Manuscript Number: BEJ-D-16-00782R1

Title: Engineering *Yarrowia lipolytica* for efficient γ -linolenic acid production

Article Type: Full Length Article

Keywords: γ -linolenic acid, *Yarrowia lipolytica*, $\Delta 6$ -desaturase, Metabolic engineering, Temperature-shift strategy

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Abstract: γ -linolenic acid (GLA) has various well-documented beneficial physiological effects and high biological significance. Because the natural supply of GLA is insufficient, microbial GLA production is a promising method for pharmaceutical and nutraceutical purposes. To establish and develop a biotechnological process for GLA production by *Yarrowia lipolytica*, the codon-optimized $\Delta 6$ -desaturase from *Mortierella alpina* was introduced into this yeast under the control of the strong hp4d promoter. A recombinant *Y. lipolytica* strain was constructed, which produced 4.6% GLA in total fatty acids. By using a temperature-shift strategy of cultivation, consisting in preliminary growth at 28 °C followed by 6-day culture at 20 °C, optimal levels of dry cell weight (DCW), lipid content and GLA concentration were obtained from the recombinant strain: 18.55 g/L, 1.16 g/L and 71.6 mg/L, respectively. These DCW, lipid and GLA values were respectively 25.7%, 19.6% and 60.9% higher than those obtained in the control cultivation experiment at the standard constant temperature of 28 °C. This work demonstrates the excellent capacity of *Y. lipolytica* for GLA production, by combining metabolic engineering with a temperature-shift strategy.

Dear Editors,

On behalf of our co-authors, we are submitting the enclosed manuscript entitled '**Engineering *Yarrowia lipolytica* for efficient γ -linolenic acid production**' for possible publication in '**Biochemical Engineering Journal**'. The classification for this manuscript should be: '120.040: metabolic engineering'.

We believe that several aspects of this paper may be of particular interests to the readers of your journal. Chiefly, the codon-optimized $\Delta 6$ -desaturase from *Mortierella alpina* was successfully introduced in *Yarrowia lipolytica* Polf and the effect of $\Delta 6$ -desaturase on cell growth, lipid accumulation, and fatty acid synthesis were systematically examined. Then using a temperature-shift strategy of cultivation, dry cell weight (DCW), lipid content and GLA concentration was improved significantly.

Firstly, the codon-optimized $\Delta 6$ -desaturase was expressed in *Y. lipolytica* using p0 vector driven by hp4d promoter. To investigate the discrepancy between the control strain and the genetically modified strain, the fermentation performance of the two strains was comparable. It was observed that GLA represented 4.6% of total fatty acids in the engineered strain, when it was not detected in the control strain Polf. In addition, the introduction of $\Delta 6$ -desaturase improved the value of DCW and increased the glucose consumption rate, but didn't affect lipid yield. Then, fermentation conditions were modified using a temperature-shift strategy. The engineered strain was consequently cultivated at 15 °C, 20 °C and 28 °C. Based on the fermentation results at different temperature, a controlled temperature-shift processes were developed: consisting in preliminary growth at 28 °C followed by 6-day culture at 20 °C, optimal levels of dry cell weight (DCW), lipid content and GLA concentration were obtained from the recombinant strain were respectively 25.7%, 19.6% and 60.9% higher than those obtained in the control cultivation experiment of 28 °C. The two-stage culture strategy proposed here, based on *Y. lipolytica* fermentation characteristics, could be applied to improving production of many other metabolites.

We certify that we have participated sufficiently in the work to take public responsibility for the appropriateness of the experimental design and method, and the collection, analysis, and interpretation of the data. We have reviewed the final version of the manuscript and approve it for publication. To the best of our knowledge and belief, this manuscript has not been published in whole or in part nor is it being considered for publication elsewhere. Also, the manuscript was not previously submitted to **Biochemical Engineering Journal**.

Please feel free to contact us if you have any questions or need additional information from us concerning this submission.

Sincerely yours,

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Detailed Response to Reviewers

Manuscript Number: **BEJ-D-16-00782R1**

Manuscript Title: **Engineering *Yarrowia lipolytica* for efficient γ -linolenic production**

Article Type: **Full Length Article**

Dear Editor,

We would like to thank the anonymous referees for their kind comments and valuable suggestions. We are pleased to answer the questions of the reviewers' and the manuscript (Manuscript number: **BEJ-D-16-00782R1**) has also been extensively revised according to the comments (resubmitted online).

The answers also have been summarized one by one as listed below.

Finally, we sincerely hope that the revised manuscript can meet the standard for publication in your journal.

