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RESEARCH ARTICLE

Engineering *Scheffersomyces segobiensis* for palmitoleic acid-rich lipid production

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Abstract

Palmitoleic acid (POA; C16:1) is an essential high-value ω-7-conjugated fatty acid with beneficial bioactivities and potential applications in the nutraceutical and pharmaceutical industries. Previously, the oleaginous yeast *Scheffersomyces segobiensis* DSM27193 has been identified as a promising production host as an alternative for POA extraction from plant or animal sources. Here, the POA-producing capacity of this host was further expanded by optimizing the fermentation process and molecular strain engineering. Specifically, a dual fermentation strategy (O-S dynamic regulation strategy) focused on the substrate and dissolved oxygen concentration was designed to eliminate ethanol and pyruvate accumulation during fermentation. Key genes influencing POA production, such as *jen*, *dgat*, *ole* were identified on the transcriptional level and were subsequently over-expressed. Furthermore, the phosphoketolase (Xpk)/phosphotransacetylase (Pta) pathway was introduced to improve the yield of the precursor acetyl-CoA from glucose. The resulting cell factory SS-12 produced 7.3 g/L of POA, corresponding to an 11-fold increase compared to the wild type, presenting the highest POA titre reported using oleaginous yeast to date. An economic evaluation based on the raw materials, utilities and facility-dependent costs showed that microbial POA production using *S. segobiensis* can supersede the current extraction method from plant oil and marine fish. This study reports the construction of a promising cell factory and an effective microbial fermentation strategy for commercial POA production.

INTRODUCTION

Unsaturated fatty acids (UFAs) are essential biological components determining membrane structures and functions. According to the position(s) of the unsaturated double bond, UFAs can be categorized into the ω-3, ω-6, ω-7 and ω-9 series. In recent years, the pharmaceutical industry has become increasingly interested in palmitoleic acid (C16:1, ω-7) (POA) in particular as an important contributor to human health. In 2008, research from the Harvard School of Public Health first confirmed that POA acts as a lipid hormone that strongly stimulates muscle insulin reaction and suppresses hepatosteatosis (Cao et al., 2008). Henceforward, emerging research suggested positive functions of ω-7 UFAs, especially of POA, regarding improving skin conditions, increasing insulin sensitivity, helping to reduce the accumulation of liver fat and inflammation, and improving coronary heart disease and hypertension (Akazawa et al., 2010; Foryst-Ludwig

et al., 2015; Gong et al., 2011; Yamamoto et al., 2015). Therefore, POA is expected to become a global nutraceutical in the near future.

However, POA is quite rare in common edible vegetable and animal oils (Porokhovinova et al., 2022). At present, it is mainly obtained from marine resources and/or some plants, mainly macadamia nuts (15%–22% POA content in macadamia oil) and sea buckthorn (30%–35% POA content in berry oil and 25%–30% in pulp/peel oil) (Hu et al., 2019; Solà Marsiñach & Cuenca, 2019). Currently, the supplement company Tersus Pharmaceuticals has launched Provinal® (C16:1n7, 50% palmitoleic acid ethyl ester) on the market, which contains POA extracted from marine anchovy and/or menhaden oil (http://tersuslifesciences.com/); another pharmaceutical company, Innovix Pharma, also launched a product from anchovies containing 50% of a POA-component, resulting in a minimum production cost of US$3000–5000/kg POA, ignoring the cost of recovering the POA from the oil and other contributing factors (Anggo et al., 2015; Koesoemawardani & Hidayati, 2018). In addition, seasonal fishing regulations and increasingly serious marine pollution also limit long-term POA extraction from marine sources. In terms of plant extraction, macadamia nut is the most expensive nut in the world. The worldwide (mainly Australia and Hawaii) macadamia nut production is currently between 16,000 and 18,000 tons per year, while most of it is used for desserts and cooking. Worldwide annual sea buckthorn production is around 400 million tons, and China contributes to over 50% of its production. However, low overall oil content (~12%) in sea buckthorn limits large-scale extraction of POA (Koskovac et al., 2017). In conclusion, the low occurrence of POA in natural sources resulted in relatively late recognition of its beneficial functions compared to the other classes of UFAs. Recently, increasing interest in understanding the physiological functions and beneficial health effects of POA and its biosynthetic pathway, promoted artificial POA production via synthetic biology (Hu et al., 2019; Wu et al., 2012).

Previous attempts using *Escherichia coli* and *Saccharomyces cerevisiae* demonstrated that non-oleaginous microorganisms are not suitable for commercial POA production. For instance, by heterologous expression of fatty acyl-ACP thioesterase and fatty acid desaturase genes, combined with the inhibition of the fatty acid catabolic pathway and the enhancement of the native acetyl-CoA carboxylase gene, the engineered *E. coli* strain finally produced 82.6 mg/L of free fatty acids (Y. Cao et al., 2014). Although POA occupied 45.6% of the total fatty acid, the total POA production amounted only to 37.7 mg/L, which is too low for industrialization. The same applied to *S. cerevisiae*. Although high POA content of up to 55% in produced lipids was obtained, the relatively low overall lipid content (<20%) in combination with low cell concentration (~3 g/L after 7 days of cultivation in shake flask fermentation) demonstrates the difficulty behind engineered non-oleaginous organisms for POA overproduction (Kamisaka et al., 2015). Hence, the adoption of an oleaginous microorganism with a certain natural POA accumulation capability might be the key to realize microbial POA production.

Some oleaginous algae (such as *Eustigmatos vischeri*, *Eustigmatos* cf. *polyphem*, and *Vischeria punctata*) are capable of high POA accumulation. However, low biomass concentrations (4–8 g/L) obtained within long cultivation periods result in overall low POA productivity (Wang et al., 2018). In terms of oleaginous yeast, POA is found usually rarely and only in low amounts (<5%) in commonly studied oleaginous species, such as *Rhodotorula glutinis* and *Trichosporum cutaneum* (Kolouchová et al., 2016). Fortunately, *Scheffersomyces segobiensis* (syn. *Pichia segobiensis*) DSM 27193, a newly reported oleaginous yeast, presented high POA accumulation abilities in its lipid profile (>10%) (Schulze et al., 2014; Zhou, Bao, et al., 2021). The complete genome sequence of *S. segobiensis* DSM 27193 is available under accession number JAG-SID000000000 in DDBJ/ENA/GenBank. The lipid and POA biosynthesis pathway and the involved genes in *S. segobiensis* DSM 27193 have also been elucidated (Zhou, Xu, et al., 2021). Owing to the fast cell growth, high cell density and relatively high content of POA, genetic engineering of *S. segobiensis* DSM 27193 cell factory to improve POA production seems to be a promising approach. In this study, the fermentation process was designed and optimized to eliminate the by-products of ethanol and pyruvate; then, genetic tools for *S. segobiensis* DSM 27193 were adapted, so that the lipid and POA synthetic pathway could be systematically optimized. Lastly, the POA production of the engineered strains was comprehensively evaluated in a 5 L bioreactor scale and compared to POA extraction from plants, aiming to provide guidance to improve POA production further by synthetic biology and enabling its industrial production.

