Original Research Article

Engineering caveolin-mediated endocytosis in *Saccharomyces cerevisiae*

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**A R T I C L E I N F O**

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**A B S T R A C T**

As a potential substitute for fatty acids, common low-cost oils could be used to produce acetyl-CoA derivatives, which meet the needs of low-cost industrial production. However, oils are hydrophobic macromolecules and cannot be directly transported into cells. In this study, caveolin was expressed in *Saccharomyces cerevisiae* to absorb exogenous oils. The expression of caveolin fused with green fluorescent protein showed that caveolin mediated the formation of microvesicles in *S. cerevisiae* and the addition of 5,6-carboxyfluorescein showed that caveola had the ability to transport exogenous substances into cells. The intracellular and extracellular triacylglycerol levels were then detected after the addition of soybean oil pre-stained with Nile Red, which proved that caveola had the ability to absorb the exogenous oils. Lastly, caveolin for oils absorption and lipase from *Bacillus pumilus* for oil hydrolysis were co-expressed in the naringenin-producing *Saccharomyces cerevisiae* strain, resulting in naringenin production increasing from 222 mg/g DCW (dry cell weight) (231 mg/L) to 269 mg/g DCW (241 mg/L). These results suggested that the caveolin-mediated transporter independent oil transport system would provide a promising strategy for the transport of hydrophobic substrates.

**1. Introduction**

High value-added nature products with a variety of chemical and biological functions can be produced by engineered microorganisms from plant oils and fatty acids [1-3]. The addition of fatty acid precursors was one of the simplest strategies to enhance fatty acid \(\beta\)-oxidation and promote the biosynthesis of acetyl-CoA derivatives. Zhang et al. demonstrated that the addition of fatty acids and the regulation of \(\beta\)-oxidation could improve the level of acetyl-CoA and promote the biosynthesis of naringenin [4]. The addition of fatty acids had many limitations such as toxicity to cells, destruction of cell membranes and reduction of intracellular pH [5-7] and the high price of the fatty acids was not suitable for industrial production. Some oils have a wide source and lower price compared with fatty acids, which meets the industrialization needs of low-cost production, they don’t disturb the pH of the medium and destroy the cell membrane and will produce free fatty acids by hydrolysis by intracellular lipase after being transported into the cell. Therefore oils are expected to replace fatty acids as precursors to promote the efficient biosynthesis of acetyl-CoA derivatives.

Because of hydrophilic lipopolysaccharides on the cell membrane, the transport of larger hydrophobic substances into cells is severely limited [8,9]. As common hydrophobic substances, fatty acids mainly depend on fatty acid transporters into cells and FadL, a fatty acid transporter located in the outer membrane of *Escherichia coli*, was expressed to improve the fatty acid biotransformation [10]. In addition, the alkane transporter AlkL derived from *Pseudomonas putida* Gpo 1 could transport aliphatic alkanes C7 to C16 and fatty acid methyl esters into cells [11,12]. However, fatty acid transport requires transporters across the inner membrane through passive diffusion, which leads to a decrease in cell viability [13]. Only the transporter with the best expression level has high transport capacity [13] and this capacity limits the intracellular transport rate of fatty acids and other hydrophobic substances. As a potential substitute for fatty acids, if soybean oil is transported into cells by similar fatty acid transporters or hydrolyzed into fatty acids extracellularly then transported into cells, it would have the same disadvantages of fatty acid transport mentioned above. Therefore, mining an independent-transporters oil transport system, which can directly transport oils into cells, would avoid the problems caused by fatty acid addition and transport.

Caveolae, formed by caveolin encoding by CAV1, are membrane...
budding structures found in many vertebrate cells. One of the important functions of caveolae is to form membrane bending and endocytic vesicles, which play important roles in endocytosis, tumorigenesis and pathogen uptake [14]. The target substance is encapsulated in the caveolae to trigger the activation of the signaling cascade, which creates the fusion and internalization of the caveolae and the release of the encapsulated substance [15]. Based on the function of endocytosis and release, caveolae had been used to engineer microorganisms. Shin et al. expressed caveolin in E. coli, making the strain more tolerant to toxic fatty acids and causing better growth ability, so that the biotransformation of the engineered strain from ricinoleic acid to (Z)-11-(heptanoyloxy) undec-9-enoic acid was 1.6 times higher than that of the control strain [13]. These results showed that caveolae-forming cells can rapidly transport fatty acids into cells, alleviate fatty acid-induced cytotoxicity, enhance the supply of acetyl-CoA and promote the biosynthesis of target products. This provided a potential strategy for the intracellular transport of other large hydrophobic substances.