PS: **The modifications were marked in red in the revised manuscript.**

Thank you very much.

Sincerely yours,

Xiao-Jun Ji

Reviewer comments:

Reviewer #1:

This manuscript by Sun et al. describes a two-prong approach to engineering a *Y. lipolytic* to produce gamma-linolenic acid (GLA). Specifically, the authors integrate a heterologous delta6-desaturase gene to convert linolenic acid to gamma-linolenic acid and use a two-stage variable temperature bioprocess to increase production. The manuscript is clear, well-written and the conclusions are justified. While the achieved titers of GLA are not overly exciting, this work advances the understanding of the process and provides new data on two-stage bioprocesses with a low-temperature production phase. Please consider the following minor points:

Answer: **Thank you for your recognition of our work. Your suggestion is very pertinent and helpful for our work. And we have revised the manuscript carefully one by one according to your suggestion as follows.**

Minor comments:

1. The genome editing tools for *Y. lipolytica* have recently been expanded to include CRISPR-Cas9 systems. Please add current references to line 49 of page 3 (e.g., DOI: 10.1021/acssynbio.5b00162)

Answer: **Thank you for your reminder. The current references below were added to line 49 of page 3 and the reference list. We have also changed the reference number in**

the reference list accordingly (marked in red).

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.

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. Biot 43 (2016) 1085-1093.

2. Please provide a reference to the hp4d promoter and its quasi-constitutive function.

Answer: Thank you for your reminder. We have added the reference in line 31 of page 9 (marked in red).

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.

3. Please expand the discussion of the increase in GLA production at low temperature. For example, what is the reasoning behind the increase in ricinoleic acid (ref 33) at low temperature, does the same reasoning apply here?

Answer: Thank you for your suggestion. I am sorry for my unclear expression before. In the revised manuscript, we have expanded the discussion of the increase in GLA production when shifted to lower temperature to line 37 of page 18 (marked in red).

“Zheng et al. [36] demonstrated specific cell growth rate are higher in the log phase than that of in the stationary phase. Thus, the cells shifted to lower temperature in the log phase were competent to produce a high level of GLA as described in the work of R. Holic et al. [35].”

The references were also added in the reference list.

M. Zheng, G.C Du, W.F. Guo, J. Chen, A temperature-shift strategy in batch microbial transglutaminase fermentation, Process Biochem. 36 (2001):525-530.

Reviewer #2:

This manuscript describes the production of GLA in *Y. lipolytica* by expression a Delta-6 desaturase, building on previous work from the authors. The main contribution of the present paper was to show that temperature shifts increase the production of GLA. Overall, I think this is a very interesting results. I never thought of temperature before as tuning variable -- in these regards, the results are novel. I recommend publication with a few minor suggestions for improving the manuscript.

Answer: Thank you for your recognition of our work. Your suggestion is very pertinent and helpful for our work. And we have revised the manuscript carefully one by one according to your suggestion as follows.

Minor comments:

1. Please provide the codon optimization table and the sequence for the Delta-6 desaturase. This is necessary so that others can reproduce the results. I did not see this unless I am missing something.

Answer: Thank you for your suggestion. We have provided the sequence for codon-optimized $\Delta 6$ -fatty acid desaturase and $\Delta 6$ -fatty acid desaturase in Supplementary Table S1.

Supplementary Table 1

The sequence for codon-optimized $\Delta 6$ -fatty acid desaturase and $\Delta 6$ -fatty acid desaturase

