De novo synthesis of nervonic acid and optimization of metabolic regulation by Yarrowia lipolytica

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Abstract

Nervonic acid, a natural fatty acid compound and also a core component of nerve fibers and nerve cells, has been widely used to prevent and treat related diseases of the brain nervous system. At present, fatty acids and their derivatives are mainly obtained by natural extraction or chemical synthesis which are limited by natural resources and production costs. In this study, the *de novo* synthetic pathway of nervonic acid was constructed in *Yarrowia lipolytica* by means of synthetic biology, and the yield of nervonic acid was further improved by metabolic engineering and fermentation optimization. Specially, heterologous elongases and desaturases derived from different organism were successfully expressed and evaluated for their potential for the production of nervonic acid in *Y. lipolytica*. Meanwhile, we overexpressed the genes involving in the lipid metabolism to increase the nervonic acid titer to 111.6 mg/L. In addition, the potential of adding oil as auxiliary carbon sources for nervonic acid production by the engineered *Y. lipolytica* were analyzed. The results indicated that supplementation with colleseed oil as an auxiliary carbon source can be beneficial for the nervonic acid productivity, which led to a highest concentration of 185.0 mg/L in this work. To summary, this study describes that the *Y. lipolytica* can potentially be used for a promising platform to produce nervonic acid and other very long chain fatty acid.

Introduction

Nervonic acid is a natural fatty acid compound and also a core component of nerve fibers and nerve cells. As a necessary fatty acid nervonic acid is essential for brain development and maintenance of neuronal biosynthesis and improvement. It can be used to prevent and treat related diseases of the brain nervous system such as mental disorders, cognitive disorders and so on (Tanaka et al. 2007). Studies have shown that nervonic acid can inhibit HIV-1 RT activity in a dose-dependent manner as a non-competitive inhibitor (Kasai et al. 2002). Individual neurotic acid levels are strongly associated with a higher risk of psychiatric disorders, and therefore several neurological disorders, such as demyelinating diseases, can be treated by neurotic acid supplementation (Amminger et al. 2012; Raoul et al. 2001; Vozella et al. 2017). Nervonic acid is also a natural component of breast milk, it can assist in the development of the infant's nervous system and promoting their growth. It has proved that increasing the content of nervonic acid in the daily diet of mice can improve the energy metabolism in mice, which may be an effective strategy for the treatment of obesity and obesity complications (Kepple Y et al. 2020). Nervonic acid has a valuable biological function, which makes it play an important role in pharmacological and nutritional applications (Li et al. 2019). Currently, nervonic acid has been extracted from plant tissues or obtained by chemical synthesis. These extraction methods have different limitations. By means of chemical synthesis, the yield of nervonic acid is very low and there are many byproducts (Rongkai et al. 2018). The most commonly used method extracting neurotic acid from plants is the at present, but the process is limited by the growth cycle and climatic condition. Therefore, it is time to explore a green and feasible way to biosynthesis nervonic acid.

In the past, great progress has been made in the biosynthesis of fatty acids by means of microorganisms (Rongkai et al. 2018). The development of synthetic biology and metabolic engineering has greatly facilitated the manipulation of microbial metabolic pathways and has significantly contributed to the production of various chemicals (Li et al. 2019). For example, the filamentous fungi *Mortierella capitata RD000969* isolated from soil can accumulated nervonic acid for 6.94% of the total fatty acid (Umemoto et al. 2014). In *Saccharomyces cerevisiae*, β-estradiol inducible expression system (EIES) was used to enhance the intracellular production of neuronic acid. Then the level of neuronic acid was further increased by overexpression of *KCS* and *ELOVL1* genes and knockout of ELO2 (Liu et al. 2020). It has been reported that through the screening and expressing of elongation genes (3-ketoacyl-CoA synthases, KCS) from different plant sources, the production of neuronic acid was realized in *Rhodosporidium toruloides* (Fillet et al. 2017b). The study has proved that the copy number of KCS gene and the push/pull strategy for KCS gene preference increased the contents of C24:1 and C22:1 fatty acid. By optimizing the fermentation conditions, the yield of erucic acid and neuronic acid in the 7 L bioreactor reached 20–30% of the yield of very-long chain fatty acids.

Yarrowia lipolytica, as a GRAS grade yeast strain, is one of the most studied "unconventional" yeast species (Bourdichon et al. 2012). *Yarrowia lipolytica*, due to its capacity for synthesizing and secreting hydrolytic enzymes like proteases and lipases (Fickers et al. 2005), is predominantly found in oily sewage and soil (Hassanshahian et al. 2012). It is also commonly exists in fatty and protein-rich foods such as cheese (Groenewald et al. 2014), dairy products, meat and sausages (Fickers et al. 2005). *Y. lipolytica* has complex intimal structure which enables it to have a high storage capacity of neutral lipids (mainly triacylglycerol), and to grow rapidly and produce lipids at a rapid rate (Beopoulos et al. 2009). *Y. lipolytica* has a wide carbon source spectrum, and since it often exists in an environment rich in hydrophobic substrates (such as alkanes or lipids), it has developed a complex mechanism to efficiently use hydrophobic substrates as the only carbon source (Fickers et al. 2005). Moreover, there are several gene families involved in the metabolic pathway of hydrophobic substrates, which are conducive to the uptake of more diverse hydrophobic substrates and lipid accumulations. Based on the above mechanism, strain can accumulate lipids that exceed 50% of cell dry weight (Beopoulos et al. 2009). The strong tolerance of *Y. lipolytica* to fluctuating pH values, salt concentrations and various organic compounds simplifies and optimizes biological processes and promotes the use of non-glucose-based feedstock (Miller et al. 2019). The genome of *Y. lipolytica* has been sequenced and gene-editing tools developed and used are becoming more sophisticated (Liu et al. 2014). There is a natural fatty acid synthesis pathway in *Y. lipolytica*. Once the glucose enters the cytoplasm, it goes through glycolytic pathway (EMP) and eventually becomes pyruvate, which has three carbon atoms, and then it goes to the mitochondria, where it ends up with acetyl-CoA (Vorapreeda et al. 2012). Acetyl-CoA which is an important precursor involved in fatty acid biosynthesis, can be produced through a variety of metabolic pathways, such as ATP citrate lyase (ACL) catalyzing the degradation of citric acid, fatty acids through β-oxidative degradation and acetyl-CoA synthase conversion to acetic acid. Under nitrogen restriction, mitochondria secrete citric acid and then forms malonyl-CoA catalyzed by acetyl-CoA carboxylase (ACC1). Acyl-CoA with 16 and 18 chain lengths which generated with acetyl-CoA as the starting point and malonyl-CoA as the elongation unit

was further extended and desaturated with 16:0 and 18:0 activated molecules as the precursor to obtain fatty acids with various chain lengths and saturation (Beopoulos et al. 2009). Each elongation consumes two molecules of NADPH, of which NADPH is derived in two ways. One is through the malate dehydrogenase catalyzed decarboxylation reaction in the cytoplasm another is pentose phosphate pathway (Wasylenko et al. 2015).