In this study, the endocytosis function of caveolae was applied to S. cerevisiae to achieve the endocytosis of soybean oil, indicating that caveolae were the ideal hydrophobic substrate transport system. The caveolae structure in S. cerevisiae was first explored by the fusion expression of caveolin and green fluorescent protein (GFP). The ability of caveolae to transport exogenous substances was then verified with 5,6-carboxyfluorescein addition, indicating that the expression of CAVI could enhance the ability to endocytose exogenous substances. Soybean oil pre-stained with Nile Red was added to the medium. The result showed that the expression of CAVI could enhance the absorption of oils. Oils can be hydrolyzed to free fatty acids as the substrates of fatty acid β-oxidation by intracellular lipases, thus could promote acetyl-CoA biosynthesis by enhancement of fatty acid β-oxidation. Because the biosynthesis of one molecule of naringenin requires three molecules of acetyl-CoA and the low level of acetyl-CoA in cells limited the efficient biosynthesis of naringenin [16], the naringenin-producing Saccharomyces cerevisiae strain was used as the chassis cell to verify the effect of caveolin-enhanced oil absorption on fatty acid β-oxidation and biosynthesis of naringenin. After expressing CAVI and BP (lipase from Bacillus pumilus) in the strain, the production of naringenin was significantly increased. The results suggested that the co-expression of CAVI and lipase could enhance the absorption and hydrolysis of oil and promote the biosynthesis of naringenin in S. cerevisiae.

2. Materials and methods

2.1. Strains and plasmids

The engineered strain ZLHB04-4 (MATa, urs3-52, trp1-289, leu2-3,112, his3Δ1, MAL2-8, SUC2, gal80::TRP1, pdc5::pGAL7-FJTAL, ar0 10::pGAL1-ARO4Δ4br-pGAL10-ARO7Δ7r-G418 and rDNA::pGAL10-CHS-PA5D5-CHI-paro7-4CL-p1DH3-FJTAL) [17] generated from S. cerevisiae CEN. PK2-1D was used as the host for pathway engineering. The engineered strain C800 derived from S. cerevisiae CEN. PK2-1D (gal80::KanMX) was used as the host for functional identification of CAVI and E. coli JM109 was used for plasmid construction and storage. Plasmid pRS423 with HIS3 selection was used to express target genes.

2.2. Strains and plasmids construction

The information of plasmids and primers used in this study are described in Table S1 and Table S2. Fragments of the TGL1 and TGL3 genes and promoter PCAV were amplified from the genomic DNA of S. cerevisiae S288c [18]. The DNA fragments of lipase from Bacillus pumilus (GenBank: JX168355) and human caveolin (GenBank: 403,908) were synthesized by Sangon Biotech Co., Ltd (Shanghai, China) after codon optimization. These sequences are shown in Table S3. The plasmids were constructed with a seamless cloning kit and all assembled plasmids were confirmed by Sanger sequencing before yeast transformation. Yeast transformations were conducted using the lithium acetate method [19,20] and the engineered recombinant strains are shown in Table 1.

2.3. Growth medium and shake-flask fermentation

The E. coli was cultured in Luria broth or agar plates and ampicillin at 100 μg/mL was added when required. The engineered S. cerevisiae strains were grown on synthetic dropout medium containing 1.74 g/L yeast nitrogen base (YNB) without amino acids (Sangon Biotech Co., Ltd), 20 g/L glucose, 5 g/L (NH4)2SO4 and 20 g/L agar was added when required, supplemented with appropriate amino acids including uracil 50 mg/L, histidine 50 mg/L, tryptophan 50 mg/L and leucine 50 mg/L depending on the auxotrophy of the strains. The shake flask cultures wereYPD medium prepared with 20 g/L glucose, 10 g/L yeast extract, 20 g/L peptone or YPDSO medium prepared with 20 g/L glucose, 10 g/L yeast extract, 20 g/L peptone, 1% [v/v] soybean oil and 2 g/L Tween 80.

