 97.3%, which is nearly 2 times higher than that of the control group (50.6%) with

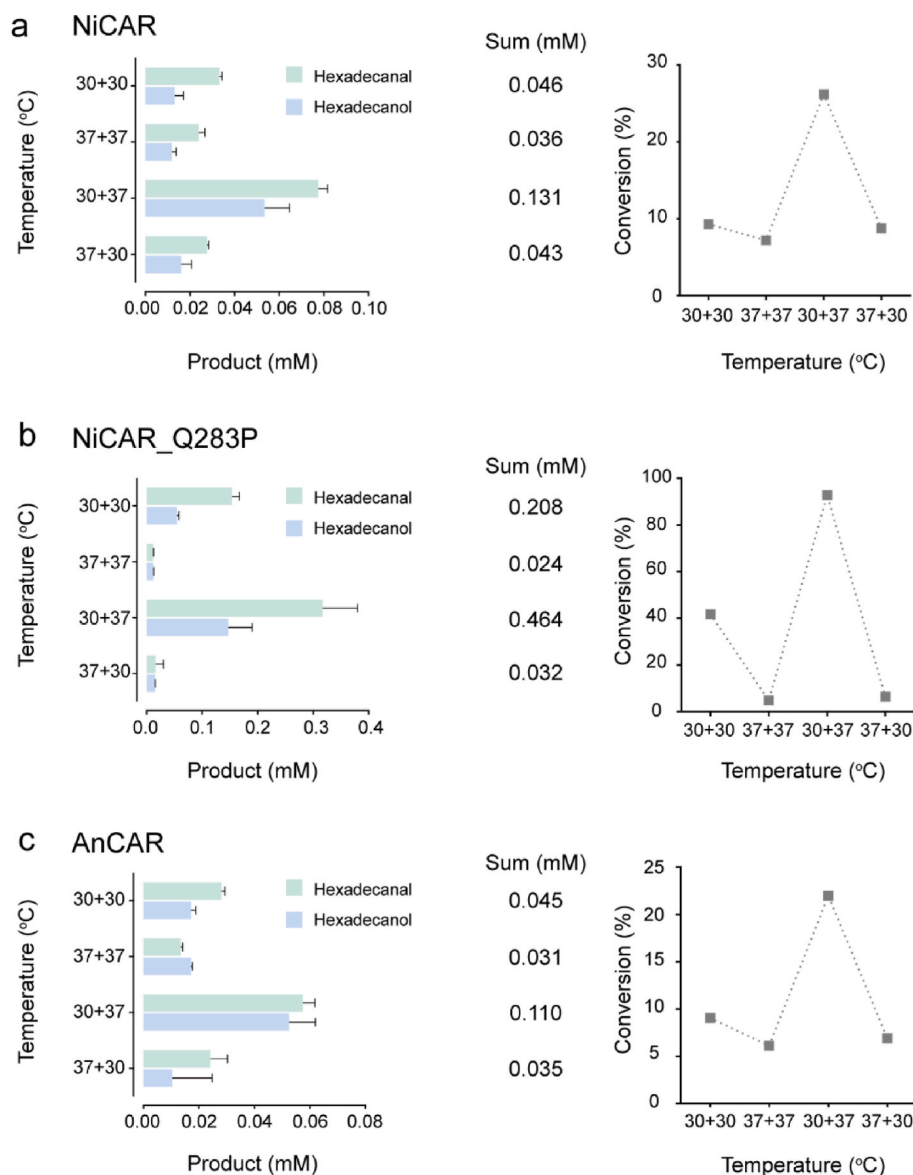

Fig. 5. Evaluation of different reaction temperature combinations on the conversion of palmitic acid. (**a**) NiCAR. (**b**) NiCAR_Q283P. (**c**) AnCAR. Values show means with error bars representing standard deviations (s.d.) of at least three independent experiments.