During the preparation of this manuscript, Wang et al. engineered *Y. lipolytica* to produce up to 57.48 g/L of microbial oil with 23.44% nervonic acid in fed-batch fermentation; the highest production titer so far described in *Y. lipolytica*. The authors combined orthogonal plant and non-plant fatty acid biosynthesis pathways in *Y. lipolytica*, used a “block-pull-restrain” strategy to increase precursor production, and strengthened TAGs synthesis to improve lipid pool (Wang et al. 2023).

In this work, we constructed the *de novo* synthesis of nervonic acid in oleaginous yeast *Y. lipolytica* (Fig. 1). In order to further improved the production of nervonic acid, the elongation genes and desaturation genes in the process of nervonic acid synthesis were screened and overexpressed in *Y. lipolytica*. Meanwhile, the expression patterns of different combinations of key genes were explored to further enhance the production of nervonic acid. Moreover, we analyzed the potential of different auxiliary carbon sources for the production of nervonic acid by *Y. lipolytica*, and first found that colleseed oil as auxiliary carbon source was helpful to increase nervonic acid production.

Materials and methods

Plasmids, strains and medium

Y. lipolytica strain ATCC MYA2613 (Po1f), which was the initial strain of the engineered strains. Construction and amplification of plasmids were dependent on *E. coli* strain JM109, which was cultured in Luria-Bertani (LB) medium and grew at 37°C. LB medium (10 g/L yeast extract, 20 g/L peptone, 10 g/L NaCl, and 15 g/L Bacto agar) was added with different resistance to construct plasmids such as 50 mg/L of kanamycin and 100 mg/L of ampicillin. The *Y. lipolytica* strains were cultivated at 30℃ in YPD medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose and 15 g/L Bacto agar). The YNB medium, which contains 6.7 g/L yeast nitrogen base without amino acids, 20 g/L glucose and 15 g/L Bacto agar, was used to screen transformants by adding 100 mg/L leucine or uracil. In this study, 5-fluoroorotic acid (1 g/L of 5-FOA) was added to YPD medium for the recovery of URA3 screening markers. All strains constructed and used in this study are listed in Table 1.

Table 1
Strains used in this study

Strains	Descriptions	Source
E. coli JM109	recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1, Δ(lac-proAB)/F[traD36, proab$^+$, lacI^q, lacZΔM15]	Invitrogen
Y. *lipolytica* Po1f	MatA, leu2-270, ura3-302, xpr2-322, axp1-2	(Nicaud 2012)
GQY-ΔPEX10	Po1f-ΔPEX10	(Qi Gao et al. 2018)
GQ06	Po1f-ΔPEX10 integrated MaELO3 by CRISPR/Cas9 at F1 site	(Gao et al. 2020b)
NA01	GQ06 integrated optimized AtKCS by CRISPR/Cas9 at A3 site	This study
NA02	NA01 integrated optimized CraKCS by CRISPR/Cas9 at F1-3 site	This study
NA03	NA01 integrated optimized CgKCS by CRISPR/Cas9 at AXP site	This study
NA04	NA03 integrated optimized CraKCS by CRISPR/Cas9 at F1-3 site	This study
NA05	NA04 cells harboring pINA1312-P_{UT}- MaD15D	This study
NA06	NA04 cells harboring pINA1312-P_{UT}- CsD15D	This study
NA07	NA04 integrated optimized CgKCS-L-MaD15D by CRISPR/Cas9 at A1-2 site	This study
NA08	NA07 integrated optimized CgKCS-L-MaD15D by CRISPR/Cas9 at E1-3 site	This study
NA09	NA04 cells harboring pINA1312-P_{UT}- DGA1	This study
NA10	NA08 cells harboring pINA1312-P_{UT}- OLE1	This study
NA11	NA08 cells harboring pINA1312-P_{UT}- DGA1-L-OLE1	This study
NA12	NA08 cells harboring pINA1312-P_{UT}- OLE1-L-DGA1	This study
NA13	NA12 cells harboring pINA1269- OLE1-L-DGA1	This study
NA14	NA10 cells harboring pINA1269-DGA1	This study
NA15	NA12 cells harboring pINA1269-DGA1	This study
NA16	NA12 cells harboring pINA1269-MaELO3	This study
NA17	NA12 cells harboring pINA1269-MaELO3-AtKCS	This study
NA18	NA12 cells harboring pINA1269-MaELO3-CraKCS	This study

Strains	Descriptions	Source
NA19	NA12 cells harboring pINA1269-CgKCS	This study
NA20	NA04 cells harboring pINA1269-ACL	This study
NA21	NA04 cells harboring pINA1269-ACS2	This study
NA22	NA04 cells harboring pINA1269-ACC1	This study
NA23	NA20 cells harboring pINA1312-ACS2	This study
NA24	NA04 integrated FAA1 by CRISPR/Cas9 at MFE site	This study

Construction of plasmids and yeast transformation

In this study, two integrative plasmids, pINA1312 and pINA1269, and CRISRPR/Cas9 system were used for metabolic engineering modification of the strains. All constructed strains are shown in Table 1. The elongation enzyme gene (*CgKCS*) from *Cardamine graeca* and the Δ15 desaturase genes (MaD15D/CsD15D) from *Mortierella alpine* and *Cannabis sativa* were synthesized and coded optimally. Primers were designed to amplify target genes by PCR, and the amplified genes were linked to plasmids pINA1312 or pINA1269 that had been digested by the ClonExpress® II One Step Cloning Kit (Vazyme Biotech, Nanjing, China). Then the recombinant plasmid with the target gene expression cassette was obtained. The primers involved in this study are all shown in Table S1. In order to achieve efficient gene expression, expression cassttes with different promoter strength and different terminator were constructed. After construction of the recombinant plasmid, it was linearized by the corresponding enzyme and then transferred into yeast cells by Frozen-EZ yeast transformation II Kit (Zymo Research, Irvine, CA).

The CRISPR/Cas9 system is able to knock out the gene and knock-in the target gene at the same time. Taking the *CgKCS* gene which was inserted into the AXP site as an example, primers with 20 bp homologous sequences at both ends of insertion site were used to obtain the amplified *CgKCS* expression cassette by PCR. Then plasmid pHR_AXP_hrGFP digested with *SpeI* and *AvrII* connected with *CgKCS* expression cassette to obtain recombinant plasmid pHR_AXP_CgKCS. Finally, the single gRNA and recombinant plasmid pHR_AXP_CgKCS were transformed into corresponding yeast cell together. All the primers used and plasmids constructed were shown in Table S2-S3.