Gene	Sequence
codon-optimized $\Delta 6$ -fatty acid desaturase	ATGGCCGCCGCCCCCTCCGTCCGAACCTTCACCAGAGCCG AAATCCTGAACGCTGAGGCCCTGAACGAGGGCAAGAAGG ACGCCGAGGCCCTTTCCTCATGATCATCGACAACAAGGT GTACGACGTGCGAGAATTCGTGCCCCGACCACCCTGGTGGT TCCGTGATTCTGACCCATGTCGGTAAGGACGGCACTGACG TCTTTGACACCTTCCACCCCGAGGCCGCTTGGGAAACCCT GGCTAATTTTTATGTTGGTGACATTCACGAGTCTGACCGA GACATCAAGAACGACGACTTTGCTGCCGAAGTCCGAAAG CTGCGAACCCTGTTTCAGTCCCTGGGTACTACGATTCTTC TAAGGCCTACTACGCCTTCAAGGTCTCCTTCAACCTGTGC ATCTGGGGCCTGTCCACCTTCGTGCGTCCGAAGTGGGGTC AAACCTCTACCCTCGCTAACGTTGTTTCTGCTGCTCTGCTG GGTCTGTTCTGGCAGCAGTGCGGTTGGCTCGCCACGATT TCCTGCACCATCAGGTCTTCCAGGACCGATTCTGGGGCGA CCTCTTTGGCGCTTTCCTCGGTGGCGTGTGTCAGGGTTTCT CCTCCTCCTGGTGGAAGGACAAGCACAATACCCACCACGC CGCCCCAACGTGCATGGTGAGGACCCCGACATCGACACT CACCCCTCCTGACCTGGTCCGAGCACGCTCTGGAGATGT TCTCCGATGTCCCGACGAGGAGCTCACCAGAATGTGGTC CAGATTCATGGTGCTGAACCAGACCTGGTTCTACTTCCCC ATCCTGTCTTTCGCCCCACTCTCCTGGTGCCTCCAGTCCAT CCTCTTCGTCATGCCCAACGGTCAGGCTCATAAACCTTCT GGTGCCCGAGTGCCATATTTCCCTGGTGGAACAGCTGTCCC TCGCCATGCACTGGACCTGGTATTTAGCCACCATGTTTCT GTTTCGTGAAGGACCCTATCAACATGTTTCGTGTACTTCTG GTGTCTCAGGCCGTGTGTGGCAACCTGCTGGCTCTGGTCT TTTCTCTGAACCACAACGGCATGCCCGTCATCTCCAAGGA

	GGAGGCCGTCGACATGGATTTCTTCACCAAGCAGATCATC ACCGGCCGAGATGTCCACCCTGGCCTGTTCCGCAACTGGT TCACCGGCGGCCTGAACTACCAGATCGAGCATCACCTGTT CCCCTCCATGCCCCGACACAACTTCTCCAAAATCCAGCCC GCCGTGGAGACCCTCTGTAAGAAGTACAACGTGCGATACC ACACTACCGGTATGATCGAGGGCACCGCCGAGGTCTTCTC CCGACTGAACGAGGTCTCTCGAGCCGCCTCTAAGATGGGT AAGGCTCAGTAA
Δ6-fatty acid desaturase	ATGGCTGCTGCTCCCAGTGTGAGGACGTTTACTCGGGCCG AGATTCTGAATGCCGAGGCCCTGAATGAAGGCAAGAAGG ATGCTGAGGACACCCTTCCTGATGATCATCGACAACAAGGT GTACGATGTCCGCGAGTTTGTCCCTGATCATCCCGGTGGA AGTGTGATTCTCACGCACGTTGGCAAGGACGGCACTGACG TCTTTGACACTTTCCACCCCGAGGCTGCTTGGGAGACTCTT GCCAACTTTTACGTTGGTGATATTCATGAGAGCGACCGCG ACATCAAGAATGATGACTTTGCGGCCGAGGTTTCGAAGCT GCGTACCTTGTTCCAGTCTCTTGGCTACTACGATTCTTCCA AGGCATACTACGCCTTCAAGGTCTCGTTCAACCTCTGCAT CTGGGGCCTGTCGACATTGTTGTTGCCAAGTGGGGCCAG ACCTCGACCCTCGCCAATGTAGTTTCGGCTGCGCTTTTGG GTCTCTTCTGGCAGCAGTGCGGATGGTTGGCGCACGACTT TTTGCATCACCAGGTCTTCCAGGATCGTTTCTGGGGCGAT CTTTTCGGTGCCCTTCTTGGGAGGTGTCTGCCAGGGTTTCTC ATCCTCCTGGTGGAAGGACAAGCACAACACTCACCACGCC GCTCCCAACGTCCACGGTGAGGATCCCGACATTGACACTC ACCCTCTGTTGACCTGGAGTGAGCATGCTCTGGAGATGTT CTCGGATGTCCCTGACGAGGAGCTGACCCGCATGTGGTTCG CGTTTCATGGTCCTCAACCAGACCTGGTTCTACTTCCCCAT TCTCTCGTTTGCCCGTCTCTCCTGGTGCCCTCCAGTCTATTC TCTTTGTTATGCCTAACGGTCAGGCCCAAGCCCTCGGG TGCGCGTGTGCCATTTCCTTGGTCGAGCAGCTGTCTCTCG CTATGCACTGGACCTGGTACCTCGCCACCATGTTCTGTTC GTGAAGGATCCCATCAACATGTTTGTGTACTTTTTGGTATC GCAGGCTGTTTGCGGCAACTTGTGGCGCTTGTGTTCTCAC TCAACCACAACGGTATGCCTGTGATTTCCAAGGAGGAGGC AGTCGATATGGATTTCTTCACCAAGCAGATCATCACGGGT CGTGATGTCCACCCTGGTCTGTTTGCCAACCTGGTTCACAG GTGGATTGAACTACCAGATTGAGCATCACTTGTTCCCTTC GATGCCCCGCCACAACCTTTTCAAAGATCCAGCCTGCTGTC GAGACCCTGTGCAAAAAGTACAATGTCCGATAACACACC ACCGGCATGATTGAGGGAACTGCAGAGGTCTTTAGCCGTC TGAACGAGGTCTCCAGGGCTGCCTCCAAGATGGGCAAGG CACAGTAA