heating at 30 °C (Fig. 6d). In addition to the substrate palmitic acid, we also observed similar results that AnCAR could catalyze lauric acid and stearic acid to lauraldehyde and octadecanal with a purity of 92.8% and 97.1%, respectively, after heat treatment at 55 °C (Figs. S11 and S12). This is significant because we can selectively accumulate fatty aldehydes by deactivating thermal sensitive ADH/AHR enzymes with a simple heating strategy. However, we noticed that the final concentrations of hexadecanal maintained relatively stable in the reactions rather than further increased as hexadecanol decreased. Although AnCAR was reported as a thermostable enzyme, it lost its activity by 50% after heating at around 65 °C (Thomas et al., 2019). That means high temperatures can still inactivate AnCAR. Here, after heating at 50–60 °C, the overall catalytic ability of AnCAR in the CFPS system was probably reduced to a similar level, which could only catalyze the formation of ~50% product relative to the control (Fig. 6d). As a result, the accumulation of fatty aldehydes was not further enhanced even if the downstream reaction catalyzed by ADHs/AHRs was blocked. On the other hand, while it is not performed in the current work, we believe that one could improve the proportion (%) of fatty alcohols in the final product mixture by overexpression of ADHs/AHRs in the cell-free system, which can further drive the complete reduction of fatty aldehydes to corresponding alcohols. Therefore, our cell-free system enables easy product selectivity, which will provide a robust and flexible platform for selective biotransformation when cell-based *in vivo* systems remain difficult or not amenable.

4. Conclusions

In this work, we demonstrated the application of CFPS-based *in vitro* systems for selective conversion of long-chain (C_{12}–C_{18}) fatty acids to corresponding alkanes, aldehydes, and alcohols, which can be often used as fuels and commodity chemicals. Due to the open nature of cell-free systems, the same substrate can be selectively converted to different target products through the rational expression of related enzymes. First, a photoenzyme CvFAP was successfully expressed *in vitro* that enabled the decarboxylation of fatty acids to form alkanes under blue light. In particular, the conversion of palmitic acid and stearic acid in the cell-free systems reached 88% and 95%, respectively. Second, three CAR enzymes were separately expressed *in vitro* for the reduction of fatty acids to form fatty aldehydes, which can be further reduced to

