Engineering *Yarrowia lipolytica* for arachidonic acid production through rapid assembly of metabolic pathway

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Abstract

Yarrowia lipolytica, a non-conventional oleaginous yeast with special traits, has attracted increasing interest for producing value-added products. Generally, the DNA fragments of these heterologous metabolic pathways are constructed *via* the classic restriction digestion and ligation method. In contrast, the one-step *in vivo* pathway assembly method has been only rarely applied to *Y. lipolytica*. Here, with arachidonic acid biosynthesis as a case study, a one-step *in vivo* pathway assembly and integration method was used for engineering *Y. lipolytica*. Using rDNA as integrative locus, this study showed that there was a relation between the assembly efficiency and the length of overlapping region. Especially, with an overlap up to 1 kb, the method was able to rapidly assemble the arachidonic acid biosynthesis pathway (nearly 10 kb) into the chromosome with high efficiency (nearly 23%). Meanwhile, the pathway assembled in *Y. lipolytica* demonstrated long-term genetic stability and the engineered strain exhibited robust growth. Furthermore, this study demonstrated that the codon-optimized genes from *Mortierella alpina* can function efficiently in *Y. lipolytica*: a high level arachidonic acid production (0.4% of total fatty acids) was produced in the engineered strain. To our knowledge, this is the first time that this method is applied to *Y. lipolytica* for functional polyunsaturated fatty acids production. This method represents a powerful tool with potential for facilitating engineering applications in non-conventional yeasts.

Keywords:

Yarrowia lipolytica, arachidonic acid, *in vivo*, pathway assembly, synthetic biology

1. Introduction

With the development of metabolic engineering and synthetic biology, the microbial-based biotechnology has been a promising strategy for production of value-added chemicals in heterologous microorganisms [1-2]. Generally, these biochemical pathways are constructed in conventional microorganisms such as *Escherichia coli* and *Saccharomyces cerevisiae* [3-4]. Meanwhile, non-conventional yeasts with special traits are viewed as important resources and have received growing attention [5]. Among them, especially, *Yarrowia lipolytica* is able to assimilate and ferment various carbon substrates and produce recombinant proteins as well as various chemicals, such as organic acids, etc [6-10]. Recently, with the development of yeast biotechnology, this non-conventional yeast has been used as a platform factory for production of various novel value-added chemicals, such as lycopene, eicosapentaenoic acid and fatty alcohol [11-14].

Interestingly, functional polyunsaturated fatty acids (PUFAs), including omega-6 and omega-3, have been proved to play important functions on human health. Especially, arachidonic acid (ARA), a typical omega-6 PUFA, is synthesized *via* the aerobic Δ -6 desaturation and elongation pathway in the fungus *Mortierella alpina* [15]. Meanwhile, based on the assembly of the desaturation and elongation pathway, ARA is also produced in various organisms, such as *Arabidopsis thaliana*, *Brassica napus* and *S. cerevisiae* [16-17]. The ARA biosynthesis pathway in these engineered chassis was constructed *via* the classic restriction digestion and ligation method. However, with the synthetic biology development, various *in vitro* and *in vivo* DNA assembly

methods are now available for constructing metabolic pathways [18]. Generally, these developed pathway assembly and integration methods, such as ePathBrick, PADOM, LCR assembly and DNA assembler, are mainly applied to *E. coli* and *S. cerevisiae* [19-20]. Compared with the classic restriction digestion and ligation method, the *in vivo* one-step pathway assembly allows for the reduction of the time and cost for constructing the biochemical pathway. However, such *in vivo* one-step pathway assembly methods have been rarely applied to the non-conventional yeast *Y. lipolytica* [21], and never to our knowledge for functional PUFAs production.

In this study, to demonstrate the possibility and efficiency of pathway assembly in the non-conventional yeast *Y. lipolytica*, we used arachidonic acid biosynthesis as a case study. The corresponding aerobic Δ -6 desaturation and elongation pathway was designed and constructed *via* one-step *in vivo* pathway assembly. Meanwhile, the relationship between the assembly efficiency and the length of overlapping region was demonstrated. Furthermore, analyses were performed throughout the time course of the fermentation in order to assess the effect of the heterologous metabolic pathway in this non-conventional yeast. It was demonstrated that the codon-optimized genes from *Mortierella alpina* can function in *Y. lipolytica*. Altogether, the engineered *Y. lipolytica* strain was demonstrated to be a potential arachidonic acid producer with long-term genetic stability and robust growth. This study shows that the *in vivo* one-step pathway assembly and integration method is capable of facilitating the engineering of *Y. lipolytica* for biotechnological applications.

2. Materials and methods

2.1 Microbial strains, media and culture conditions

E. coli DH5 α was used for cloning and plasmid amplification. *E. coli* DH5 α was grown at 37 °C with constant shaking in Luria-Bertani Broth supplemented with 100 $\mu\text{g mL}^{-1}$ of ampicillin for plasmid amplification. *Y. lipolytica* Po1f, a leucine and uracile auxotrophic strain devoid of any secreted protease activity, was used as the base strain for all studies [22]. The strains and plasmids used in the study are listed in Table 1.