EXPERIMENTAL PROCEDURES

Microorganism and cultivation medium

S. segobiensis (syn. *Pichia segobiensis*) DSM 27193 used in this study was deposited at DSMZ culture collection in Braunschweig, Germany. The selection medium for gene integration was YPD medium (1% yeast extract, 2% peptone and 2% glucose) at 25°C with appropriate antibiotics: 200 μg/mL hygromycin B and 100 μg/mL nourseothricin. For POA and/or lipid production, YM medium (0.3% yeast extract, 0.3% malt

extract, 0.3% peptone and 5% glucose) and mineral salt medium were used (Zhou, Bao, et al., 2021), containing (g/L): KH_2PO_4 8.99, $Na_2HPO_4{\cdot}2H_2O$ 0.12, sodium citrate$\cdot 2H_2O$ 0.1, yeast extract 0.1. In addition, the culture was supplemented with 2 mL trace element solutions (g/L: $CaCl_2{\cdot}2H_2O$ 4, $FeSO_4{\cdot}7H_2O$ 0.55 and citric acid 0.475, $ZnSO_4{\cdot}7H_2O$ 0.1, $MnSO_4{\cdot}H_2O$ 0.076, 96% H_2SO_4 100 μL) and 2 mL salt solution (g/L: $MgSO_4{\cdot}7H_2O$ 20.0, yeast extract 10.0) per 100 mL of cultivation medium directly before inoculation. *E. coli* DH5α was used for the construction of plasmids and cultivated in LB medium supplemented with 100 μg/mL ampicillin at 37°C.

Construction of plasmids and strains

Plasmids in this study are listed in Table S1. All primers were synthesized by Genewiz Inc. (China) and are listed in Table S2. The coding sequence of heterologous genes and antibiotic marker genes was codon-optimized (Table S4) and synthesized by GenScript (China). The endogenous genes and all promoters and terminators were amplified using the genomic DNA of *S. segobiensis* DSM 27193 and *S. stipites* CBS 6054. The expression cassettes P_{TKT}-T_{TKT} and $P_{TKT'}$-$T_{TKT'}$ used for gene expression were constructed for substitution of the original promoter and terminator elements in 113-GPD-TEF to get plasmids 113-GPD-TKTO and 113-GPD-TKT'O, respectively. Competent yeast cells were prepared using Frozen-EZ Yeast Transformation II Kit™. Electroporation was used to introduce *Not* I linearized plasmids into *S. segobiensis* DSM 27193 after protocol optimization (Shao & Zhao, 2014). All engineered strains are listed in Table 1.

Shake flask and fed-batch fermentation

For shake flask fermentation, a single colony either of wild type or the engineered strain was picked from a YPD agar plate and subsequently inoculated into a 20 mL YM medium as pre-culture. The cultivation was carried out at 25°C and 200 rpm for approximately 24 h. For the main culture, a certain amount of pre-culture was inoculated into 50 mL of the same medium to obtain an initial OD_{600} of 0.5–1.0. The main cultures were cultivated at 200 rpm for 120 h.

For fed-batch fermentation, cultivation was performed in a 5 L fermenter (T&J intelli-ferm Btype; Minifors fermenter) with working volume of 2 L. The initial batch phase was carried out in a mineral salt medium with 50 mL of salt solution as well as 50 mL of trace element solution which were fed into the medium daily. A constant pH of 5.0 was maintained automatically by titration with 4 M H_3PO_4 and 4 M NaOH. In each fermenter, Contraspum A 4050 HAC was applied as an antifoam agent. The cultivation temperature was 25°C, and the initial OD_{600} was 0.5–1.0. For the optimized fermentation strategy, the initial agitation was set to 400 rpm and increased to maximally 800 rpm after 48 h. During the fed-batch cultivation, the cells were automatically fed with a 500 g/L glucose solution to maintain a constant biomass-specific glucose consumption rate. The whole fermentation was divided into three periods: cell proliferation (0–42 h), lipid accumulation (42–186 h) and lipid degradation (after 186 h). Glucose feed rate was automatically adjusted according to the

TABLE 1 Engineered strains used in this study.

Strain	Description	Source
S. segobiensis DSM27193	Wild type	Donated by A/Prof. Katrin Ochsenreither Karlsruhe Institute of Technology
SS-1	*S. segobiensis* DSM27193/P_{TKT}-*jen11*-T_{TKT}	This study
SS-2	*S. segobiensis* DSM27193/P_{TKT}-*jen12*-T_{TKT}	This study
SS-3	*S. segobiensis* DSM27193/P_{TKT}-*Scjen*-T_{TKT}	This study
SS-4	*S. segobiensis* DSM27193/P_{TKT}-*gpdh*-T_{TKT}	This study
SS-5	*S. segobiensis* DSM27193/P_{TKT}-*cat2*-T_{TKT}	This study
SS-6	*S. segobiensis* DSM27193/$P_{TKT'}$-*pgat*-$T_{TKT'}$	This study
SS-7	*S. segobiensis* DSM27193/$P_{TKT'}$-*dgat*-$T_{TKT'}$	This study
SS-8	*S. segobiensis* DSM27193/$P_{TKT'}$-*ole1*-$T_{TKT'}$	This study
SS-9	*S. segobiensis* DSM27193/$P_{TKT'}$-*ole2*-$T_{TKT'}$	This study
SS-10	*S. segobiensis* DSM27193/$P_{TKT'}$-*Scole*-$T_{TKT'}$	This study
SS-11	*S. segobiensis* DSM27193/P_{TKT}-*jen12*-T_{TKT}, $P_{TKT'}$-*dgat*-$T_{TKT'}$ and $P_{TKT'}$-*ole1*-$T_{TKT'}$	This study
SS-12	*S. segobiensis* DSM27193/P_{TKT}-*jen12*-T_{TKT}, $P_{TKT'}$-*dgat*-$T_{TKT'}$, $P_{TKT'}$-*ole1*-$T_{TKT'}$ and $P_{TKT'}$-*xpk*-$T_{TKT'}$- P_{TKT}-*pta*-T_{TKT}	This study

calculated glucose consumption rates within the respective phase so that the glucose concentration was maintained below 50 g/L throughout the fermentation process.

Determination of biomass

Cell concentration was determined by the optical density at 600 nm (OD_{600}) with the initial culture medium as blank. All samples were diluted to an optical density of 0.2–0.8 prior to measurement.