Growth condition and auxiliary carbon source

Y. lipolytica strains were cultured in 2 mL YPD at 30°C (220 rpm) and then inoculated in 250 mL triangular flask containing 50 mL YPD with an initial OD_{600} of 0.01. The strains were cultured for 72 hours under the same conditions. Adding 0.25 mL of different carbon sources (ω-9 octadecanoic acid, soybean oil, colleseed oil, sunflower seed oil, waste cooking oil) to 50 mL YPD. On this basis, gradient experiments of colleseed oil supplemental levels were designed, such as 0, 0.25, 0.5, 0.75, 1.0, and 1.25 mL added to 50 mL YPD.

Extraction of VLCFAs

20 mL of the fermentation medium was taken into a 50 mL centrifuge tube and centrifuged at 6,000 rpm for 5 min. The supernatant was discarded, and then 15 mL of ddH_2O was added to the centrifuge tube. The mixture was thoroughly mixed and subjected to centrifugation under the same conditions. After repeating the above procedure, added 5 mL 4 M HCl to the collected cells. The mixture was oscillated and then held for 30 minutes at 37℃ at 220 rpm. Next, the test tube was kept in boiling water bath and ice for 5 minutes, and the operation was repeated again. Then 20 mL of methanol and chloroform mixed solution was added into the test tube, in which the volume ratio of methanol to chloroform was 1:2. After 30 minutes at 37°C, the underlying liquid was centrifuged (4800 rpm, 5 min) and then sucked into a glass tube and dried in an oven at 105°C. After about 12 h, taking the test tube out and then adding 3 mL of 0.5 mol/L methanol potassium hydroxide solution into the test tube when the test tube is restored to room temperature. Ultrasound was used to dissolve the oil in the tube, and the tube was kept in a water bath at 75℃ for 20 min. Adding 3 ml of 14% boron trichloride solution to the test tube and keep the same condition for 20 min. Then taking out the tube, and adding 1 mL saturated NaCl and 0.5 mL n-hexane in it. The mixture was thoroughly mixed and the upper solution was centrifuged at 12000 rpm for 2 min. Then dilute the upper liquid and mix it with the internal standard at a volume ratio of 1:4 to get the sample to be tested.

Gas chromatography coupled with mass spectrometry (GC – MS) analysis of VLCFAs

The sample was analyzed by GC-MS which was carried out using an Agilent System 6890 gas chromatograph (GC) with an Agilent 5975 quadrupole mass selective detector (MSD) equipped with a HP-5 column (30 m × 0.25 mm × 0.25 μm, Agilent, Santa Clara, CA, USA). The initial temperature of GC was held at 150°C for 2 min, and then at a rate of 20°C/min to 180°C. And then it went up to 200°C at a rate of 8°C/min. Then in 18 minutes the temperature reached 218°C, raised to 250°C at 8°C/min. The temperature subsequently raised to 300°C in 3.4 min. The split ratio was 20:1. The quantitative analysis was carried out by the corresponding fatty acid methyl ester standards.

Results and discussion

De novo synthesis of nervonic acid in Y. lipolytica

We previously engineered *Y. lipolytica* to produce VLCFAs with carbon chain lengths up to 24 by co-expression heterologous C16/18-elongase from *Mortierella alpina* (MaELO3), β-ketoacyl-CoA synthases (KCSs) from *Arabidopsis thaliana* (AtKCS) and *Crambe abyssinica* (CraKCS) combining with the deletion of *PEX10* (Gao et al. 2020). Although VLCFAs metabolism was successfully engineered, the resulting strain GQ07 only accumulates marginal nervonic acid (C24:1), and the titer needs to be further improved. Owing to the limitation of auxotrophic markers of plasmids, here, we re-engineered VLCFAs metabolism pathway into chromosome using the recently established CRISPR/Cas9 technology without the selection

marker (Schwartz et al. 2016). The use of hybrid promoter UAS4B-TEF (UT) provided an excellent platform for high gene expression in *Y. lipolytica* (Gao et al. 2018). We therefor used this for the over-expression of the codon-optimized *MaELO3*, *AtKCS*, and *CraKCS* genes into the integration sites F1, A3, F1-3 of *Y. lipolytica* GQY-ΔPEX10 strain, respectively. Previous studies confirmed that *Cardamine graeca* KCS enzyme has the ability to elongate erucoyl-CoA (C22:1-CoA) to nervonic acid by in vitro activity assays (Taylor et al. 2009). In another work, heterologous expression of *C. graeca* KCS in *Rhodosporidium toruloides* efficiently catalyzed all elongation steps to produce nervonic acid (Fillet et al. 2017a). To elucidate the effects of *CgKCS* overexpression on nervonic acid production in *Y. lipolytica*, the codon-optimized *CgKCS* was integrated into the AXP stie by CRISPR/Cas9 technology in the *MaELO3*, *AtKCS*-expressing background strain (NA01), yielding strain (NA03). As shown in Fig. 2, strain NA03 can produce about 18.2 mg/L of nervonic acid, which is approximately 4-fold than that of strain NA01. These results clearly showed that the chain length of VLCFs could be selectively modulated by engineering different source of KCS. Consisting with previous reports, *CgKCS* gene could efficiently push elongation of the erucoyl-CoA pool to nervonic acid (Fillet et al. 2017a). Simultaneously overexpression *MaELO3*, *AtKCS*, *CraKCS* and *CgKCS* genes obtaining strain NA04 led to the production of 20.8 mg/L neurotic acid, this strain was used as a host strain for the following genetic manuscript.

Explore desaturase of neurotic acid synthesis

The fatty acid profile of the engineered *Y. lipolytica* NA04 strains revealed that the rewritten the elongation pathway can improve the accumulation of nervonic acid. However, cells engineered also resulted in high amounts of C24:0 saturated fatty acid (lignoceric acid), which indicated that the desaturation step from lignoceric acid to nervonic acid was rate limiting. We thus speculated that introduction of heterologous desaturation pathway would further enhance nervonic acid production. Nervonic acid is produced from lignoceric acid catalyzed by the enzyme Δ-15 desaturase (D15D). Several D15D have been identified until now, out of which we selected two D15D from *Mortierella alpina* (MaD15D) and *Cannabis sativa* (CsD15D) for expression and characterization in *Y. lipolytica* NA04 strain under the control of hybrid promoter UAS4B-TEF (UT) using plasmid pINA1312(Wang et al. 2011; Bielecka et al. 2014). To ensure efficient expression of the D15D, the gene sequences were codon optimized for expression in *Y. lipolytica*. As shown in Fig. 3, CsD15D gave the less effects on titer of nervonic acid, while MaD15D gave the better performance on production of nervonic acid with a titer of 49.4 mg/L, 2.4-fold increase. These results illustrated that both of the elongation pathway and desaturation pathway are important for nervonic acid biosynthesis in *Y. lipolytica*.