YPD media was prepared with 20 g L $^{-1}$ glucose, 10 g L $^{-1}$ yeast extract and 20 g L $^{-1}$ tryptone. Selective YNB-Ura media was prepared with 20 g L $^{-1}$ glucose, 6.7 g L $^{-1}$ yeast nitrogen base (without amino acids) (Difco), 0.77 g L $^{-1}$ CSM-Ura (Complete Supplement Mixture minus uracil) and 20 g L $^{-1}$ agar. Fermentation media was prepared with 50 g L $^{-1}$ glucose, 9 g L $^{-1}$ yeast extract, 8 g L $^{-1}$ yeast nitrogen base (without amino acids) (Difco).

Shake flask culture was performed in 500 mL flask containing 100 mL fermentation media. Generally, yeast seed from frozen stocks were inoculated into 5 mL YPD media at 28 °C at 180 rpm. Overnight culture was inoculated into 50 mL YPD media at 28 °C at 180 rpm in 250 mL shake flask for 24 h. Yeast cells were re-inoculated into 50 mL YPD media with inoculation rate of 5% under the same condition with an optical density of 0.1 and cultivated during 48 h. The culture seed were inoculated into fermentation media with an inoculation rate of 10% for 72 h (28 °C, 180 rpm), after which pH, biomass and glucose content were measured, and fatty acid profiles were analyzed, every 24 h.

2.2 DNA manipulation

To prepare individual gene expression cassettes, yeast promoters (TEF1p, 406 bp; FBA1p, 800 bp) and terminators (LIP2t, 563 bp; XPR2t, 422 bp) were *de novo* synthesized. The functional Δ -6 desaturase, Δ -6 elongase and Δ -5 desaturase encoding genes from *M. alpina* were codon-optimized (Genscript, Nanjing, China). All the codon-optimized genes used are listed in Supplementary Table 1. The selective marker *URA3* was *de novo* synthesized. The integration site 28S rDNA coding sequence was PCR-amplified from the genomic DNA isolated from *Y. lipolytica* Po1f. All the primers used are listed in Supplementary Table 2. Each pathway part (over 200 ng), including promoter, functional coding gene, terminator, selective marker and integration site, was prepared by overlap extension PCR (OE-PCR) according to a previously described procedure [23]. Specifically, the first gene cassette fragment was amplified with the 28s rDNA (L)-F and LIP2t-R primer, the second gene cassette fragment was amplified with the FBAP-F and XPRt-R primer, the third gene cassette fragment was amplified with the TEFp-F and LIP2t-R primer, the fourth gene cassette fragment was amplified with the *URA3*-F and 28s rDNA (R)-R primer. The PCR products of each individual gene expression cassette, obtained *via* OE-PCR, were ligated to pMD18-T vector for cloning and sequencing. A DNA mixture of individual cassettes (over 300 ng) was used to transform *Y. lipolytica* Po1f strain cells.

2.3 Yeast transformation

Transformation of *Y. lipolytica* was performed by the modified lithium acetate procedure previously described [24-25]. Briefly, *Y. lipolytica* Po1f was inoculated

from glycerol stock directly into 10 mL YPD media and shaken at 28 °C at 180 rpm for 24 h. Yeast cells were inoculated with a ratio of 5% into 50 mL YPD media and shaken at 28 °C at 180 rpm until OD₆₀₀ reached 0.7. Then, a sample of 2.5 mL ice-precooled 1 M hydroxyurea was added into the culture. Yeast cells were shaken and harvested when OD₆₀₀ reached 1.0 to 1.5, by centrifugation at 4 000 rpm for 10 min. Yeast cells were washed twice in ice-precooled sterile water. Then, yeast cells were resuspended in 50 mL ice-precooled 100 mM LiOAc (pH 6.0) and incubated at 28 °C for 60 min without shaking. Next, yeast cells were resuspended and rinsed in different conditions (the first time with 25 mL of ice-precooled 100 mM LiOAc (pH 6.0) by centrifugation at 4 000 rpm for 10 min, the second time with 2 mL of ice-precooled 100 mM LiOAc (pH 6.0) by centrifugation at 4 000 rpm for 10 min, the third time with 1 mL of ice-precooled 100 mM LiOAc (pH 6.0) by centrifugation at 4 000 rpm for 10 min). At last, yeast cells were resuspended in 100 µL of 100 mM LiOAc (pH 6.0) and used for transformation directly. A sample of 2-5 µg of DNA mixture of each gene expression cassette was added to transformation cells, followed by 10 µL of 100 mg mL⁻¹ boiled salmon sperm DNA. Yeast cells were incubated at 28 °C for 15 min with shaking at 150 rpm, followed by addition of 720 µL of PEG buffer (40% PEG-3500, 100 mM LiOAc, pH 6.0) and 100 µL of 1 M dithiothreitol. Yeast cells were incubated at 28 °C for 60 min with shaking at 220 rpm, then heat shocked for 10 min at 39 °C, spun down and resuspended in 1 mL of pre-cooling 100 mM LiOAc (pH 6.0). 200 µL of cells were grown on YNB-Ura plates at 28 °C for 2-5 days for selection of the engineered transformants.

2.4 PCR analysis of the assembled pathway

Genomic DNAs were extracted from the original *Y. lipolytica* Po1f and the engineered *Y. lipolytica* YL 6-1 strains, following shake flask cultivation for 48 h, using a yeast genomic DNA kit (Omega Bio-tech, USA). For ARA biosynthesis from Δ -6 desaturation pathway, confirmation of the correct DNA assembly was performed by PCR amplification of each structural gene from the genomic DNAs. All the primers used are listed in Supplementary Table 2.