2. I am very intrigued by the temperate result. How do the temperature shifts affect the distribution of fatty acyl chains. Ideally, the authors would provide results similar to table 2 for the polf control.

Answer: Thank you for your suggestion. We have provided results similar to table 2 for the polf control as table3 (marked in red).

Table 3 Fatty acid profiles of the control strain *Yarrowia lipolytica* Polf under various temperature control strategies.

Temperature (°C)	Fatty acid composition (%)					
	C16:0	C16:1	C18:0	C18:1	C18:2	C20:0
15	29.12	6.23	17.65	24.10	19.23	3.67
20	34.02	5.03	18.25	22.54	17.19	3.05
28	40.62	4.12	23.27	18.39	11.23	2.56
28 (0-24h) 20 (after 24h)	31.21	6.02	19.99	24.51	16.46	1.81
28 (0-48h) 20 (after 48h)	34.25	4.83	22.46	21.95	13.62	2.89
28 (0-24h) 15 (after 24h)	33.12	4.67	21.45	23.89	14.19	2.68
28 (0-48h) 15 (after 48h)	35.31	3.89	20.96	23.25	13.17	3.42

3. The authors should avoid auxotrophic strains. Have they looked at the prototrophic parent? Are the results the same?

Answer: Thank you for your suggestion. Auxotrophic strains have not looked at the prototrophic parent. Recently, leucine auxotrophy has been reported to affect lipogenesis of *Yarrowia lipolytica* because leucine may be an intracellular trigger to stimulate lipogenesis. The control and engineered strain in this study are all leucine auxotrophic strains. We think the two strains devoid of leucine can eliminate the effects of Leucine defect. Although the leucine auxotrophic strains were used in this research, compared to the original Polf, the engineered strain performed a higher glucose uptake and biomass production.

On the other hand, we are working on GLA synthesis of the engineered strain by addition of leucine stimulation. Maybe in the future we can reveal the link between leucine and GLA synthesis.

4. As the review form asks the question, I do not think the highlights accurately reflect the contributions of the manuscript. In particular, expression of the desaturase was previously done. The contribution of the present paper was to show the beneficial effect of temperature shifts.

Answer: Thank you for your suggestion. We have rewritten the highlights to reflect the beneficial effect of temperature shifts (marked in red).

Highlights

- A recombinant *Y. lipolytica* for GLA production was constructed.

- The effect of temperature on production of GLA by the recombinant strain was investigated.
- DCW, lipid and GLA production were enhanced by temperature shift strategies.
- The best culture condition was 28 °C followed by 6-day culture at 20 °C.

Highlights

- A recombinant *Y. lipolytica* for GLA production was constructed.
- The effect of temperature on production of GLA by the recombinant strain was investigated.
- DCW, lipid and GLA production were enhanced by temperature shift strategies.
- The best culture condition was 28 °C followed by 6-day culture at 20 °C.

Engineering *Yarrowia lipolytica* for efficient γ -linolenic acid production

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Huang^{3, 4, 5}, Xiao-Jun Ji ^{1, 5*}

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1 **Abstract:**

2
3 γ -linolenic acid (GLA) has various well-documented beneficial physiological
4 effects and high biological significance. Because the natural supply of GLA is
5 insufficient, microbial GLA production is a promising method for pharmaceutical and
6 nutraceutical purposes. To establish and develop a biotechnological process for GLA
7 production by *Yarrowia lipolytica*, the codon-optimized $\Delta 6$ -desaturase from
8 *Mortierella alpina* was introduced into this yeast under the control of the strong hp4d
9 promoter. A recombinant *Y. lipolytica* strain was constructed, which produced 4.6%
10 GLA in total fatty acids. By using a temperature-shift strategy of cultivation,
11 consisting in preliminary growth at 28 °C followed by 6-day culture at 20 °C, optimal
12 levels of dry cell weight (DCW), lipid content and GLA concentration were obtained
13 from the recombinant strain: 18.55 g/L, 1.16 g/L and 71.6 mg/L, respectively. These
14 DCW, lipid and GLA values were respectively 25.7%, 19.6% and 60.9% higher than
15 those obtained in the control cultivation experiment at the standard constant
16 temperature of 28 °C. This work demonstrates the excellent capacity of *Y. lipolytica*
17 for GLA production, by combining metabolic engineering with a temperature-shift
18 strategy.