Biomass concentration was analysed gravimetrically. Specifically, 1 mL aliquot of culture broth was transferred to a predried and preweighed 1.5 mL reaction tube and centrifuged at 13,000 rpm for 5 min. The supernatant was collected and used for the determination of glucose. The cell pellet was washed with 1 mL saline (0.9% NaCl), dried at 60°C for 24 h and was then weighed.

Glucose, ethanol and pyruvate determination

For glucose, ethanol and pyruvate determination, the supernatant was used for HPLC analysis. The analysis was performed with a standard HPLC device (Agilent 1100 Series, Agilent, China) with a Rezex ROA organic acid H^+ (8%) column (300 by 7.8 mm, 8 m; Phenomenex) protected by a Rezex ROA organic acid H^+ (8%) guard column (50 by 7.8 mm). Applying 5 mM H_2SO_4 under isocratic conditions at 55°C (column temperature) as the mobile phase at a constant flow rate of 0.4 mL/min. Detection of glucose was achieved via an Agilent 1200 series refractive index detector at 55°C, ethanol and pyruvate were tested with an ultraviolet-visible light absorbance detector at 210 nm, 55°C.

Lipid analysis

Quantification of lipid content and determination of fatty acid composition was achieved by direct acidic transesterification of freeze-dried yeast cell mass to fatty acid methyl esters (FAME) and subsequent GC analysis based on the method modified previously (Zhou, Bao, et al., 2021). Briefly, washed cell mass from a 20 mL sample was freeze-dried at −30°C and 0.370 mbar for 24 h. Approximately 20 mg of freeze-dried cells were transferred to a 15 mL glass tube with Teflon cap. Approximately, 1.5 mL of hexane, 0.5 mL of 2 mg/mL internal standard (methyl benzoate) dissolved in hexane and 2 mL of 15% H_2SO_4 in methanol were added as a solvent for the lipid extraction and a catalyst for the esterification reaction. Samples were incubated at 100°C for 2 h with continuous shaking. After cooling on ice, 1 mL of demineralized water was added, and phase separation was facilitated by centrifugation for 5 min at 2500 rpm. One microlitre of the upper phase, containing the FAMEs, was analysed via gas chromatography (Agilent Technologies, 6890 N Network GC-System) equipped with a DB-Wax column (L: 30 m d: 0.25 mm, Agilent Technologies Deutschland GmbH, Böblingen, Germany; 122-7032) and a flame ionization detector working with a pressure of 1.083 bar and an initial temperature of 40°C. Column temperature was increased from 40 to 250°C with a rate of 8 °C/min which was held at 250°C for 10 min before cooling down to 40°C. For identification and quantification of FAMEs, the RM3 FAME Mix standard (Art. No. 07256-1AMP; Sigma–Aldrich; Taufkirchen; Germany) was used.

Quantitative RT-PCR analysis

Strain samples for RT-PCR analysis were collected in the exponential growth phase (at 70 h) and shock-freeze in liquid nitrogen. RNA was extracted and purified using a FastPure Cell/Tissue Total RNA Isolation Kit (Vazyme, China) according to the instructions of the manufacturer. After removing DNA, the total mRNA was reverse transcribed to cDNAs using a HiScript® II Q Select RT SuperMix for qPCR (Vazyme, China) according to the instructions. A quantitative real-time PCR (qPCR) assay was performed with 1 μL cDNA template (~25 ng), specific primers (Table S3) and a StepOne Plus instrument (Applied Biosystems, USA). The procedure was as follows: initial activation was performed at 95°C for 3 min, followed by 30 cycles of thermal denaturation at 95°C for 10 s and then annealing and elongation at 60°C for 30 s, then maintaining at 95°C for 15 s, 60°C for 60 s and 95°C for 15 s. The transcription levels of related genes were calculated using the $2^{-\Delta\Delta Ct}$ method. The gene expression levels were normalized using 18S rRNA as an internal control. At least three technical replicates were performed for each cDNA sample analysed.

RESULTS AND DISCUSSION

O-S dynamic regulation strategy design to reduce by-product accumulation

Fermentation in shake flask and bioreactor showed that a considerable amount of ethanol and pyruvate was accumulated during the fed-batch fermentation process by *S. segobiensis* DSM27193 (Figure S1). Although ethanol and pyruvate were mostly re-consumed in the later stage of fermentation, the accumulation of these by-products consequentially extended the fermentation period and may have caused stress. Genome

analysis displayed that *S. segobiensis* DSM27193 is closely related to *Scheffersomyces stipites*, which is a Crabtree negative yeast capable of fermenting a wide range of sugars to ethanol (Papini et al., 2012; Zhou, Xu, et al., 2021). Flux states of *S. stipitis* metabolism declared that increasing dissolved oxygen (DO) levels led to a decrease in ethanol synthesis (Granados-Arvizu et al., 2021; Unrean & Nguyen, 2012). Previous attempts to improve DO levels through regulation of agitation and aeration rates during fermentation were indeed conducive to decrease ethanol accumulation (Zhou et al., 2023).

In addition, ethanol concentration was also demonstrated to be dependent on substrate concentration, as high substrate concentration led to high ethanol production (Farias et al., 2014). Based on this, glucose concentration in the medium was maintained at three ranges: 30–50, 50–70 and 70–90 g/L (Table 2). The results showed that maintaining low substrate concentrations could effectively reduce ethanol and pyruvate accumulation. Especially, when the glucose concentration was controlled between 30 and 50 g/L, no ethanol was detected during the whole fermentation process, and pyruvate accumulation was also reduced by nearly half. In return, an increase of 63% in dry cell weight (DCW) and an increase of 42% in lipid accumulation were obtained. Furthermore, POA content in total lipids was enhanced by 46%, resulting in an overall 2.3-fold increase in POA production.

Considering the effectiveness of DO level and substrate concentration regulation, a dual fermentation strategy (O-S dynamic regulation strategy) was designed. Figure 1 illustrates that the glucose concentration was indeed maintained at a consistent level between 20 and 50 g/L. Finally, 55.1 g/L of DCW containing 27.4% lipid was obtained with a POA content of 14.2% (Figure 1). Overall, the POA titre reached 2.14 g/L, corresponding to a 2.5-fold increase compared to non-optimized fermentation conditions. While the O-S dynamic regulation strategy completely eliminated ethanol accumulation, however, over 6 g/L of pyruvate was still determined, indicating an obstacle lying on the pathway from pyruvate to the lipid synthesis precursor acetyl-CoA.