2.5 Copy number analysis

To determine the relative copy number of the gene expression cassettes of pathway assembly in the engineered *Y. lipolytica*, qPCR analysis was performed on the genomic DNA using *ACT1* as the internal control. Genomic DNAs were extracted from the original *Y. lipolytica* Po1f and the engineered *Y. lipolytica* YL 6-1 strains, following cultivation for 48 h, using a yeast genomic DNA kit (Omega Bio-tech, USA). qPCR was performed on an ABI 7900 instrument (Applied Biosystems, Foster City, CA, USA) and the primers used are listed in Supplementary Table 1. Fold changes were determined by the $2^{-\Delta\Delta C_t}$ method and normalized to the *ACT1* gene. All experiments were performed in triplicate.

2.6 Analysis of fatty acid profiles

Fatty acid methyl esters of *Y. lipolytica* strains were prepared as previously described [26]. GC analysis of fatty acid methyl esters was performed with GC-FID (GC-2010, Shimadzu, Japan) equipped with a capillary column (DB-23, 60 m $\times$ 0.22 mm) to analyze fatty acid profiles of sample. Using nitrogen as the carrier gas, a

sample of 1 μL was injected with the injector at 250 $^{\circ}\text{C}$. The column temperature was raised from 100 to 196 $^{\circ}\text{C}$ at 25 $^{\circ}\text{C min}^{-1}$ and then increased to 220 $^{\circ}\text{C}$ at 2 $^{\circ}\text{C min}^{-1}$, keeping this temperature for 6 min. The FID detector temperature was set to 280 $^{\circ}\text{C}$. Fatty-acid standards for methyl palmitate (C16:0), methyl stearate (C18:0), methyl oleate (C18:1), methyl linoleate (C18:2), methyl octadecatrienoate (C18:3), methyl eicosatrienoate (C20:3) and methyl arachidonate (C20:4) were purchased from Sigma Aldrich and used as standard to identify fatty-acid peaks. Total lipid content of samples with different fatty-acid methylesters was calculated using fatty-acid standards.

2.7 pH, biomass and residual glucose quantification

pH was measured using a pH electrode (Mettler-Toledo, Urdorf, Switzerland). Cell biomasses were harvested by centrifugation at 12 000 rpm for 10 min, washed twice with distilled water and dried at 70 $^{\circ}\text{C}$ to a constant weight. Glucose content was measured by a biosensor with glucose oxidase electrode (SBA-40C, Institute of Biology, Shandong Academy of Sciences, China).

3. Results and discussion

3.1 Design of One-step pathway assembly for arachidonic acid production in *Yarrowia lipolytica*

We provide here a proof of concept of the possibility and efficiency of *in vivo* one-step pathway assembly in a non-conventional yeast chromosome, using *Y. lipolytica* as chassis. Generally, with rDNA as integration locus, a metabolic pathway assembly experiment is designed as shown in Fig. 1. Briefly, each functional gene

cassette, consisting of a promoter, a structural gene and a terminator, is synthesized *via* an OE-PCR step. The 5'-end of the first gene cassette is designed to present homology with the chromosome loci of rDNA in order to promote integration, while the 3'-end of the first gene cassette is designed to present homology with the 5'-end of the second cassette, and so-on, and the 3'-end of the last gene cassette is designed to present homology with the chromosome loci of rDNA. Each gene cassette is designed to share a same sequence overlap with the neighboring one, in order to promote their ordered integration by homologous recombination into the chromosome.

We use here ARA biosynthesis as a case study for this one-step pathway assembly method. ARA is synthesized in engineered *Y. lipolytica* *via* a three steps reaction: the first step is the Δ -6 desaturation of linoleic acid to gamma-linolenic acid, the second step is the Δ -6 elongation of gamma-linolenic acid to dihomo-gamma-linolenic acid and the last step is the Δ -5 desaturation of dihomo-gamma-linolenic acid to ARA (Fig. 2). With *Y. lipolytica* as a chassis, targeted ARA has previously been synthesized by the DuPont Company using integrative plasmids constructed *via* the classic restriction digestion and ligation method [27]. As shown in Supplementary Fig. 1, our scheme of *in vivo* one-step assembly is capable of reducing the time and cost of constructing the entire biosynthesis pathway, compared with the classic assembly strategy. In our study, the ARA biosynthesis pathway, designed *via in vivo* one-step assembly, consists of four gene cassettes, namely 28S rDNA (L)-TEF1p- Δ -6 desaturase-LIP2t, FBAP- Δ -6

elongase-XPR2t, TEF1p- Δ -5 desaturase-LIP2t, *URA3*-28S rDNA(R). The three ARA synthesis pathway genes, Δ -6 desaturase, Δ -6 elongase and Δ -5 desaturase encoding genes from *M. alpina*, were codon-optimized for expression in *Y. lipolytica*. Moreover, a Kozak sequence was added before each structural gene to improve the transcription level.