To optimize the KCS and D15D expression, and release the auxotrophic markers as well, we tried to fuse CgKCS with MaD15D with a (GSG) linker between CgKCS and MaD15D (*CgKCS-L-MaD15D*) in the chromosome of *Y. lipolytica* NA07 strain using established CRISPR/Cas9 technology. However, while one copy of *CgKCS-L-MaD15D* was introduced into the A1-2 site of *Y. lipolytica* NA07 strain, not necessarily improve nervonic acid production was found, instead a slight decrease in nervonic acid titer was observed. The reason could be due to the low expression of the fusion. As such, an extra copy of *CgKCS*-

L-MaD15D was introduced into the E1-3 site of *Y. lipolytica* NA07 strain resulting strain NA08. As shown in Fig. 3A, increasing the fusion copy of *CgKCS-L-MaD15D* in strain NA04 significantly enhanced the production of nervonic acid to 32.1 mg/L in shake flask culture. At the meantime, the amount of lignoceric acid produced by NA08 were 255.1 mg/L, which were 7.3-fold than that for control strain NA04. The FA profiles of the new engineering strain and the control strain were compared. The strain NA08 was found to synthesize more VLCFA (C20-C24) than the control strain NA04, while the C18:2/1 fatty acid content was reduced (Fig. 3B).

Overexpression of genes OLE1 and DGA1 leads to significant increases in nervonic acid accumulation.

Diacylglycerol-acyltransferase (DGAT) catalyzes the acylation of diacylglycerol using acyl-CoA as the acyl donor. This enzyme has been postulated to be a main enzyme in boosting lipogenesis because it catalyzes the last step in TAG synthesis (Blazeck et al. 2014; Gajdos et al. 2016; Tai et al. 2013). The integrative vector pINA1312 carrying the *DGA1* gene under the control of hybrid promoter UAS4B-TEF (UT) was successfully integrated into the chromosome of NA04 strain. After 96 h cultivation, homologous recombinant of *DGA1* significantly enhanced neurotic acid-producing level, which increase 1.8-fold compared to the NA04 strain (Fig. 4A). Meanwhile, percentage of FA distribution showed a very different between the two engineered *Y. lipolytica* strains NA04 and NA09. A large reduction in C16:0 and C18:1/2 content was observed in strain NA09 resulting in an increase in the VLCFA fraction (Fig. 4B). Therefore, the target gene *DGA1* was selected for subsequent genetic modification.

OLE1 of *Y. lipolytica* encodes the sole and essential Δ-9 stearoyl-CoA desaturase catalyzing the conversion of saturated to unsaturated fatty acids. Previous studies have shown that OLE1 is important for lipogenesis (Flowers et al. 2008; Qiao et al. 2015). Therefore, *OLE1* serve as an attractive engineering target to overproduce nervonic acid. To implement the identified target, we overexpressed the *OLE1* in the *Y. lipolytica* NA08 strain by introducing a native copy of the *OLE1* gene through integrated plasmid pINA1312 under the control of strong promoter UT resulting stain NA10. As shown in Fig. S1, overexpression of *OLE1* led to 24.4% increase in nervonic acid level over the control strain NA08. Acetyl-CoA is a critical metabolite carbon and energy metabolism involving in multiple key metabolic function (Gao et al. 2018; Huang et al. 2018). Here, we investigated the effects of overexpressing the key genes in acety-CoA metabolic pathway on the nervonic acid productivity in *Y. lipolytica*. The *ACL*, encoding the ATP-dependent citrate lyase, the *ACC1*, encoding the acetyl-CoA carboxylase from *Y. lipolytica*, and *ACS2*, encoding the acetyl-CoA synthetase gene, from *S. cerevisiae* were overexpressed in the background strain through integrated plasmid pINA1269. Though no obviously different of nervonic acid production was observed among the engineered strain, the overexpression of *ACC1* led to a C24:0 titer 4-fold higher than the control strain NA04 (Fig. S2). We wanted to evaluate whether increased supply of the precursor acetyl-CoA level could increase nervonic acid production. The main fatty acyl-CoA synthetase encoding gene *FAA1* was thus overexpressed on the *MFE* loci, which involving in -oxidation, in the background strain NA04 by CRISPR/Cas9 system. The resulting strain NA22 produce 28.3 mg/L nervonic acid in shake flasks, which was 1.36-fold higher than that of the control strain NA04 (Fig. S3). This strategy might be a potential way to improve nervonic acid production in *Y. lipolytica*.

Since the single overexpression of *DGA1* or *OLE1* boosted the titer of nervonic acid in flask culture, we then reasoned that simultaneous co-overexpression of *DGA1* and *OLE1* would further increase nervonic acid accumulation. And we also performed the fusion strategy to evaluate if the covalent joining of these two enzymes could improve the productivity level of nervonic acid. DGA1 and OLE1 were fused with an artificial flexible linker (GSG) as either DGA1-L-OLE1 or OLE1-L-DGA1, but only the OLE1-L -DGA1 fusion protein resulted in a 1.7-fold increased acid in engineered *Y. lipolytica* NA08 (Fig. 4C). We also tried different combinations of *DGA1*, *OLE1* and *OLE1-L-DGA1* obtained three different strains. In comparison, strain NA15, which simultaneously overexpressed *DGA1*and *OLE1-L-DGA1*, had the highest yield of nervonic acid (111.6 mg/L) among all combinations.

Elongation kcs gene copy number adjustment increased nervonic acid production in Y. lipolytica

To further develop a high-level nervonic acid production strain, we evaluated the impact of adjusting the gene dosage on nervonic acid yield. For this purpose, we adding an extra copy of four elongation genes *MaELO3*, *CraKCS*, *AtKCS* and *CgKCS* thought integrated plasmid pINA1269 to the strain NA12. As shown in Fig. 5A, only the extra copy of *MaELO3* enhanced the production of nervonic acid, the yield of nervonic acid increased by 63.9% and reached 90.6 mg/L. Meanwhile, the production of fatty acids C20:1 and C22:1 was significantly improved in the strain with extra copy of *CgKCS*. Since previous reports showed that increasing the copy number of *CgKCS* could boost the concentration of nervonic acid in *R. toruloides* (Fillet et al. 2017b), the inconsistent results might be caused by different genetic background of the stains.

Effect of oily substrates as auxiliary carbon sources for nervonic acid production by the engineered Y. lipolytica.