For shake flask fermentation, the recombinant yeast was precultured in 5 mL of synthetic dropout medium on a shaker (Zhichu, Shanghai, China) (30 °C, 220 rpm). After 20 h growth, 300 μL of preculture was inoculated into 250 mL shake flask with 30 mL YPD or YPDSO medium for 72 h at 30 °C with shaking at 220 rpm. A microplate reader (synergyH1, Bio-Soar, Nanjing, China) was used to measure the cell density. All experiments were performed with biological replicates.

2.4. Analysis of the structure and transportation of caveolae

For analysis of structure, the engineered strains Z01, Z10, Z11, Z12 and Z13 were precultured in 5 mL of YNB medium for 20 h. Precultured cells were inoculated into YNB medium and cultured for 72 h. The cells were harvested and washed four times with cold phosphate buffered saline method [19,20] and the engineered recombinant strains are shown in Table 1.

| Description | Source |
|-------------|--------|
| CEN.PK2-1D, MAL2-8, SUC2, gal80::TRP1, pdc5::pGAL7-FJTAL, ar0 10::pGAL1-ARO4Δ4br-pGAL10-ARO7Δ7r-G418 and rDNA::pGAL10-CHS-PA5D5-CHI-paro7-4CL-p1DH3-FJTAL | [18] |
| L20 ZLHB04-4 pRS423-PgAL-CAV1-PgAV | This study |
| L21 ZLHB04-4 pRS423-PgAL-CAV1-PgAV-TGLI | This study |
| C800 | gal80::KanMX | [45] |
| Z01 Z800 pRS423-PgAL-CAV1-PgAV | This study |
| Z04 Z800 pRS423-PgAL-GFP-PgAV | This study |
| Z10 Z800 pRS423-PgAL-CAV1-GFP-PgAV | This study |
| Z11 Z800 pRS423-PgAL-CAV1-GFP-PgAV | This study |
| Z12 Z800 pRS423-PgAL-CAV1-GFP-PgAV | This study |
| Z13 Z800 pRS423-PgAL-GFP-PgAL-GFP-PgAL-CAV1-PgAV | This study |
saline (PBS) buffer and were resuspended in PBS buffer, then the cells were observed using fluorescence microscopy (Nikon, Tokyo, Japan).

To analyze fluorescence dye transportation, the dye transport method was performed according to a previous method [14,21]. The control strain C800 and the engineered strain Z01 were precultured in 5 mL of YNB medium for 20 h. Precultured cells were inoculated into YNB medium and cultured for 16 h then the strains were cultured for an additional 6 h after the addition of 10 mM 5,6-carboxyfluorescein. Cells were harvested and washed four times with cold PBS buffer until the fluorescent intensity of the supernatant reached blank level, then resuspended in PBS buffer and observed using laser confocal microscopy (Leica stellaris 5, Germany).

For analysis of hydrophobic substance transportation, the control strain C800 and engineered strain Z01 were precultured in 5 mL of YNB medium for 20 h. Precultured cells were inoculated into YPDSO medium pre-stained with Nile Red for 24 h. The cells were harvested and washed four times with cold PBS buffer until the fluorescent intensity of the supernatant reached blank level, then resuspended in PBS buffer and observed using laser confocal microscopy.

2.5. Lipid extraction and thin layer chromatography analysis

The control strain C800 and engineered strain Z01 were cultured in YPD supply with soybean oil (YPDSO) medium and 5 mL of fermentation broth and harvested after 48 h by centrifugation at 12,000×g for 15 min. The supernatants and pellets were used for lipid extraction and thin layer chromatographic (TLC) analysis [22–24]. The pellets were washed three times with distilled water (ddH₂O) and extracted with 1 mL of 1:1 (v/v) methanol and chloroform supplemented with an internal standard of triheptadecanoylglycerol (Nu-chek-prep; Elysian, MN, USA). The mixture was then supplemented with 500 μL of ddH₂O and centrifuged at 12,000×g for 15 min after vortexing for 15 min. The lower chloroform layer containing the lipids was transferred into a new 1.5 mL Eppendorf tube and extracted with 500 μL of 1:1 (v/v) methanol and chloroform by vortex for 15 min. The mixture had 250 μL of ddH₂O added and was centrifuged at 12,000×g for 15 min after vortexing for 15 min. The chloroform extract was then subjected to TLC analysis. The supernatants were pretreated with 500 μL of acetic acid, 500 μL of 12% (w/v) sodium chloride solution and 2 mL of ethyl acetate, supplemented with an internal standard of triheptadecanoylglycerol and vortexed for 15 min. The mixture was vortexed at 12,000×g for 15 min, then resuspended in chloroform for TLC analysis after the upper organic phase was collected and dried with nitrogen.