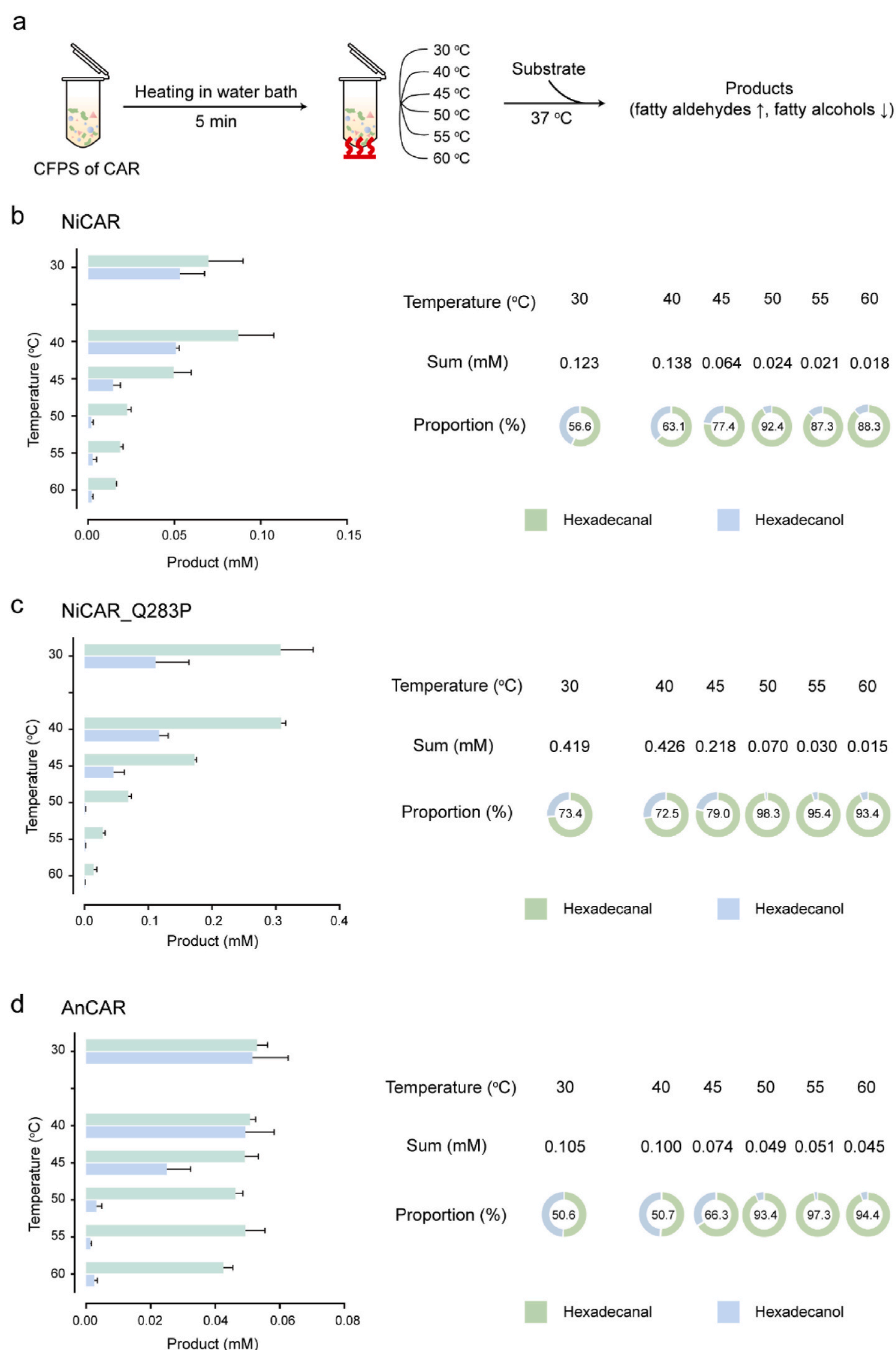

Fig. 6. Selective biotransformation of palmitic acid to hexadecanal. (**a**) Schematic diagram of cell-free selective bioconversion by thermal regulation. Accumulation of hexadecanal in cell-free reactions catalyzed by (**b**) NiCAR, (**c**) NiCAR_Q283P, and (**d**) AnCAR after heating deactivation of endogenous ADH/AHR enzymes. Values show means with error bars representing standard deviations (s.d.) of at least three independent experiments.

corresponding alcohols by endogenous ADH/AHR enzymes derived from *E. coli* cell extracts. In this CAR-based biotransformation system, we were especially able to selectively produce aldehydes and/or alcohols in one pot by choosing suitable CAR enzymes. For example, using a thermostable AnCAR, the proportion of fatty aldehydes such as hexadecanal and octadecanal could be accumulated more than 97% in the product mixture by heating deactivation of ADHs/AHRs. Since the activity of cell-free expressed CvFAP and CARs has been demonstrated *in vitro*, we expect that CFPS will be a promising and complementary approach for rapid expression and screening of improved enzymes from the engineered enzyme libraries, which were only carried out using *in vivo* expression systems as reported previously (Kramer et al., 2020; Li et al., 2021).

In summary, our cell-free platform has several key features. First, cell-free reaction is fast. It requires only hours to obtain target products, whereas a few days or weeks might be needed to grow cells for product formation *in vivo*. Second, the use of cell-free systems allows for fine tuning of reaction conditions and easy optimization, making the whole platform more flexible and productive. Lastly but most importantly, our cell-free strategy enables one-pot selective biotransformation by just adding different plasmids to construct relevant metabolic pathways *in vitro*. However, for cell-based production, laborious steps are needed to

engineer strains and often one strain can only be used to produce one product without the property of selectivity as showcased with our cell-free systems. Taken together, our results highlight the flexibility of cell-free system for tunable and selective biotransformation with remarkable bioconversion efficiency. Looking forward, we envision that future efforts will continue to expand the types of biotransformation pathways and thus the products that can be reconstituted and synthesized *in vitro*.

Author contribution statement

Yushi Liu: Conceptualization, Investigation, Methodology, Visualization, Writing – original draft. **Wan-Qiu Liu:** Methodology, Project administration. **Shuhui Huang:** Methodology, Validation. **Huiling Xu:** Methodology, Formal analysis. **Haofan Lu:** Investigation. **Changzhu Wu:** Conceptualization, Writing – review & editing. **Jian Li:** Conceptualization, Supervision, Funding acquisition, Resources, Writing – review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mec.2022.e00217.

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