As an oleaginous yeast, *Y. lipolytica* can quickly grow to high densities with a high lipid content and utilize a large number of renewable substrates and inexpensive materials such as hydrophobic substrates, crude glycerol and lignocellulosic biomass as carbon sources (Ledesma-Amaro et al. 2016; Nambou et al. 2014; Poli et al. 2014). In order to screen the most suitable carbon source for the production of neuronic acid by *Y. lipolytica*, an auxiliary carbon sources such as colleseed oil, soybean oil, sunflower seed oil, waste cooking oil or oleic acid was supplemented to YPD medium (Fig. 6A). In this screening experiment of the auxiliary carbon sources, the strain NA02 was first used as the fermentation strain, and 0.25 mL of the auxiliary carbon source was added into the 50 mL YPD medium. As shown in Fig. 6A, the culture with colleseed oil as auxiliary carbon exhibited the highest nervonic acid productivity among all the auxiliary substrates used. In the medium with colleseed oil added, the yield of nervonic acid in strain NA09 reached 132.6 mg/L, which was 2.5-fold higher than that of control YPD medium. The effect of the concentration of colleseed oil as an auxiliary carbon source on neurotic acid production was evaluated by supplementation with colleseed oil at 0.25 to 1.25 mL in shake flask (Fig. 6B). The fermentation results showed that when the colleseed oil supplemental level was 0.5 mL or 0.75 mL, the yield of neuronic acid was the highest, which was 132.6 mg/L and 138.4 mg/L, respectively, about 3.6-fold improvement over the level observed in the medium without colleseed oil. Considering the cost

efficiency, for the following experiments we selected 0.5 mL colleseed oil adding into 50 mL fermentation medium.

To explore the reason why colleseed oil was the most suitable auxiliary carbon source for neuronic acid production in this study, the VLCFA profile of colleseed oil was analysis. For colleseed oil used here, C20:1 and C22:1 were the most abundant portion of the VLCFA, little amount of C24:1 and C24:0 were observed. *Y. lipolytica* GQY-ΔPEX10 was the strain that only deleted *PEX10*, a gene encoding a major peroxisomal matrix protein, from *Y. lipolytica* Po1f. When this strain was cultured in YPD supplemented with colleseed oil, for without any modification on elongation and desaturation, its profile of VLCFA was similar to that of colleseed oil. After metabolic engineering of the strain, the carbon flux was significantly drain to VLCFA, which demonstrated that the successful of our strategies to generate high neuronic acid production (Fig. 4S).

Finally, the performance of the best neuronic acid-producing strain NA15 in this study was assessed in YPD medium with or without colleseed oil. After 3 days fermentation, a neuronic acid production of 185.0 mg/L was achieved in the medium adding colleseed oil, approximately 1.6-fold higher than that without colleseed oil, which was the highest yield of neurotic acid in this study (Fig. 6C). Despite complex multistep engineering efforts, production titers in this study still lower than that of previous report.[24] However, the systematic engineering strategies of *Y. lipolytica* introduced in this study may provide a deep understanding of the biosynthesis of neurotic acids and other VLCFAs. It should nevertheless be pointed out that further improvements of neurotic acids production in *Y. lipolytica* will be expected using higher biomass concentrations and controlled bioreactor.

Conclusion

In summary, we engineered the oleaginous yeast *Y. lipolytica* following multi-level strategies for efficient accumulation of neurotic acid production. Specifically, we reconstructed the elongation pathway as well as desaturation pathway, optimized the key gene expression in fatty acid metabolism through adding gene copy and protein fusion. Furthermore, we first demonstrated that supplementing the colleseed oil as auxiliary carbon benefited the neurotic acid production. The yeast engineering strategy of pathway assembling presented in this study may be employed to optimize microbial production of other valuable VLCFA chemistry.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All of the authors have read and approved to submit it to *Bioresources and Bioprocessing*.

Availability of data and materials

All data generated or analyzed during this study are included in this article.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

XRZ designed experiments; XRZ and XLC conducted experiments, XRZ, XLC and JLY collected data; XRZ, XLC, JLY, GQ and JTS analyzed data; QH and LJW conceived the idea and supervised the research; XRZ, XLC and LJW drafted the manuscript and contributed to data interpretation. All authors read and approved the final manuscript.