Establishment of genome editing protocol and carboxylic acid transporter expression

Considering that no precedent for genetic modification of *S. segobiensis* has been reported so far, exploitation of an efficient genetic modification method was particularly important for further construction of the cell factory. Genome analysis implied that *S. segobiensis* belongs to the CUG-Ser clade yeasts, which use an alternative codon system that decode CUG for serine instead of leucine as common in the classical genetic code (Cao et al., 2018), and thereby limiting the availability of heterologous drug resistance markers for transformation. To date, most of the work for gene expression in *Scheffersomyces* species like *S. stipites* and *Candida tanzawaensis* exploited either nutritional auxotrophy markers or their sensitivity towards adapted antibiotics like bleomycin or hygromycin (Laplaza et al., 2006). Screening pressure tests showed that *S. segobiensis* DSM 27193 was highly sensitive towards hygromycin and nourseothricin. Herein, both resistance genes were codon-optimized (CTG to TTG) synthesized and then inserted into plasmid 113-GPD-TEF by replacing the *ura3* position.

Like many nonconventional microbial species, the transformation efficiency of *S. segobiensis* was initially very low. Specifically, the transformation of 1 μg of 5 kb linearized DNA fragment resulted in less than five colonies when using a standard electroporation protocol (0.75 kV/mm) (Cao et al., 2018). By optimizing the transformation conditions to voltages of >2.5 kV for 5 ms, however, significantly increased colony counts (>40) were routinely obtained. Additionally, commonly used promoters like P_{TEFin} and P_{G6PD} seemed not to work in

TABLE 2 The result of *S. segobiensis* DSM27193 fermentation with different glucose concentrations.

	Glucose			
Concentration results	**Control**	**70–90 g/L**	**50–70 g/L**	**30–50 g/L**
DCW (g/L)	30.13 ± 1.6	43.25 ± 1.8	47.10 ± 2.9	49.60 ± 1.2
Lipid content (w/w, %)	19.59 ± 0.8	27.31 ± 1.1	26.84 ± 1.4	27.25 ± 0.9
Lipid titre (g/L)	5.93 ± 0.3	11.88 ± 0.4	12.64 ± 0.5	13.02 ± 0.3
POA content in total lipid (%)	10.3 ± 0.6	14.8 ± 1.4	13.9 ± 0.3	15.0 ± 0.3
POA titre (g/L)	0.61 ± 0.1	1.63 ± 0.2	1.76 ± 0.3	1.99 ± 0.2
Maximal ethanol concentration (g/L)	13.46 ± 1.0	6.38 ± 0.7	4.67 ± 0.6	0
Maximal pyruvate concentration (g/L)	12.89 ± 1.1	5.89 ± 0.6	6.89 ± 0.5	7.31 ± 0.4

Note: The glucose concentration was maintained at different ranges during the whole fermentation process. The maximal ethanol and pyruvate concentrations were obtained in the mid-fermentation.

Abbreviations: DCW, dry cell weight; POA, palmitoleic acid.

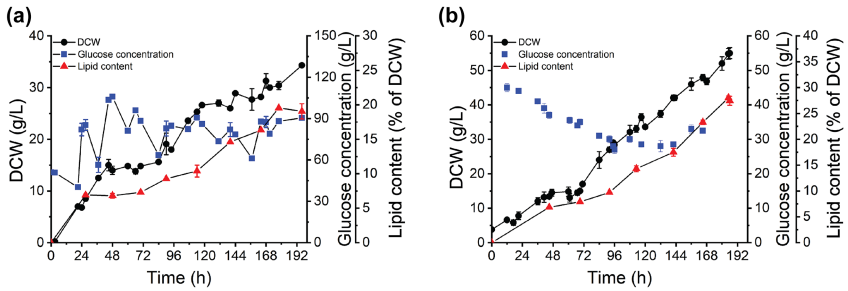

FIGURE 1 *S. segobiensis* DSM 27193 fermentation without (A) and with (B) O-S dynamic regulation strategy. In the O-S dynamic regulation strategy, agitation speed and aeration rate were set at 400 rpm and 1 vvm, respectively, in the first 42 h of fermentation to avoid the potential damage caused by shear force. Subsequently, agitation speed and aeration rate were regulated to 800 rpm and 1.5 vvm, respectively, to guarantee a minimum 40% of DO in the fermentation broth. To regulate the substrate concentration, the whole fermentation was divided into three periods: cell proliferation (0–42 h), lipid accumulation (42–186 h) and lipid degradation (after 186 h). The glucose feed rate was automatically adjusted according to the calculated glucose consumption rates within the respective phase so that the glucose concentration was maintained below 50 g/L throughout the fermentation process. The experiment was performed in triplicates.

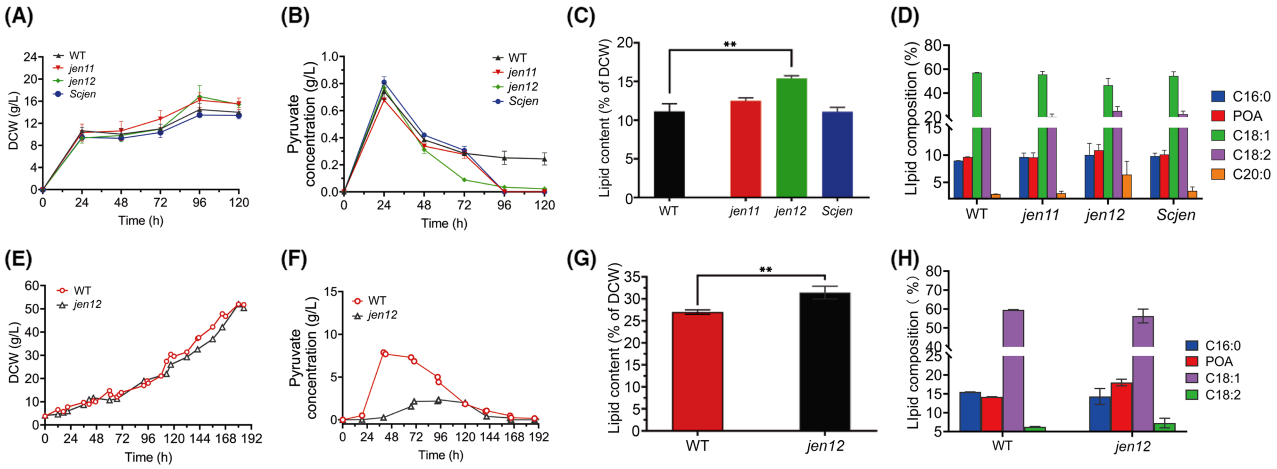

FIGURE 2 The influence of monocarboxylate transporter introduction on cell growth, pyruvate accumulation and POA production of *S. segobiensis* DSM 27193 in shake flask (A–D) and 5 L fermenters (E–H). The experiment was performed in triplicates.