In addition, rDNA sequence was selected as integration target sequence for metabolic pathway integration. We choose in our study to use rDNA as a target for integration, preferably to “zeta” sequences that are often used for *Y. lipolytica* engineering, since Po1f strain is devoid of Ylt1 retrotransposon and of dispersed repeated “zeta” sequences [28]. Although the DNA fragments with “zeta” borders can be integrated at random in *Y. lipolytica* strain (no “zeta”) via a non-homologous end joining process, the transformation frequency was low [29-31]. Interestingly, the partial functional cassettes of mixtures were detected in the transformants based on PCR analysis (data not shown). That is maybe that the partial genes were integrated in non-homologous locations. Meanwhile, it was noticed that the transformation efficiency changed as a function of gene concentration in the transformation mix. Especially, if the gene concentration was exceeded over 5 ng, the transformation efficiency declined (data not shown). The integration events in our research were similar to previously report [31]. On the basis of the PCR screening, a positive engineered *Y. lipolytica* strain, YL 6-1, was selected for measurement of ARA production. As shown in Supplementary Fig. 2, the engineered *Y. lipolytica* YL 6-1 strain was able to produce a significant ARA amount, during a 48 h shake flask

fermentation, whereas the original *Y. lipolytica* Po1f strain was not able to synthesize ARA.

3.2 Relation between the length of overlapping region and assembly efficiency

To test the effect of various lengths of overlapping regions on the efficiency of pathway assembly, different combinational lengths of overlaps were designed. As shown in Table 2, for the type one assembly design, overlaps between each gene cassette were 50-60 bp and homology with chromosomal rDNA sequence was 300 bp. The total size of the three genes together with the *URA3* marker was nearly 9.2 kb. For the type two, overlaps between cassettes were 50-60 bp and homologous rDNA was 900-1000 bp. The total size of the three genes plus *URA3* was nearly 10.4 kb. For the type three, overlaps between cassettes were 400-500 bp and homologous rDNA was 900-1000 bp. The total size of the three genes plus *URA3* was nearly 10.4 kb. Typically, in transformation experiments performed with these DNA mixtures, 20-50 colonies appeared after 2-4 days at 28 °C. In order to confirm the correct assembly and integration of the pathway, 30 clones of each assembly type were randomly picked for genomic DNA extraction. PCR analysis was used to confirm the presence of each gene of the biosynthesis pathway, namely Δ -6 desaturase, Δ -6 elongase and Δ -5 desaturase encoding genes (data not shown). Obviously, the highest correct assembly efficiency, of 23%, was obtained with the type three assembly design. Meanwhile, the correct assembly efficiency for type one and type two reached nearly 7% and 13%, respectively. It is supposed that the efficiency of pathway assembly is mainly affected by the length of overlaps. Recently, the same research on the effect of

homology length on the frequency of homologous integration in *Y. lipolytica* was reported. Verbeke et al. [30] performed that, with homology length of 500 bp, only 2% of the frequency of homologous integration is reported in *Y. lipolytica* Po1d. However, with flanking shorter regions (50, 100 and 250 bp, respectively), the homologous recombination is impossible in *Y. lipolytica* Po1d.

When we compare our results with the higher efficiency of *in vivo* pathway assembly (60-80%) in *S. cerevisiae*, it appears that the reason of this large difference in assembly efficiency between *S. cerevisiae* and *Y. lipolytica* is that *Y. lipolytica* is a non-conventional yeast with a higher efficiency of non-homologous recombination than of homologous recombination [32]. Recently, Gao et al. [21] performed that beta-carotene is synthesized *via* one-step pathway assembly in *Y. lipolytica* with an assembly efficiency of 12-21%. The difference in integration efficiency is maybe attributed to the different lengths of homologous sequences, the special traits of *Y. lipolytica* strains and the different biosynthesis pathway of drop-in products. To improve the efficiency of homologous recombination in *Y. lipolytica*, the use of strains deleted for *KU70* and *KU80* has been reported [33-34]. However, the efficiency of homologous recombination was mainly affected by the homologous integration region in the engineered *Y. lipolytica*. Especially, a dramatic increase in the frequency of homologous recombination (60.0–63.3%) was found for homologous integration sequences with a length of 750 and 1000 bp in *Y. lipolytica* H222-SW2 ($\Delta ku70$) [34]. Recently, the treatment of competent cells of *Y. lipolytica* with hydroxyurea, which has a function of cell cycle arrest, has been reported to increase gene targeting [25]. In the

present study, hydroxyurea-treated yeast cells have been used to improve the efficiency of pathway assembly.

3.3 Copy number estimation

To verify the copy number of the heterologous genes within the entire pathway integrated into the chromosome of YL 6-1, qPCR was performed on genomic DNAs of the engineered strain. The relative copy number of Δ -6 desaturase, Δ -6 elongase and Δ -5 desaturase encoding genes of the engineered YL 6-1 strain have been determined to be roughly 1 (Supplementary Fig. 3). In fact, with the relative copy number of Δ -6 desaturase, Δ -6 elongase and Δ -5 desaturase encoding genes of the engineered YL 6-1 as control, the relative copy number of functional genes in Polf was determined to be roughly 0. It is maybe that the entire ARA biosynthesis pathway is not present in Polf. A series of genetic tools, including integrative plasmids, have been developed for using *Y. lipolytica* as a chassis for various applications [11]. Generally, the integration of a heterologous gene cassette can be targeted, by using bordering regions of homology, to a chosen chromosomal site. Several loci have been used for that purpose in *Y. lipolytica*, including a single-copy gene such as *XPR2*, the copies of tandemly repeated sequences of rDNA or the dispersed copies of repeated elements such as the “zeta” sequence of Ylt1 retrotransposon [35]. Notably, there are at least 100 potential “zeta” sequence integration sites and more than 200 copies of rDNA clusters in *Y. lipolytica* [35-36].