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Figures

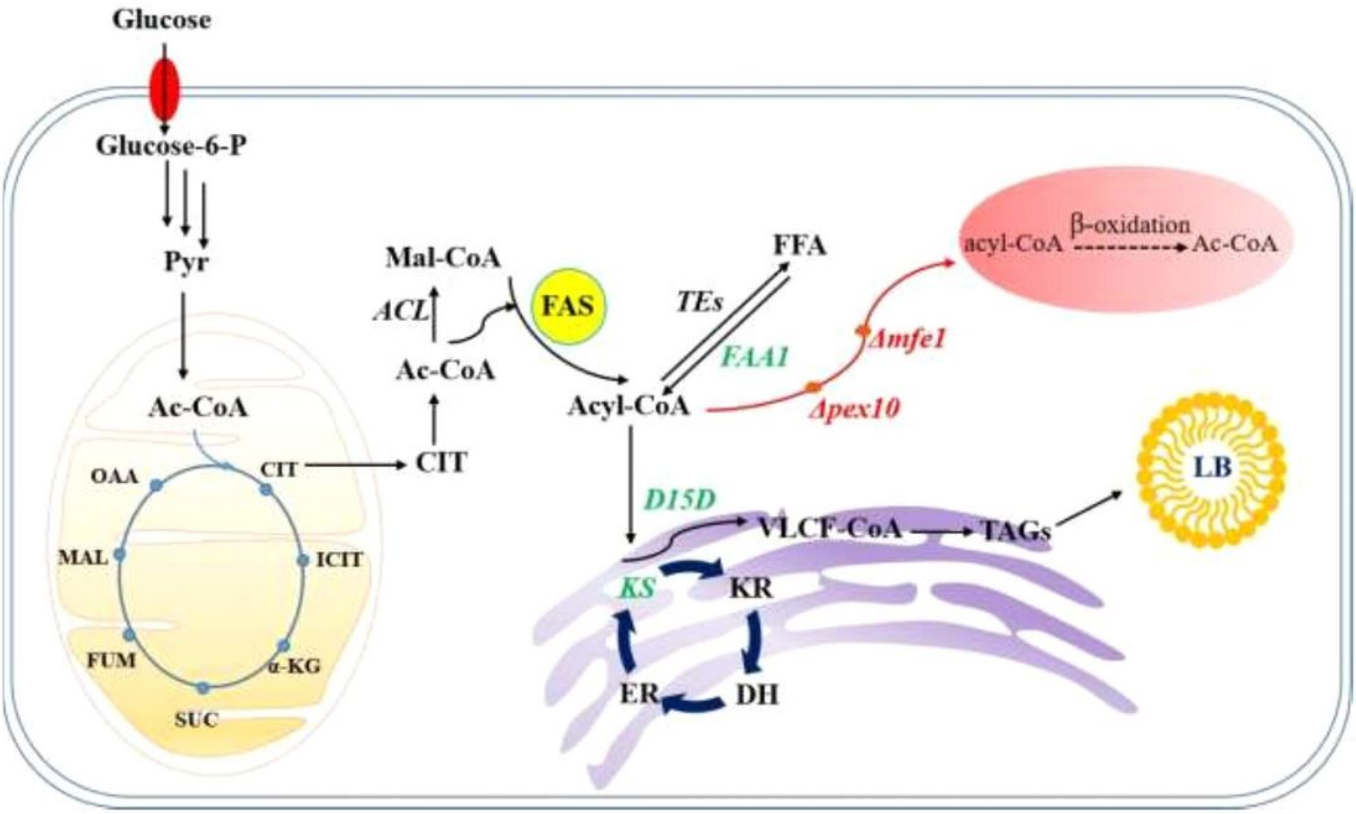

Fig 1

Figure 1

Biosynthesis pathway for neurotic acid production in the yeast *Y. lipolytica*. Neurotic acid is biosynthesized from the initiation unit of acetyl-CoA and the extension unit of malonyl-CoA with fatty acid synthetase. Green words represent the heterogeneous expression pathways; black words represent the native pathways; and red words represent selected for disruption in this study. Gene abbreviations: Pyr, pyruvic acid; Ac-CoA, acetyl coenzyme A; CIT, citric acid; Mal-CoA, Malonyl coenzyme A; FAS, fatty acid synthase; TEs, thioesterase; ACL, ATP-citrate lyase; FAA1, acyl-CoA synthetase; FFA, free fatty acid; KS, ketoacyl-CoA synthase; KR, 3-ketoacyl-CoAreductase; DH, 3-hydroxyacyl-CoA dehydratase; ER, enoyl-CoA reductase; D15D, Δ15 desaturase; TAG, triacylglycerol.

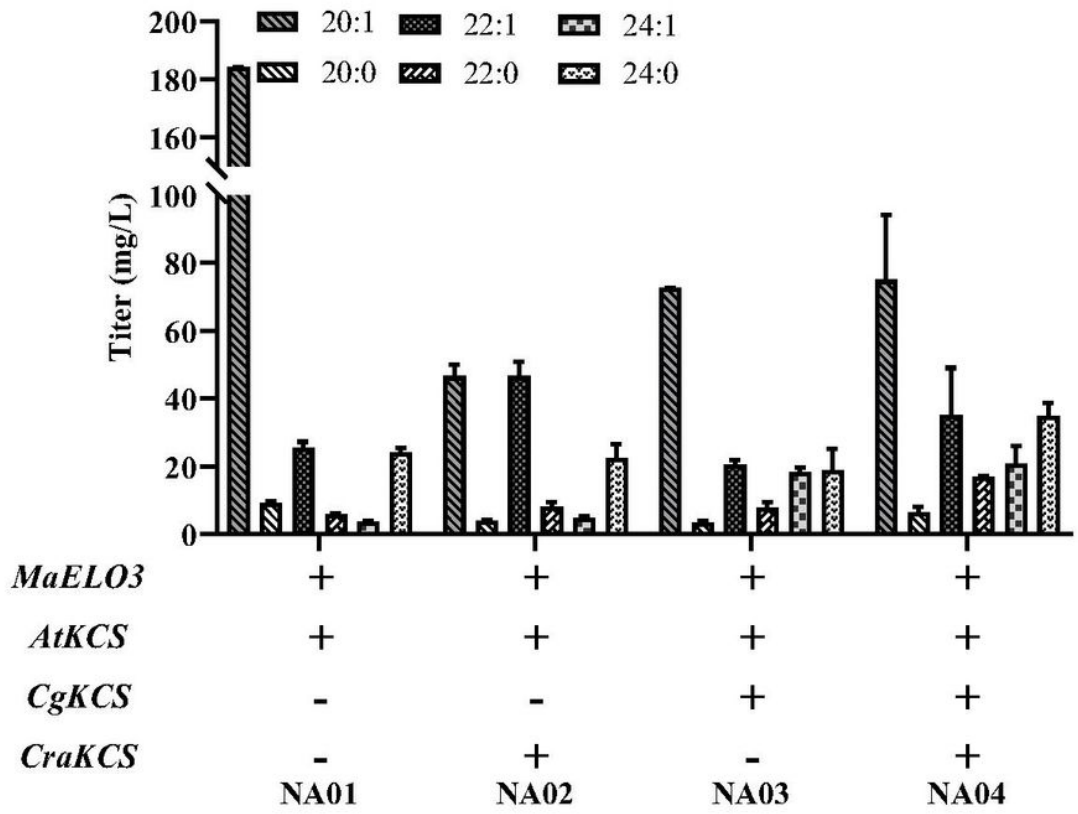

Fig 2

Figure 2

Effects of overexpression of elongation genes from different sources on nervonic acid production on solid medium (YPD). The data shows the average of two independent experiments, with the error bars representing standard deviations. *MaELO3*: *Mortierella alpina ELO3* gene; *AtKCS*: *Arabidopsis thaliana KCS* gene; *CgKCS*: *Cardamine graeca KCS* gene; *CraKCS*: *Crambe abyssinica KCS* gene.