S. segobiensis. Therefore, a homologous transketolase promoter (P_{TKT}) and another exogenous transketolase promoter ($P_{TKT'}$) from *S. stipites* CBS 6054 have been examined by monitoring the expression of a reporter gene. The results showed an 11–23-fold increase in fluorescence intensity promoted by P_{TKT} and $P_{TKT'}$ compared with P_{TEFin} and P_{G6PD} (Figure S3).

De novo fatty acid synthesis requires a constant supply of the metabolic precursor cytosolic acetyl-CoA. In yeast, glucose is converted to cytosolic acetyl-CoA through the combined reactions of the glycolytic cycle, mitochondrial pyruvate dehydrogenase (Pdh) and ATP: citrate lyase (Acl). Pyruvate accumulation especially in the exponential growth phase might be caused by the imbalance of fast glycolysis and relatively slow downstream pathways. Therefore, three monocarboxylate transporters, including endogenous Jen11, Jen12 and exogenous *Sc*Jen from *S. cerevisiae*, were introduced into *S. segobiensis* DSM27193, respectively, to accelerate pyruvate transport from cytoplasm to mitochondria. In shake flask fermentations, the introduction of all monocarboxylate transporters did, however, accelerate the consumption of the accumulated pyruvate rather than decrease its maximal concentration around 24 h (Figure 2B). In particular, the engineered strain SS-2 over-expressing endogenous Jen12 did best in accelerating pyruvate consumption. Correspondingly, the lipid content was significantly increased to 15% in the engineered strain SS-2, corresponding to a 36% increase compared to the original strain. More pronounced improvement was observed in a 5 L bioreactor using the O-S dynamic regulation strategy, where the accumulation of pyruvate was retarded, and <2 g/L of pyruvate was detected after 96 h fermentation of strain SS-2, while 7.7 g/L of pyruvate was accumulated after 24 h fermentation of the wild type (Figure 2F). As expected, the lipid content was increased to 32.3% containing 17.9% POA. As a

result, 2.97 g/L of POA was obtained, corresponding to a significant increase of 38.8% compared to the original strain.

Enhancing neutral lipid production in combination with fatty acid profile regulation to improve POA production

Further improvement of POA production in *S. segobiensis* DSM 27193 might be achieved by enhancing the total lipid production and/or by increasing the POA content in the lipid profile. Prior work dealing with engineering neutral lipid production and fatty acid composition has led to the construction of efficient cell factories (Liu et al., 2022; Tai & Stephanopoulos, 2013). The synthesis pathway for lipid accumulation is complex and involves a variety of enzymes and regulatory genes. The most commonly used targets for regulating lipid production are Acl, acetyl-CoA carboxylase (Acc), diacylglycerol acyltransferase (Dgat), glycerol-3-phosphate acyltransferase (Gpat), glycerol-3-phosphate dehydrogenase (Gpdh), among others (Figure 3A). In order to accurately predict the limiting step during lipid production in *S. segobiensis* DSM 27193, the transcriptional levels of the involved genes in exponential phase cells between different cultivations were analysed. The results showed that the transcription of *cat* (coding for

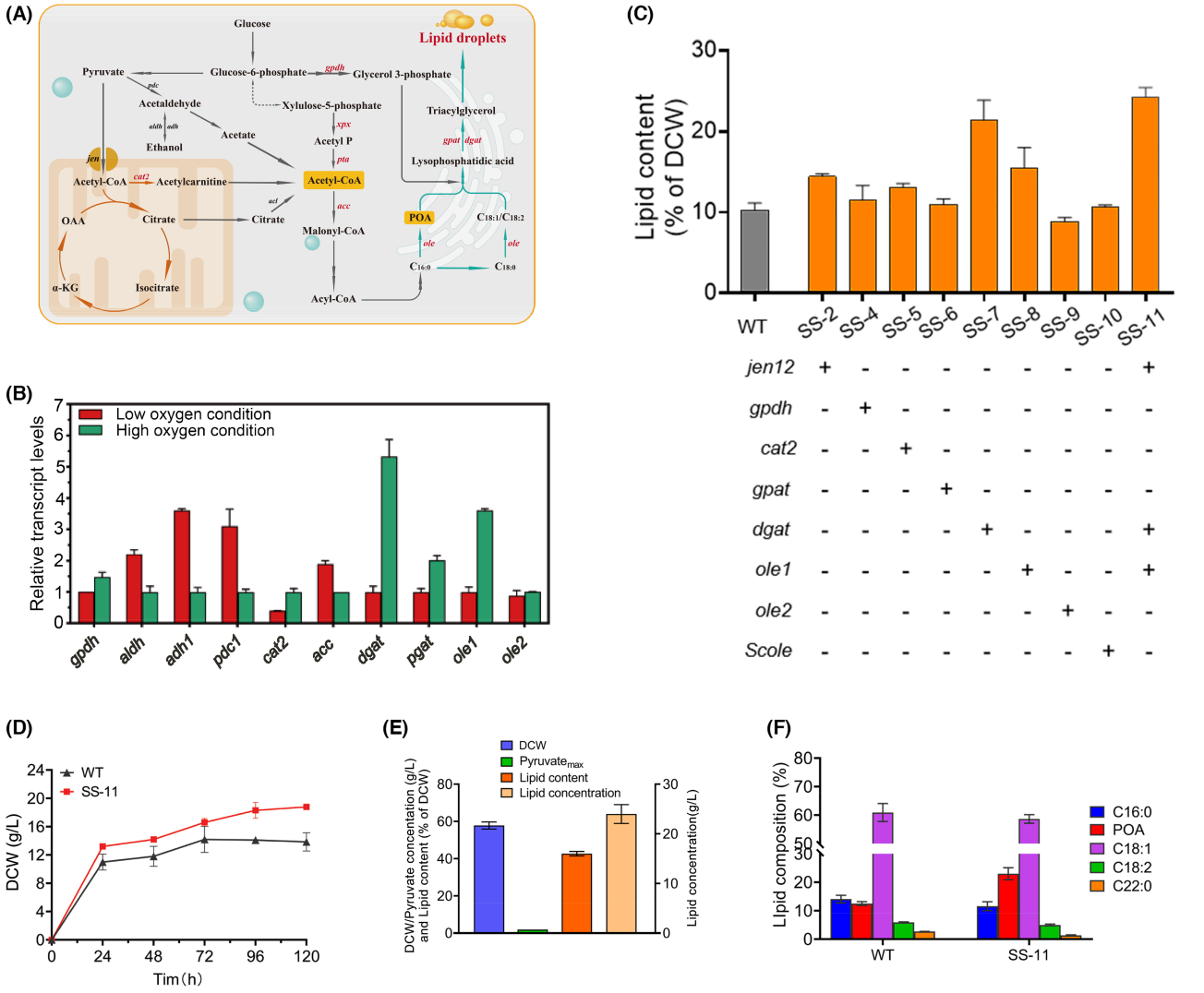

FIGURE 3 Engineered *S. segobiensis* DSM 27193 factory for POA-rich lipid production. (A) Summary of the key lipid metabolism fluxes and genes in *S. segobiensis* DSM 27193. Enzymes marked in red colour are over-expressed, the reaction lines with different colours occurring in different places (black in cytoplasm, orange in mitochondria and green in endoplasmic reticulum); (B) transcription levels of the key genes involved in lipid and POA production under the condition of different DO levels in exponential phase, (C) lipid accumulation by different engineered *S. segobiensis* DSM 27193 strains, (D) cell growth of engineered SS-11 strain in shake flask and (E, F) fermentation of engineered SS-11 in a 5 L bioreactor. The experiment was performed in triplicates.