Interestingly, there is a relation between the copy number and the selection marker convention rate. Previously, the integrants carrying up to 60 copies of

transformants were obtained *via* the defective *URA3* gene in rDNA-based *Y. lipolytica* strain [37]. Especially, when using the *ura3d4* allele for selection, the higher multicopy transformants were obtained, regardless of whether the “zeta” or rDNA, or the single copy XPR2 locus, were used as targeting sites [29]. As demonstrated, the multiple integrations are generally obtained only when a defective selection marker is used, regardless of the number of copy of the target used [30-31]. Therefore, obtaining only one integrated copy of the new pathway was the result expected in our case, since the fully functional *URA3* gene has been used as selective marker. Previously, another pathway engineering experiment using a non-defective marker and homologies of 300 bp with 28S rDNA has also lead to single copy integration [38].

3.4 Stability assessment

In the following step, genomic stability of the engineered *Y. lipolytica* strain YL 6-1 capable of producing ARA was assessed under non-selection culture. The engineered YL 6-1 strain was cultivated over 40 generations. Based on PCR analysis, shown in Supplementary Fig. 4, the fortieth generation of the engineered *Y. lipolytica* YL 6-1 was found to have conserved the three heterologous gene of the engineered pathway. Accordingly, it had also been showed previously that engineered *Y. lipolytica* with 28S rDNA as target site for pathway integration was stable even over 40 generations [38]. In the present study, we demonstrated that our method allowed high assembly efficiency and long-term genetic stability of the engineered *Y. lipolytica* strain.

3.5 Fermentation performance of engineered *Yarrowia lipolytica*

With linoleic acid (C18:2) as a substrate, the ARA can be accumulated in *Y. lipolytica* via the *in vivo* one-step assembled ARA biosynthesis pathway. To verify the possible effect of the *de novo* ARA biosynthesis pathway on the fatty acid profiles of *Y. lipolytica*, GC analysis of the main fatty acid profiles, including palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), gamma-linolenic acid (C18:3), dihomo-gamma-linolenic acid (C20:3) and arachidonic acid (C20:4), was performed.

As shown in Fig. 3, the C16:0 and the C18:0 content of the original Po1f strain was slightly higher than those of the engineered YL 6-1 under fermentation at 72 h, respectively. Interestingly, when compared with the original Po1f strain, the engineered YL 6-1 strain exhibited a higher consumption rate of C18:1. The C18:2 content of the original and the engineered strain was detected, respectively. Especially, when compared with that of the original Po1f strain, the C18:2 content of the engineered YL 6-1 strain was significantly lower. Due to the absence of native Δ -6 desaturase, Δ -6 elongase and Δ -5 desaturase functions, *Y. lipolytica* is not able to synthesize C20:4 and intermediates C18:3 and C20:3. As shown in Fig. 3, Po1f was not able to produce C18:3. Whereas, using C18:2 as a substrate, C18:3 was synthesized in the engineered *Y. lipolytica* strain via the novel ARA biosynthesis pathway. For YL 6-1 strain, the C18:3 content reached to 2.8% of total fatty acids at 72 h. Recently, we have constructed an engineered *Y. lipolytica* capable of producing C18:3 (6% of TFA) under the optimized fermentation process [39]. Using C18:3 as a

substrate, this engineered strain was thus able to produce C20:3 *via* the novel ARA biosynthesis pathway. Especially, the C20:3 content of the engineered YL 6-1 strain was at 0.7% of total fatty acids at 72 h. In contrast to the original Polf strain, the engineered YL 6-1 strain was able to synthesize C20:4. Especially, a high C20:4 content of 0.4% of total fatty acids was produced at 72 h. With the novel functions of the heterologous ARA biosynthesis pathway, the targeted ARA and intermediates, gamma-linolenic and dihomo-gamma-linolenic acid, were produced in the engineered *Y. lipolytica*. It was concluded that the ARA biosynthesis pathway from *M. alpina* can function efficiently in *Y. lipolytica*.

With glucose at 50 g L⁻¹ as an initial carbon substrate, the pH value is shown in Fig. 4. Obviously, the pH value of the fermentation culture in the engineered YL 6-1 strain was higher than that of the original Polf strain at 72 h. Especially, the pH value of the fermentation culture in the original Polf was 2.1 at 72 h. For the fermentation culture in the engineered YL 6-1, the pH value was 3.3 at 72 h. In fact, there is a relation between the pH and the secreted organic acids in the fermentation culture. Especially, citric acid and malic acid are viewed as important metabolic intermediates with potential for providing the precursors of fatty acid biosynthesis. Future work is to further evaluate the metabolic flux of the engineered and original strains, based on the analysis of organic acids production in the fermentation culture. Moreover, the biomass titer of the engineered YL 6-1 strain was significantly higher than that of the original Polf strain (10.2 vs 6.9 g L⁻¹) (Fig. 4). As shown in Fig. 4, the rate of glucose consumption of the engineered YL 6-1 strain was thus slightly higher than that of the

original Polf strain. Recently, it was reported that the leucine can be viewed as an intracellular trigger to promote lipogenesis [40]. Although the leucine auxotrophic strains were used in this research, compared to the original Polf, the engineered strain YL 6-1 performed a higher glucose uptake and biomass production. It was concluded that more cellular substrates maybe are diverted toward the biomass production and lipogenesis in the leucine auxotrophic strain YL 6-1. Especially, the heterologous ARA biosynthesis pathway was able to direct the metabolic flux to the desired product. Based on our analysis of the fermentation performances of the original and engineered strain, it was demonstrated that the ARA biosynthesis pathway in *M. alpina* can function well in *Y. lipolytica* without adverse effects.