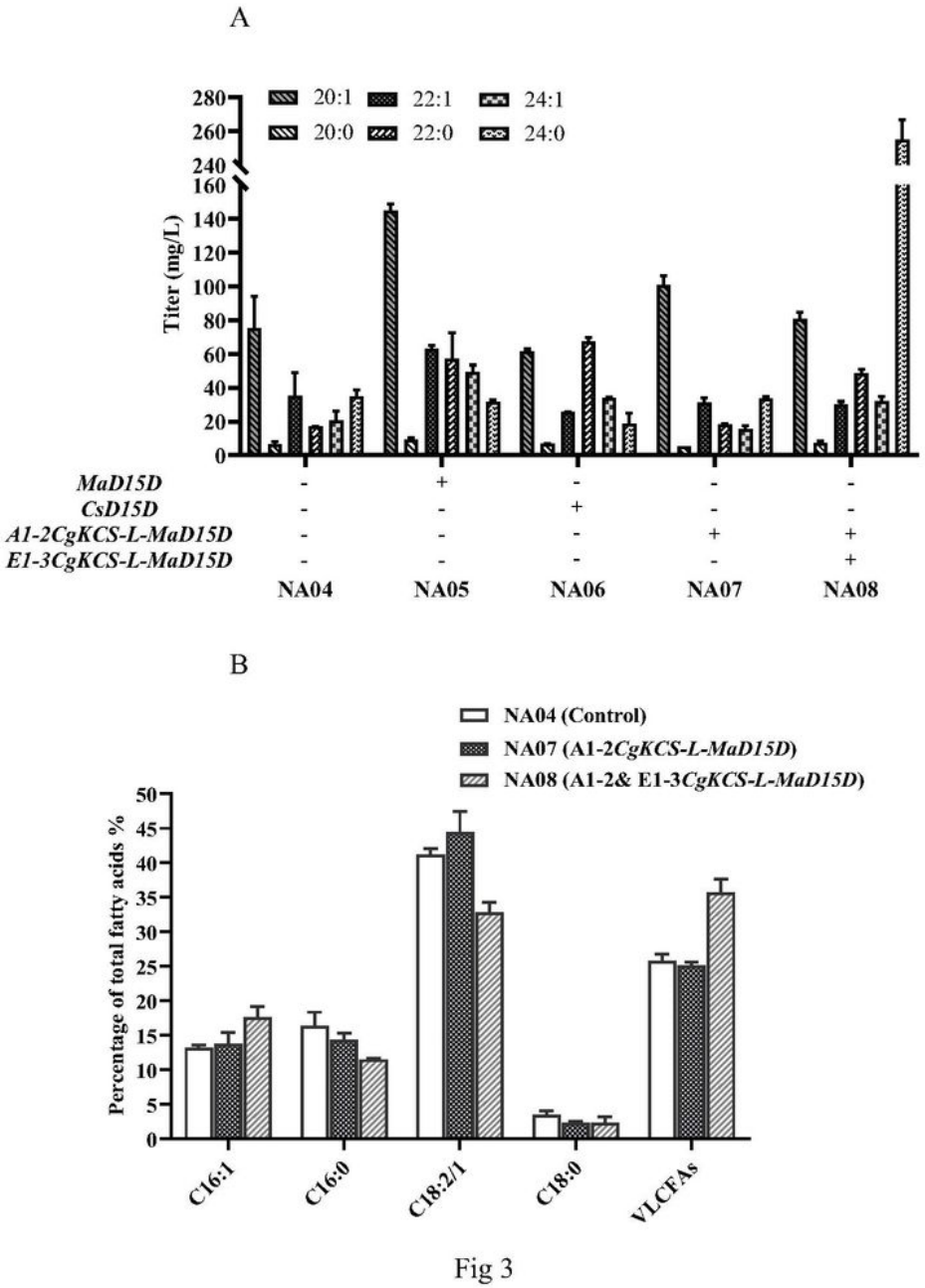

Fig 3

Figure 3

Heterologous desaturase expression in *Y. lipolytica*. (A) Screening the vary version of Δ15 desaturase and elongase for nervonic acid production. *MaD15D*: *Mortierella alpine* Δ15 desaturase gene; *CsD15D*: *Cannabis sativa* Δ15 desaturase gene. (B) Percentage of FA distribution in the engineered *Y. lipolytica* strains NA04 and NA08. The data are the averages of two biological replicates with error bars representing standard deviations.

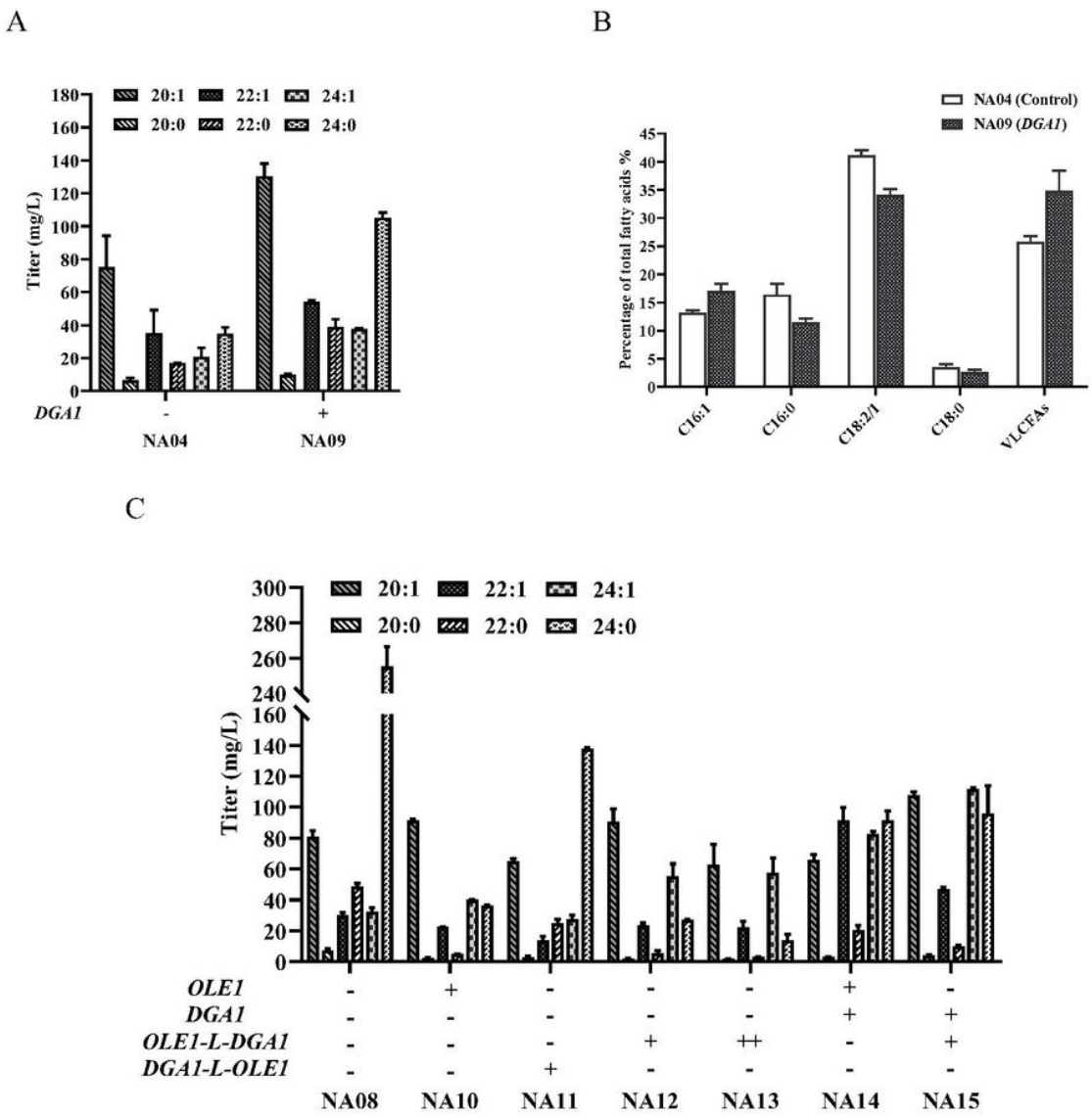

Fig 4

Figure 4

Effects of overexpression of genes *OLE1*and *DGA1* for the biosynthesis of nervonic acid in *Y. lipolytica*. (A) Overexpression of *DGA1* gene to improve nervonic acid production. (B) Percentage of FA distribution in the engineered *Y. lipolytica* strains NA04 and NA09. (C) Different combinations of *DGA1*and *OLE1* to increase nervonic acid production. The data are the averages of two biological replicates with error bars representing standard deviations.