2.6. Metabolite quantification

Fermentation broth (500 μL) was mixed with methanol (500 μL) and vortexed vigorously for 5 min. Analysis and quantification were performed after centrifugation at 12,000×g for 6 min and 0.22 μm filtration. The supernatants were quantified through a high-performance liquid chromatography (HPLC) system (Shimadzu, Kyoto, Japan) equipped with a 250 × 4.6 mm, 5 μm C18 column (Thermo Fisher Scientific, Massachusetts, United States) [25]. The detection wavelength of (2S)-naringenin was 350 nm with an SPD-M20A UV detector (Shimadzu) at 40 °C. The samples were eluted with solvent A (water with 0.1% trifluoroacetic acid) and solvent B (acetonitrile with 0.1% trifluoroacetic acid) at a flow rate of 1 mL/min. The elution gradient was 10%–40% solvent B for 0–10 min, 40%–90% solvent B for 10–30 min, 90% solvent B for 30–35 min and 90%–100% solvent B for 30–35 min. To measure the soybean oil content, the supernatant was collected after centrifugation of culture broth, then drying in a conventional condition and measuring the weight of the soybean oil residue.

3. Results

3.1. The structure analysis of caveolin-mediated microvesicles

The previous study showed that caveolin encoded by the CAVI gene could form caveolae in E. coli [13]. To analyze the structure of caveolin-mediated microvesicles and characterize the microvesicles structure in S. cerevisiae, the GFP was fused to the N-terminal or C-terminal of CAVI. As shown in Fig. 1, when the fluorescent protein was expressed alone, the fluorescence distribution filled the entire cell. While the fluorescent protein was fused with CAVI, the fluorescence formed small circular structures, suggesting that caveolin could mediate the formation of caveolae structure in S. cerevisiae. To further characterize the three-dimensional structure, the caveolae were observed by the Z-stack of the laser confocal microscope as small circles stacked together. There was no obvious effect on the structure of the caveolae when the green fluorescent protein was fused to the N-terminal or C-terminal of CAVI with or without a flexible linker.

3.2. The endocytosis of caveolae

Caveolin mediated the formation of caveolae by invading the phospholipid bilayer membrane, which may be a transport carrier for endocytosis of substances in S. cerevisiae. To verify the uptake ability of caveolae, 5,6-carboxyfluorescein was used as the indicator and the intracellular fluorescence was observed using fluorescence microscopy (Fig. 2). The results showed that fluorescent intensity in the engineered strains was stronger than that of the control strain, indicating that the cells had a stronger ability to uptake 5,6-carboxyfluorescein with expression of CAVI. The weak fluorescence of the control strain may be due to the penetration of fluorescein into the cells and these results suggested that caveolae could strengthen the ability of taking up substances from the surrounding media.

3.3. Effect of engineered caveolae on the transportation of hydrophobic substances

To further verify the ability of caveolae to import extracellular oils, soybean oil was stained with Nile Red and then added to the culture medium. As shown in Fig. 3, the engineered cells showed red fluorescence due to oil absorption, while the control cells did not, indicating that caveolae could assist the absorption of exogenous oil droplets. Soybean oil is rich in triacylglycerol (TAG), which can be hydrolyzed by lipases to produce free fatty acids (FFAs) [26,27]. The intracellular and extracellular TAG and FFAs are detected by TLC to characterize the transport and absorption capacity of caveolin to soybean oil. As shown in Fig. 4, the extracellular TAG content of the CAVI-expressing cells in YPDSO medium was lower than that of the control cells and the intracellular TAG content of the CAVI-expressing cells was higher than that of the control cells, which further showed that caveolae could promote the absorption of oils. In YPD medium, the intracellular TAG content of the CAVI-expressing cells was equivalent to that of the control cells. There was no difference in the intracellular and extracellular FFAs content between the CAVI-expressing cells and the control cells in YPD or YPDSO media.