carnitine-acetyltransferase), *gpdh*, *dgat* and *gpat* were significantly increased under high dissolved oxygen conditions, where relatively higher lipid production was obtained (Figure 3B). Therefore, the four genes were further over-expressed. The over-expression of *dgat* resulted in a lipid content up to 19% of DCW, which is nearly twice as much as for the wild type, while over-expression of the other genes resulted only in a slight improvement in lipid production. Interestingly, all the engineered strains showed enhanced cell growth (Figure S4A). Especially, over-expression of *dgat* led to a 29% increase in DCW in the recombinant SS-7 strain compared to the wild type, indicating a positive correlation between cell growth and lipid titre. In terms of POA production, its content remained fairly constant around 10% in total lipid regardless of these genes' regulation, and the highest POA production of 0.34 g/L was obtained by SS-7 (Figure S4C,D).

Another key and highly regulated enzyme that is required for the synthesis of monounsaturated fatty acids, mainly palmitoleate (C16:1n-7) and oleate (C18:1n-9), is stearoyl-CoA desaturase (Scd), which catalyses the delta-9-*cis* desaturation of a range of fatty acyl-CoA substrates (Liu et al., 2013). Lipid profile comparison between oleaginous and non-oleaginous microorganism exhibits that POA composition was relatively higher in non-oleaginous yeast, like 38.9% in *S. cerevisiae* compared to <10% in *Yarrowia lipolytica*, despite overall lower lipid titers in non-oleaginous yeasts (Kolouchová et al., 2016). Therefore, Scd from *S. cerevisiae* coded by *Scole* was cloned and heterologously expressed in *S. segobiensis* DSM 27193 after codon optimization. In addition, previous genome analysis deduced that there are two Scds (Ole1 and Ole2) in *S. segobiensis* DSM 27193 (Zhou, Xu, et al., 2021). Hence, both endogenous Scds, Ole1 and Ole2 were also over-expressed. The results showed a slight increase in cell mass when expressing Scd, corresponding to an increase in glucose consumption especially in the exponential growth phase (Figure S5). Surprisingly, over-expression of the endogenous Ole1 resulted in a 50% increase in total lipid accumulation and a 44% increase in POA content in the lipid profile, at the expense of palmitate (C16:0, from 9% to 6.7%) and oleate (C18:1, from 57.1% to 54.3%). Similar observations have also been reported for *Y. lipolytica* stains engineered for lipid production: improving monosaturated fatty acids synthesis can be beneficial both for cell growth and total lipid accumulation (Qiao et al., 2015). Finally, POA production by SS-8 over-expressing Ole1 reached 0.36 g/L, indicating a 1.4-fold increase compared with the original strain. Although the heterogeneous expression of ScOle resulted in a positive regulation of lipid composition to form more POA, no enhancement in total lipid accumulation has been observed. However, neither lipid nor POA accumulation was enhanced via the over-expression of Ole2, while its expression led to an obvious shift towards palmitate and linoleate (C18:2) at the sacrifice of POA and oleate (C18:1). Therefore, Ole1 might be the main Scd in *S. segobiensis* DSM 27193. Compared to the Scds in other oleaginous yeast and fungi that prefer to catalyse the desaturation of stearate (C18:0) (Wan et al., 2013), Ole1 in *S. segobiensis* DSM 27193 seems to have a relatively higher substrate preference for palmitate. More random mutations and rational design focused on Ole1 might therefore further enhance the POA synthesis ability of *S. segobiensis* DSM 27193.

According to the aforementioned results, *jen12*, *dgat* as well as *ole1* were selected for further combinational expression to enhance POA biosynthesis. The resulted engineered strain SS-11 accumulated up to 24.3% DCW of lipid, presenting a 143% increase compared to the wild type (Figure 3C). Furthermore, the cell growth of SS-11 was also observed to be better than the wild type. In the 5-L bioreactor scale, 57.2 g/L of DCW was obtained, with a lipid content of 42.3% per DCW containing 22.8% of POA. Overall, 5.52 g/L of POA was obtained (Figure 4F).

Xpk/Pta route introduction further improved POA economical production

The theoretical lipid yield from glucose is only 0.27 g/g (Kamineni et al., 2021). To improve the

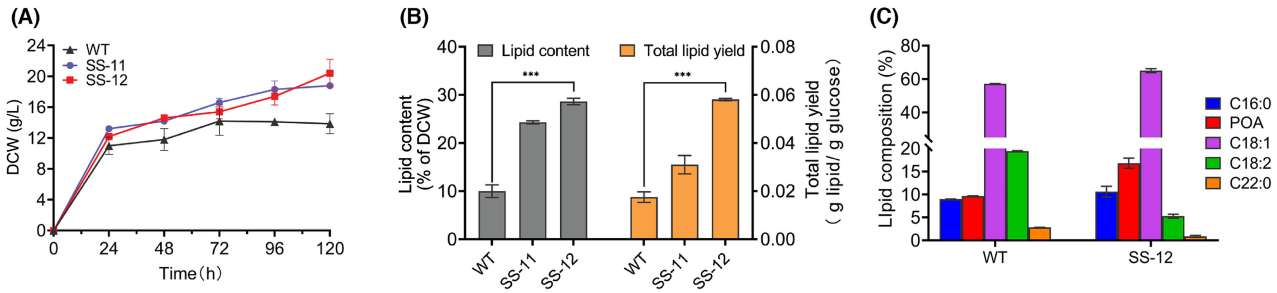

FIGURE 4 POA production by engineered SS-12 strain in shake flask. Comparison of cell growth (A) and lipid accumulation (B) among wild type *S. segobiensis* DSM 27193, engineered strain SS–11 and SS–12. (C) Lipid composition of engineered strian SS–12.