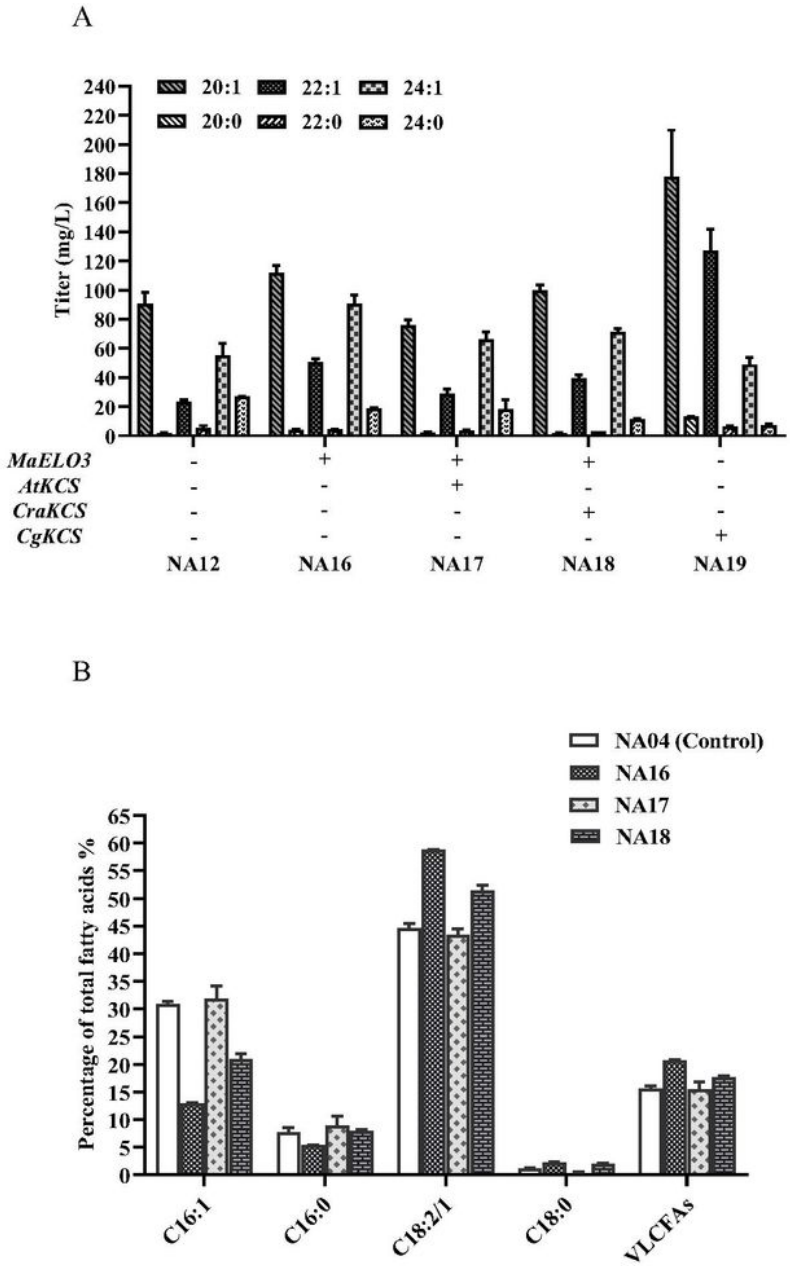

Fig 5

Figure 5

Improvement of nervonic acid production in *Y. lipolytica* through elongation *kcs* gene copy number adjustment. (A) An extra copy of *KCS* gene was overexpressed in NA12. *MaELO3*: *Mortierella alpina ELO3* gene; *AtKCS*: *Arabidopsis thaliana KCS*gene; *CgKCS*: *Cardamine graeca KCS* gene; *CraKCS*: *Crambe abyssinica KCS* gene. (B) Percentage of FA distribution in the engineered *Y. lipolytica* strains NA04, NA16-

18. The data are the averages of two biological replicates with error bars representing standard deviations.

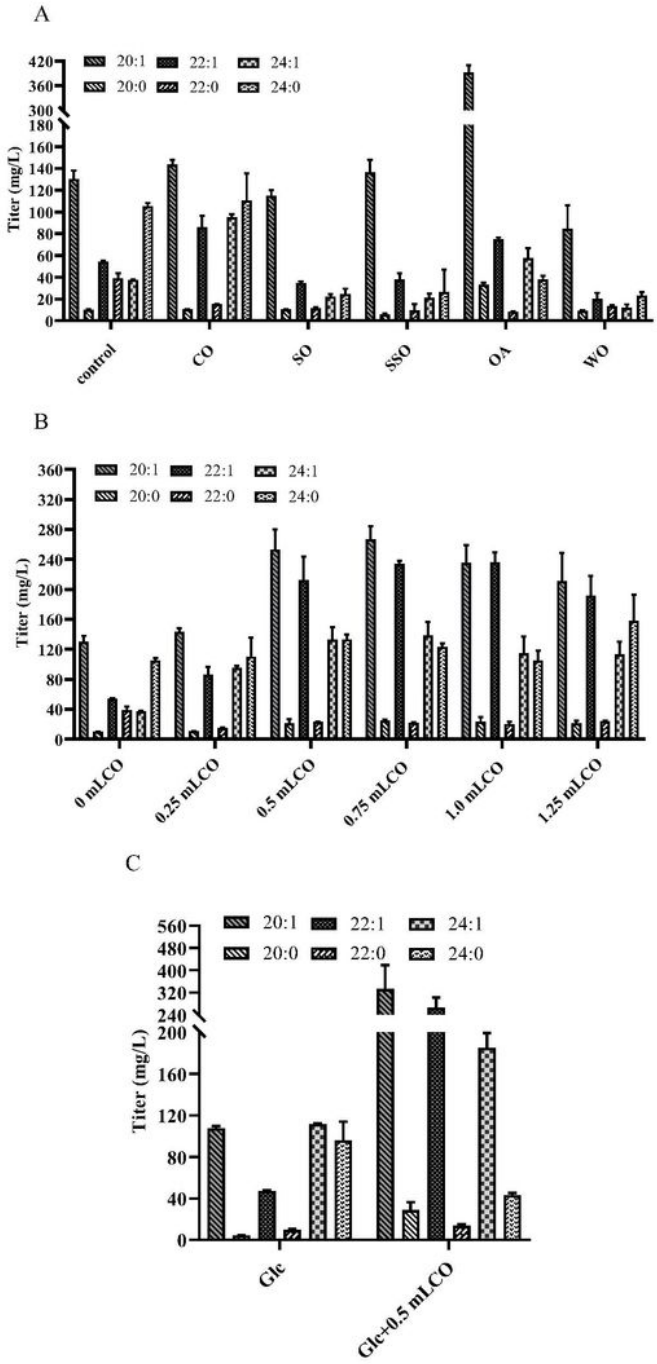

Fig 6

Figure 6

Effect of oil/oleic acid addition on nervonic acid production in engineered *Y. lipolytica*. (A) Adding different auxiliary carbon sources on nervonic acid production. (B) colleseed oil supplemental level on

the yield of nervonic acid. (E) Fermentation of strains NA15 in the YPD medium with 0.5 mL colleseed oil. The data are the averages of two biological replicates with error bars representing standard deviations.

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