3.4. Effect of engineered caveolae and lipases on the production of (2S)-naringenin

Caveolae mediated by caveolin possess a transport function that does not depend on transporters and was expected to enhance the endocytosis and hydrolysis of exogenous oil through the overexpression of CAVI and/or lipase genes, to promote fatty acid β-oxidation (Fig. 5A). As shown in Fig. 5B, in YPDSO medium, the naringenin production of the engineered strain L08 with the overexpression of CAVI at 240 mg/g DCW was higher than that of the control strain at 222 mg/g DCW,
indicating that the overexpression of CAV1 could promote the transport of oils into cells and improve the production of naringenin, an acetyl-CoA derivative.

To further promote the hydrolysis of endocytosed oils and enhance the process of β-oxidation, lipase from Bacillus pumilus (BP), lipase from S. cerevisiae (encoded by TGL3) and sterol ester hydrolase from S. cerevisiae (encoded by TGL1) were overexpressed. The results showed that the production of naringenin in engineered strains were higher than that of the control strain regardless of whether lipases were overexpressed alone (engineered strains L17, L18 and L19) or co-overexpressed with CAV1 (engineered strain L20, L21 and L22), indicating that lipases could promote the biosynthesis of naringenin in YPDSO medium as seen in Fig. 5B. The naringenin production reached 262 mg/g DCW in engineered strain L22 expressing TGL3 and CAV1, which was significantly higher than that of engineered strain L19 with the overexpression of TGL3 at 252 mg/g DCW. Compared with the production of naringenin of engineered strain L17 at 264 mg/g DCW with the overexpression of BP lipase, the production of naringenin of engineered strain L20 with the co-overexpression of BP and CAV1 increased to 269 mg/g DCW. Similarly, the production of naringenin of engineered strain L21 with co-overexpression of TGL1 and CAV1 was 264 mg/g DCW, but the naringenin production of engineered strain L18 with the overexpression of TGL1 was 257 mg/g DCW. Different from the effect in YPDSO medium, there was no significant difference in naringenin production between

Fig. 1. Confocal microscopy observation of caveolae formed in S. cerevisiae.
Figure A, B, C, D and E: Representative images in control strain Z04, engineered strains Z10, Z11, Z12 and Z13, respectively. A-1, B-1, C-1, D-1 and E-1 are under fluorescence microscopy; A-2, B-2, C-2, D-2 and E-2 are under bright field microscopy; A-3, B-3, C-3, D-3 and E-3 are merged images of fluorescence microscopy and bright field microscopy.
Moreover, the extracellular soybean oil content of the engineered strains was lower than that of the control strain, which proved that the absorption and utilization capacity of soybean oil by the engineered strains was better than that of the control strain (Fig. 5D). It was concluded that the biosynthesis of acetyl-CoA derivatives was promoted by enhancing the expression of caveolin and lipases supplied with soybean oil supply. However, biomass of engineered strains were lower than that of control strain, which may be due to the endocytosis of soybean oil and/or the metabolic burden caused by heterologous genes overexpression and

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**Fig. 2.** Endocytosis of 5,6-carboxyfluorescein in *S. cerevisiae*.  
A: The endocytosis of 5,6-carboxyfluorescein in control strain C800, A-1, A-2 and A-3 under fluorescence field microscopy, bright field microscopy and a merged image of the two, respectively.  
B: The endocytosis of 5,6-carboxyfluorescein in engineered caveolin-mediated strain Z01, B-1, B-2 and B-3 under fluorescence microscopy, bright field microscopy and a merged image of the two, respectively.