glucose-to-lipid yield, many strategies involving rewire the central carbon metabolism to improve the supply of lipid precursor acetyl-CoA and redox cofactor NADPH were designed (Cai et al., 2022; Gao et al., 2022; Ku et al., 2020; Qiao et al., 2017; Zhou et al., 2016). One such pathway is the phosphoketolase (Xpk)/phosphotransacetylase (Pta) pathway, in which, glycolysis intermediate fructose 6-phosphate (F6P) and/or the pentose phosphate pathway intermediate xylulose-5-phosphate (X5P) can be directly converted into acetyl phosphate (AcP) via carbon rearrangement catalysed by phosphoketolase (Xpk). Subsequently, AcP can be reversibly transformed into acetyl-CoA by phosphotransacetylase (Pta) (van Rossum et al., 2016). The Xpk/Pta route has been proven to provide acetyl-CoA and NADPH more efficiently than the route via glycolysis and Acl. Consequently, the theoretical lipid yield from glucose via Xpk/Pta route can theoretically reach up to 0.31 g/g (Kamineni et al., 2021). Previous investigation and genome analysis revealed that *S. segobiensis* DSM 27193 possesses a strong PPP pathway (Zhou, Xu, et al., 2021). The introduction of the Xpk/Pta pathway might therefore efficiently reallocate the carbon flow. Herein, the genes *xpk* (AN4913.2) from *A. nidulans* and *pta* (AIY95081.1) from *B. subtilis* were synthesized after codon modification and then over-expressed in SS-11.

The resulting recombinant strain SS-12 exhibited encouraging performance in cell growth, lipid accumulation and POA synthesis. Specifically, 20.4 g/L of DCW were obtained by SS-12 in shake flask fermentation within 120 h fermentation, corresponding to a 43.7% increase in the wild type (Figure 4A). The upward trend of cell growth indicated that higher DCW could be achieved with prolonged fermentation time. The intracellular lipid content reached 28.5% of DCW, showing a 156.8% increase compared to the wild type, leading to a ~5-fold increase in total lipid production. In terms of POA production, 16.7% POA in total intracellular lipid corresponding to a titre of 0.97 g/L was achieved by strain SS-12, exhibiting also a ~5-fold increase compared with the wild type (Figure 4B,C). Further, scale-up in a 5 L bioreactor under O-S dynamic regulation strategy control resulted in 59.6 g/L of final biomass concentration and 49.6% of lipid accumulation, corresponding to a lipid titre of 29.6 g/L (Figure 5A,B). In addition, no ethanol has been detected during the whole fermentation process, and <1.9 g/L of pyruvate as the main by-product was accumulated. The lipid composition showed that POA content reached 24.9%, which was 1.5-fold higher than that in the parent strain. As a sacrifice, the content of palmitate was decreased from 14.1% to 9.6%, and the content of oleate also exhibited a slight decrease from 61.6% to 58.1% Finally, strain SS-12

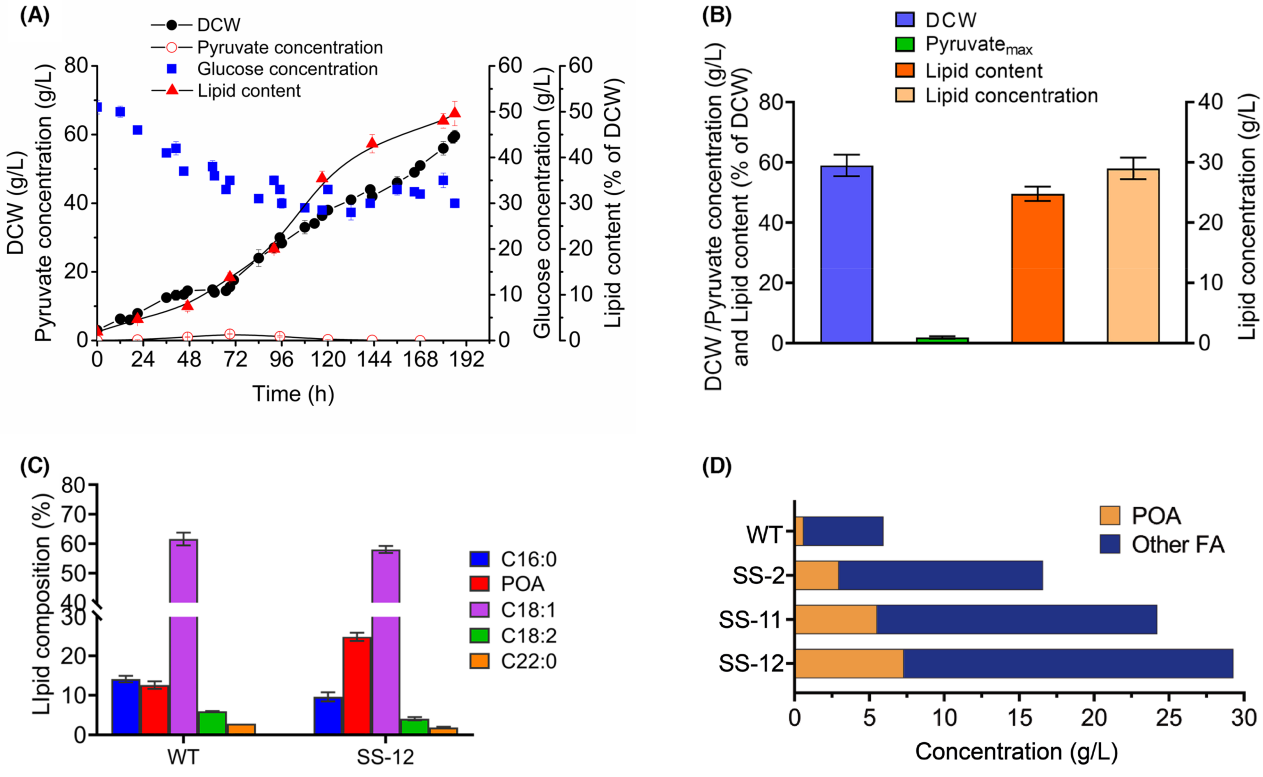

FIGURE 5 POA production by engineered SS-12 strain in 5 L bioreactor using O-S dynamic regulation strategy. (A) The fermentation process, (B) the final results of the fermentation, (C) lipid accumulation, (D) a comparison of POA and total lipid production between SS-12 and the original strain. The experiment was performed in triplicates.