4. Conclusions

In this work, with ARA biosynthesis as a case study, the *in vivo* one-step pathway assembly strategy was successfully developed for the non-conventional yeast *Y. lipolytica*. Based on the analysis of the effect of different overlapping regions, this study showed that the efficiency of pathway assembly was mainly affected by the length of overlaps. Especially, the highest efficiency (nearly 23%) was obtained when using overlaps of roughly 1 kb, in accordance to previous data on homologous recombination in *Y. lipolytica* [41]. Furthermore, the accumulation of ARA and intermediate gamma-linolenic and dihomo-gamma-linolenic acid between the original and engineered *Y. lipolytica* was demonstrated. Our results show that the engineered strain can produce ARA, up to a content of 0.4% of total fatty acids, during the fermentation. In addition, this study showed that the newly integrated heterologous

ARA biosynthesis pathway was stably maintained through at least 40 generations and that it was sufficient to allow obtaining a higher titer of biomass during fermentation. One can hypothesize that this increased growth performance of the ARA pathway bearing cells could contribute, as a selective advantage, to the maintenance of this modification through the generations, during long term cultivation. Altogether, this study showed that the one-step *in vivo* assembly and integration method enables reliable assembly of many DNA parts in *Y. lipolytica* via a rapid and convenient workflow. Future work focusing on the utilization of other integration sites, such as MFE locus and Pex10 locus, should further induce and improve the accumulation of ARA. With the development of synthetic biology, the CRISPR-Cas9 genome editing tools for *Y. lipolytica* have recently been expanded [42-43]. Combined with these well-developed genetic tools, the one-step *in vivo* assembly and integration method will facilitate the use of non-conventional yeasts in many applications.

References

- [1] J. Nielsen, J.D. Keasling, Engineering Cellular Metabolism, Cell. 164 (2016) 1185-1197.
- [2] P.P. Peralta-Yahya, F. Zhang, S.B. del Cardayre, J.D. Keasling, Microbial engineering for the production of advanced biofuels, Nature. 488 (2012) 320-328.
- [3] R. Jain, X. Sun, Q. Yuan, Y. Yan, Systematically engineering *Escherichia coli* for enhanced production of 1,2-propanediol and 1-propanol, ACS. Synth. Biol. 4 (2015) 746-756.
- [4] K. Thodey, S. Galanie, C.D Smolke, A microbial biomanufacturing platform for natural and semisynthetic opioids, Nat. Chem. Biol. 10 (2014) 837-844.
- [5] N. Bati, E.G. Hammond, B.A. Glatz, Biomodification of fats and oils: trials with *Candida lipolytica*, J. Am. Oil. Chem. Soc. 61 (1984) 1743-1746.
- [6] R. Ledesma-Amaro, J.M. Nicaud, Metabolic engineering for expanding the engineering for expanding the substrate range of *Yarrowia lipolytica*, Trends. Biotechnol. 2016, doi: 10.1016/j.tibtech.2016.04.010.
- [7] H.H. Liu, X.J. Ji, H. Huang, Biotechnological applications of *Yarrowia lipolytica*: past, present and future, Biotechnol. Adv. 33 (2015) 1522-1546.
- [8] C. Madzak, *Yarrowia lipolytica*: recent achievements in heterologous protein expression and pathway engineering, Appl. Microbiol. Biotechnol. 99 (2015) 4559-4577.
- [9] H. Guo, P. Liu, C. Madzak, G. Du, J. Zhou, J. Chen, Identification and application

- of keto acids transporters in *Yarrowia lipolytica*, Sci. Rep. 5 (2015) 8138.
- [10] P. Ferreira, M. Lopes, M. Mota, I. Belo, Oxygen mass transfer impact on citric acid production by *Yarrowia lipolytica* from crude glycerol, Biochem. Eng. J. 110 (2016) 35-42.
- [11] F. Matthäus, M. Ketelhot, M. Gatter, G. Barth, Production of lycopene in the non-carotenoid-producing yeast *Yarrowia lipolytica*, Appl. Environ. Microbiol. 80 (2014) 1660-1669.
- [12] Z. Xue, P.L. Sharpe, S.P. Hong, N.S. Yadav, D. Xie, D.R. Short, H.G. Damude, R.A. Rupert, J.E. Seip, J. Wang, D.W. Pollak, M.W. Bostick, M.D. Bosak, D.J. Macool, D.H. Hollerbach, H. Zhang, D.M. Arcilla, S.A. Bledsoe, K. Croker, E.F. McCord, B.D. Tyreus, E.N. Jackson, Q. Zhu, Production of omega-3 eicosapentaenoic acid by metabolic engineering of *Yarrowia lipolytica*, Nat. Biotechnol. 31 (2013):734-740.
- [13] G. Wang, X. Xiong, R. Ghogare, P. Wang, Y. Meng, S. Chen, Exploring fatty alcohol-producing capability of *Yarrowia lipolytica*, Biotechnol. Biofuels. 9 (2016) 107.
- [14] P. Xu, K. Qiao, W.S. Ahn, G. Stephanopoulos, Engineering *Yarrowia lipolytica* as a platform for synthesis of drop-in transportation fuels and oleochemicals, Proc. Natl. Acad. Sci. U. S. A. 113 (2016) 10848-10853.
- [15] X.J. Ji, L.J. Ren, Z.K. Nie, H. Huang, P.K. Ouyang, Fungal arachidonic acid-rich oil: research, development and industrialization, Crit. Rev. Biotechnol. 34 (2014) 197-214.