**Fig. 3.** Endocytosis of hydrophobic substances in *S. cerevisiae*.  
A: The endocytosis of soybean oil stained with Nile Red in control strain C800, A-1 and A-2 under bright field and fluorescence microscopy.  
B: The endocytosis of soybean oil stained with Nile Red in engineered caveolin-mediated strain Z01, B-1 and B-2 under bright field and fluorescence field microscopy.
Fatty acid transport requires the participation of fatty acid transporters, but the transport of fatty acids across the cell membrane into the cytosol impairs the integrity of the membrane and limits the availability of substrates [28, 29], so an ideal transport system independent of fatty acid transporters is required to improve the transport of hydrophobic substrates. In this study, the structure of caveolae in S. cerevisiae was explored by expressing the CAV1 gene derived from humans. It was found that caveolae in S. cerevisiae formed a hollow structure with the scattered accumulation of caves. Fluorescein and soybean oil in the media were transported into cells, which proved the endocytosis function of caveolae. The caveolae were then applied to the endocytosis system is expected to become a green tool to solve environmental problems and achieve the biosynthesis of high-value acetyl-CoA derivatives. Although the production of naringenin only slightly increased from 231 mg/L in control strain ZLHB04-4 to 241 mg/L in the engineered strain L20 (data not shown), which may be due to the insufficient downstream pulling force of fatty acid β-oxidation. Therefore, the next work is to overexpress key genes of fatty acid β-oxidation to further strengthen fatty acid β-oxidation. In addition, CAV1 was expressed by different promoters to analyze the effect of different expression levels of cavelonin on naringenin production. Opposite to the effect of strong promoter (pGAL1), the naringenin production was no significant difference when CAV1 was expressed with medium and weak promoters (data not shown). Therefore, the strong promoter was more conducive to the expression of CAV1, which may be due to the formation of more microvesicles to transport and absorb soybean oil.

Compared with soybean oil, the waste oil produced in industry and life (such as waste cooking oil or leaked crude oil [40]) is not friendly to the environment and food safety [41, 42]. Therefore, exploring the utilization of waste oil and crude oil to produce high value-added biochemical products is crucial. In this study, caveolae-forming S. cerevisiae had the ability to absorb soybean oil. If caveolae-forming S. cerevisiae was applied for endocytosis of these waste and crude oil to promote high value-added acetyl-CoA derivatives production, it would not only address the problems of waste oil reproccessing and crude oil spilling, but also promote the production of high-value products. The endocytosis system is expected to become a green tool to solve environmental problems and achieve resource reuse to produce high value-added products.

CRediT authorship contribution statement

Qian Zhang: Conceptualization, the study, Methodology, performed the experiments, Formal analysis, Writing – original draft, designed and coordinated the project. Ning Li: Conceptualization, the study, Writing – original draft, designed and coordinated the project. Yunbin Lyv: Conceptualization, the study, performed the experiments, Formal analysis, designed and coordinated the project. Shiqin Yu: conceived the study, designed and coordinated the project. Jingwen Zhou: Conceptualization, the study, Methodology, Writing – original draft, designed and coordinated the project, All authors discussed the results and

5,6-carboxyfluorescein was supplemented in the media and the fluorescence intensity of CAV1-expressing cells was significantly higher than that of the control, suggesting that expression of CAV1 gene could promote the transport of exogenous substrates in S. cerevisiae. This agreed with previous studies, which showed that expression of CAV1 in E. coli formed caveolae to facilitate 5,6-carboxyfluorescetin to transport into cells [13,14]. Soybean oil that was pre-stained with Nile Red, was used to show that the caveolae formed in S. cerevisiae had the ability to transport hydrophobic substrates and these results confirmed that the expression of CAV1 had endocytic function in microorganisms such as E. coli and S. cerevisiae.
commented on the manuscript.

Declaration of competing interest

The authors declare there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2022.06.008.

Fig. 5. Effects of CAV1 and lipases on naringenin production.

A: The endocytosis process of soybean oil by caveolae and (2S)-naringenin biosynthetic pathway in S. cerevisiae. The dotted line represents multistep reactions. TAL, tyrosine ammonia lyase; 4CL, 4-hydroxy-3-cinnamoyl-CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase. B: The naringenin production of engineered strains in YPDSO medium. C: The naringenin production of engineered strains in YPD medium. BP: lipase from Bacillus pallidus, TGL1: the gene of sterol ester hydrolase from S. cerevisiae, TGL3: the gene of lipase from S. cerevisiae. Cyan columns represent OD600 yellow columns represent the production of naringenin. D: The residues of soybean oil in engineered strains. All data are means ± SD (n ≥ 3). *P < 0.05; **P < 0.01; ***P < 0.001; n. s. P > 0.05. (Student’s t-test: two-tailed, two-sample equal variance).

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