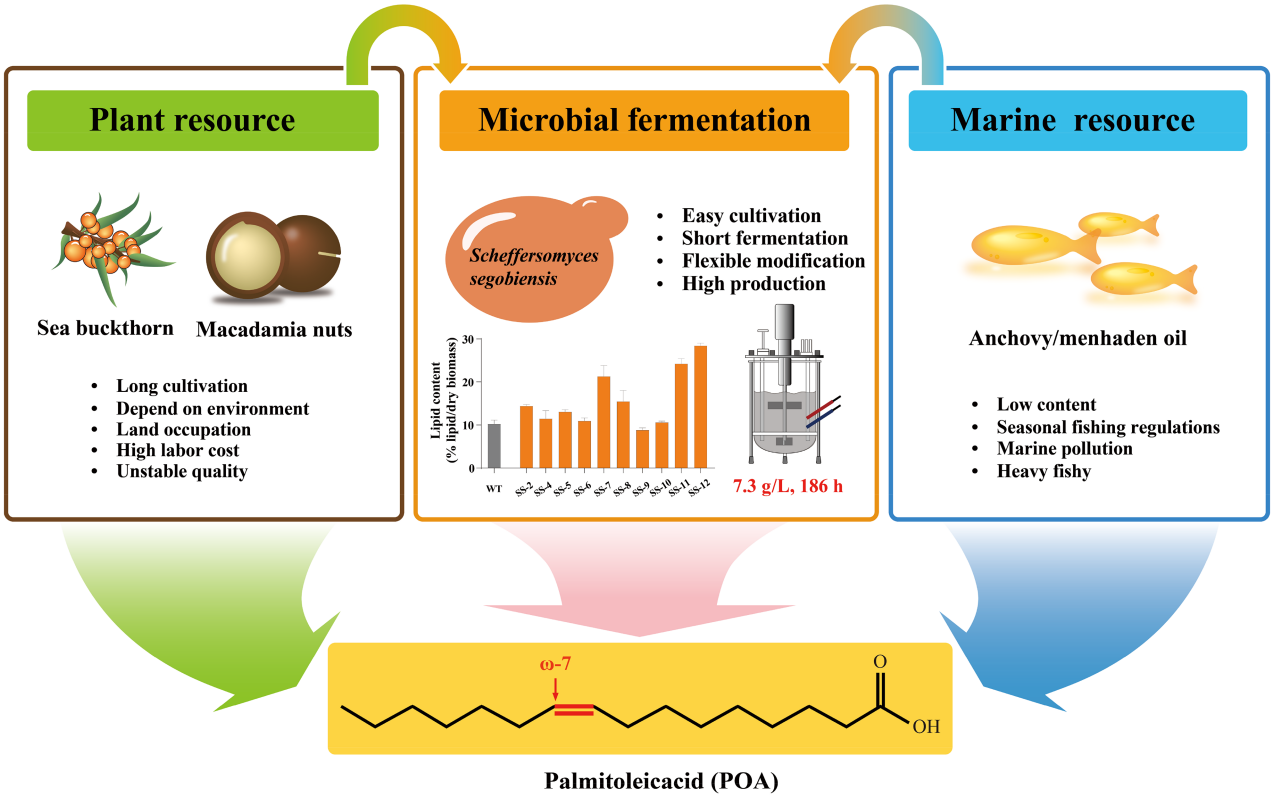

FIGURE 6 Microbial fermentation offers another promising approach for POA production.

produced 7.3 g/L of POA in the bioreactor process, implying an 11-fold improvement towards the original strain (Figure 5d).

Economic feasibility of POA biological production through fermentation

The companies located in China, such as Puredia (http://ynwkoem.com/cpzx) and Wuhan Chemstan Biotechnlogy Co., Ltd. (http://www.chemstan.com/intro/1.html), have attempted to extract POA-rich oil from Sea Buckthorn. Here, we did an evaluation of POA extraction from this resource. The total planting area of Sea buckthorn in China is 12,736 km^2, among which 7056 km^2 is artificial cultivation (http://www.gov.cn/xinwen/2022-06/10/content_5695086.htm). Every year, 80,000–100,000 tons of Sea buckthorn are picked and further processed. The calculation results show that 70–88 square meters of land are needed to harvest 1 kg of Sea buckthorn. In addition, Sea buckthorn grows very slowly and usually bears fruit after 6 years of cultivation, which can be harvested once a year. Consequently, the adoption of valuable purified POA from Sea buckthorn is not an economical approach, which may be one of the main reasons why POA-containing pharmaceuticals have not been exploited in China.

In order to better estimate the cost benefits of microbial POA production by using engineered *S. segobiensis*, a preliminary cost assessment based on the raw materials, utilities and facility-dependent costs was performed. Except for the small demand for trace elements, the industrial prices of the raw materials used in the fermentation medium are listed in Table S5. The purchase cost of electricity and cooling water were ¥1.2/kWh (186 h of fermentation) and ¥4.1/*t* (~1 t/day/bioreactor), respectively. The facility-dependent costs were estimated as 8% of direct fixed costs. Except for the labor cost, the cost for materials is calculated to be ¥38,000 for 1 kg of POA adoption, which was similar to the plant extraction. However, the whole fermentation process is time saving, space efficient, stable and controllable, presenting multiple advantages compared to the plant/fish extraction (Figure 6). However, the labor cost always accounts for a considerable cost assessment. Herein, more genetic modifications and advanced fermentation scale-up strategies are still needed to further improve POA titers and shorten fermentation time in the future.

CONCLUSIONS

Microbial POA production has the potential to replace current fish and/or plant extraction. In this study, a

highly efficient microbial cell factory for the artificial production of POA was developed using *S. segobiensis* as a host. In combination with the fermentation process regulation, the final POA production of 7.3 g/L was obtained. To the best of our knowledge, this is the highest report for POA production by fermentation. Except for the POA synthesis capability, *S. segobiensis* DSM 27193 is also attractive with respect to high cell density, broad substrate spectrum (especially for pentoses) and strong tolerance to fermentation environment. In the future, metabolic engineering focusing on POA excretive production from cheap biomass substrates will further reduce or eliminate the cost of cell harvest, disruption, lipid extraction and product separation, which cannot be realized by plant production.

AUTHOR CONTRIBUTIONS

Xiujuan Qian: Data curation (equal); funding acquisition (lead); investigation (lead); methodology (lead); project administration (lead); writing – original draft (lead); writing – review and editing (lead). **Huirui Lei:** Investigation (supporting); writing – review and editing (supporting). **Xinhai Zhou:** Data curation (supporting); formal analysis (supporting); investigation (supporting). **Lili Zhang:** Formal analysis (supporting); investigation (supporting). **Wenxing Cui:** Data curation (supporting); investigation (supporting). **Jie Zhou:** Investigation (supporting). **Fengxue XIn:** Funding acquisition (supporting); writing – original draft (supporting); writing – review and editing (supporting). **Weiliang Dong:** Project administration (supporting); supervision (equal); writing – review and editing (equal). **Min Jiang:** Methodology (equal); supervision (equal); writing – review and editing (supporting). **Katrin Ochsenreither:** Conceptualization (supporting); resources (equal); writing – review and editing (supporting).

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

DATA AVAILABILITY STATEMENT

Data that support the findings of this study are available upon reasonable request.

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SUPPORTING INFORMATION

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