- [16] J.R. Petrie, P. Shrestha, S. Belide, M.P. Mansour, Q. Liu, Horne J, P.D. Nichols, S.P. Singh, Transgenic production of arachidonic acid in oilseeds, *Transgenic. Res.* 21 (2012):139-147.
- [17] F. Beaudoin, L.V. Michaelson, S.J. Hey, M.J. Lewis, P.R. Shewry, O. Sayanova, J.A. Napier, Heterologous reconstitution in yeast of the polyunsaturated fatty acid biosynthetic pathway, *Proc. Natl. Acad. Sci. U. S. A.* 97 (2000) 6421-6426.
- [18] R.E. Cobb, J.C. Ning, H. Zhao, DNA assembly techniques for next-generation combinatorial biosynthesis of natural products, *J. Ind. Microbiol. Biotechnol.* 41 (2014) 469-477.
- [19] P. Xu, A. Vansiri, N. Bhan, M.A. Koffas, ePathBrick: a synthetic biology platform for engineering metabolic pathways in *E. coli*, *ACS Synth. Biol.* 1 (2012) 256-266.
- [20] Z. Shao, H. Zhao, H. Zhao, DNA assembler, an *in vivo* genetic method for rapid construction of biochemical pathways, *Nucleic Acids Res.* 37 (2009) e16.
- [21] S. Gao, L. Han, L. Zhu, M. Ge, S. Yang, Y. Jiang, D. Chen, One-step integration of multiple genes into the oleaginous yeast *Yarrowia lipolytica*, *Biotechnol. Lett.* 36 (2014) 2523-2528.
- [22] C. Madzak, B. Tréton, S. Blanchin-Roland, Strong hybrid promoters and integrative expression/secretion vectors for quasi-constitutive expression of heterologous proteins in the yeast *Yarrowia lipolytica*, *J. Mol. Microbiol. Biotechnol.* 2 (2000) 207-216.
- [23]. R.M. Horton, H.D. Hunt, S.N. Ho, J.K. Pullen, L.R. Pease, Engineering hybrid

- genes without the use of restriction enzymes-gene-splicing by overlap extension, *Gene*. 77 (1989) 61-68.
- [24] D.C. Chen, J.M. Beckerich, C. Gaillardin, One-step transformation of the dimorphic yeast *Yarrowia lipolytica*, *Appl. Microbiol. Biotechnol.* 48 (1997) 232-235.
- [25] V. Tsakraklides, E. Brevnova, G. Stephanopoulos, A.J. Shaw, Improved gene targeting through cell cycle synchronization, *PLoS One*. 10 (2015) e0133434.
- [26] E.B. Cahoon, K.G. Ripp, S.E. Hall, A.J. Kinney, Formation of conjugated delta8, delta10-double bonds by delta12-oleic-acid desaturase-related enzymes: biosynthetic origin of calendic acid, *J. Biol. Chem.* 276 (2001) 2637-2643.
- [27] H.G. Damude, P.J. Gillies, D.J. Macool, W. Pollak, J.J. Ragghianti, Z.X. Xue, N.S. Yadav, H.X. Zhang, Q.N. Zhu, High arachidonic acid producing strains of *Yarrowia lipolytica*, United. States. Patent. 2009, US 7, 588, 931.
- [28] L. Liu, H.S. Alper, Draft genome sequence of the oleaginous yeast *Yarrowia lipolytica* PO1f, a commonly used metabolic engineering host, *Genome Announc.* 2 (2014) e00652-14.
- [29] T. Juretzek, M. Le Dall, S. Mauersberger, C. Gaillardin, G. Barth, J.M. Nicaud, Vectors for gene expression and amplification in the yeast *Yarrowia lipolytica*, *Yeast*. 18 (2001) 97-113
- [30] G. Pignède, H.J. Wang, F. Fudalej, M. Seman, C. Gaillardin, J.M. Nicaud, Autocloning and amplification of LIP2 in *Yarrowia lipolytica*. *Appl. Environ. Microbiol.* 66 (2000) 3283-3289.

- [31] F. Bordes, F. Fudalej, V. Dossat, J.M. Nicaud, A. Marty, A new recombinant protein expression system for high-throughput screening in the yeast *Yarrowia lipolytica*, J. Microbiol. Methods. 70 (2007) 493-502.
- [32] G.F. Richard, A. Kerrest, I. Lafontaine, B. Dujon, Comparative genomics of hemiascomycete yeasts: genes involved in DNA replication, repair, and recombination, Mol. Biol. Evol. 22 (2005) 1011-1023.
- [33] Verbeke J, A. Beopoulos, J.M. Nicaud, Efficient homologous recombination with short length flanking fragments in Ku70 deficient *Yarrowia lipolytica* strains, Biotechnol. Lett. 35 (2013) 571-576.
- [34] A. Kretzschmar, C. Otto, M. Holz, S. Werner, L. Hübner, G. Barth, Increased homologous integration frequency in *Yarrowia lipolytica* strains defective in non-homologous end-joining, Curr. Genet. 59 (2013) 63-72.
- [35] N. Schmid-Berger, B. Schmid, G. Barth. Ylt1, a highly repetitive retrotransposon in the genome of the dimorphic fungus *Yarrowia lipolytica*, J. Bacteriol. 176 (1994) 2477-2482.
- [36] S. Casarégola, C. Feynerol, M. Diez, P. Fournier, C. Gaillardin, Genomic organization of the yeast *Yarrowia lipolytica*, Chromosoma. 106 (1997) 380-390.
- [37] M.T. Le Dall, J.M. Nicaud, C. Gaillardin, Multiple-copy integration in the yeast *Yarrowia lipolytica*, Curr. Genet. 26 (1994) 38-44.
- [38] E. Celińska, W. Grajek, A novel multigene expression construct for modification of glycerol metabolism in *Yarrowia lipolytica*, Microb. Cell. Fact. 12 (2013) 102.
- [39] M.L. Sun, C. Madzak, H.H. Liu, P. Song, L.J. Ren, H. Huang, X.J. Ji,

- Engineering *Yarrowia lipolytica* for efficient γ -linolenic acid production, Biochem. Eng. J. 117 (2017) 172-180.
- [40] J. Blazeck, A. Hill, L. Liu, R. Knight, J. Miller, A. Pan, P. Otoupal, H.S. Alper, Harnessing *Yarrowia lipolytica* lipogenesis to create a platform for lipid and biofuel production, Nat. Commun. 5 (2014) 3131.
- [41] G. Barth, C. Gaillardin, *Yarrowia lipolytica*, in: Wolf, K. (Eds.), Nonconventional Yeasts in Biotechnology, Springer-Verlag, Berlin Heidelberg, 1996, pp. 313-388.
- [42] C.M. Schwartz, M.S. Hussain, M. Blenner, I. Wheeldon, Synthetic RNA polymerase III promoters facilitate high-efficiency CRISPR–Cas9-mediated genome editing in *Yarrowia lipolytica*, ACS. Synth. Biol. 5 (2016) 356-359.
- [43] S. Gao, Y. Tong, Z. Wen, L. Zhu, M. Ge, D. Chen, Y. Jiang, S. Yang, Multiplex gene editing of the *Yarrowia lipolytica* genome using the CRISPR-Cas9 system, J. Ind. Microbiol. Biotechnol. 43 (2016) 1085-1093.

Tables and Figure Legends

Table 1: Plasmids and strains used in this study.

Table 2. The efficiency of *in vivo* one-step assembly and integration of arachidonic acid biosynthesis pathway with different lengths of overlap in *Yarrowia lipolytica*. (For each type, 30 colonies were randomly picked and analyzed.)

Fig. 1. A one-step method for assembly and integration of arachidonic acid biosynthesis pathway using *in vivo* homologous recombination in *Yarrowia lipolytica*. OE-PCR, overlap extension PCR.

Fig. 2. Arachidonic acid biosynthesis *via* three steps reaction.

Fig. 3. Fatty acid profiles of the original strain *Yarrowia lipolytica* Po1f and the recombinant strain *Y. lipolytica* YL 6-1. C16:0, palmitic acid; C18:0, stearic acid; C18:1, oleic acid; C18:2, linoleic acid; C18:3, gamma-linolenic acid; C20:3, dihomo-gamma-linolenic; C20:4, arachidonic acid.

Fig. 4. Fermentation performance of the original strain *Yarrowia lipolytica* Po1f and the recombinant strain *Y. lipolytica* YL 6-1.

Table 1.

Strains	Genotype and/or heterologous expression cassette	Reference
<i>E. coli</i>		
DH5 α	<i>fhuA2 (argF-lacZ) U169 phoA glnV44 ϕ80 $\Delta(lacZ)M15$ <i>gyrA96 recA1 relA1 endA1 thi-1 hsdR17</i></i>	Vazyme Biotech
<i>Y. lipolytica</i>		
Po1f	<i>MatA, leu2-270, ura3-302, xpr2-322, axp1-2</i>	[20]
YL 6-1	<i>MatA, leu2-270, ura3-302, xpr2-322, axp1-2, URA3</i> <i>TEFp-Δ-6-D-LIP2t, FBAp-Δ-6-E-XPR2t, TEFp-Δ-5-D-LIP2t</i>	This work
Plasmid		
pMD18-T		TAKARA
pMD18-YL-1	rDNA (L)- <i>TEFp-Δ-6-D- LIP2t 2</i>	This work
pMD18-YL-2	<i>FBAp -Δ-6-E- XPR2t</i>	This work
pMD18-YL-3	<i>TEFp-Δ-5-D- LIP2t</i>	This work
pMD18-YL-4	<i>URA3- rDNA (R)</i>	This work

Codon-optimized *M. alpina* genes: Δ -6-D, Δ -6 desaturase; Δ -6-E, Δ -6 elongase; Δ -5-D, Δ -5

desaturase.

Table 2.

Length of various overlap	Total colonies (PCR analysis)	Correct colonies (PCR analysis)	Cumulative probability (%)
Type one	30	2	6.7
Type two	30	4	13.3
Type three	30	7	23.3

Fig. 1

Fig. 2

Fig. 3

Fig. 4