19 **Keywords:**

20 γ -linolenic acid, *Yarrowia lipolytica*, $\Delta 6$ -desaturase, Metabolic engineering,
21 Temperature-shift strategy
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1. Introduction

Polyunsaturated fatty acids (PUFAs) are important metabolites which have great potential in pharmaceutical and nutraceutical applications. They are significant cellular components conferring fluidity to membranes and influencing signaling process in living cells [1,2]. As a consequence, the demand for PUFAs, for use as health supplements, has continually increased in recent decades [3]. Although PUFAs can be isolated from natural resources, such as plant seeds and marine organisms, the productivity of many of these systems is extremely low due to long growth cycles, to dependence on climate and weather variations (e.g. drought), and to expanding marine pollution. It is therefore highly desirable to produce PUFAs from alternative sources that are economical and sustainable. Nevertheless, economic production of targeted PUFA utilizing microorganisms will require optimizing native regulatory networks or assembling heterologous metabolic pathways, which can only be achieved through molecular, genetic and combinatorial approaches [4].

In recent decades, the non-conventional yeast *Yarrowia lipolytica* has been an attractive system for microbial oil production and has been used in a broad range of industrial applications, such as organic acid production, protein production and bioremediation [5-7]. The use of *Y. lipolytica* also benefits from a fully sequenced genome [8] and well-developed genetic tools [9-11], therefore, engineering this yeast can be achieved with relative ease. Moreover, *Y. lipolytica* has also been shown to be rather robust and able to grow on a variety of substrates [7].

The metabolic pathways for *de novo* fatty acids synthesis from glucose in *Y. lipolytica* are beginning to be thoroughly understood, as illustrated in Fig. 1. PUFA

1 biosynthesis is generally associated with a variety of pathways, catalyzed by a
2
3 complex series of desaturation and elongation steps. All the genes encoding
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5 desaturation and elongation activities involved in PUFA biosynthesis have been
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7 cloned and identified from multiple organisms, and the availability of these enzymes
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9 made it possible to accumulate tailored fatty acids in heterologous hosts [12]. The
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11 Δ^6 -fatty acid desaturase is the rate-limiting enzyme in the biosynthesis of PUFAs,
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13 and is responsible for the conversion of the essential fatty acids linoleic acid (LA, Δ^9 ,
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15 ^{12} C18:2) and α -linolenic acid (ALA, $\Delta^9, 12, 15$ C18:3) to γ -linolenic acid (GLA, $\Delta^{6, 9, 12}$
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17 C18:3) and stearidonic acid (STA, $\Delta^{6, 9, 12, 15}$ C18:3), respectively. Many physiological
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19 and pathological diseases related to low levels of PUFA can be efficiently alleviated
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21 by dietary supplementation of GLA [1]. Moreover, it has been reported that GLA is
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23 unique among the (n-6) PUFA family members (LA, GLA, arachidonic acid) in its
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25 potential to suppress tumor growth and metastasis [2]. These beneficial effects made
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27 GLA a much valuable and sought-after product. The principal sources for GLA are
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29 vegetable oils derived from evening primrose, borage and blackcurrant. However,
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31 given the finite supply of arable land and increasing demand for GLA, it is unlikely
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33 that GLA from plant oils will contribute significantly to meeting future food
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35 supplementation needs.

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37 The most significant unsaturated fatty acids produced by *Y. lipolytica* are oleic
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39 acid (C18:1) and linoleic acid (C18:2), whereas unsaturated fatty acids with more
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41 than two double bonds, so-called PUFAs, are not synthesized by this microorganism
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43 naturally [13]. However, *Y. lipolytica* has been proposed as a preferred host for PUFA
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1 production [14], and engineering of PUFA biosynthesis pathways in this yeast has
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3 already been attempted. Xue et al [15] produced tailored fatty acids comprising
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5 eicosapentaenoic acid (EPA, C22:5) at 15% of dry cell weight (DCW) by metabolic
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7 engineering of *Y. lipolytica* and commercial products have been developed using this
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9 recombinant yeast [16]. Zhang et al. [17] successfully produced *trans*-10, *cis*-12
10
11 conjugated linoleic acid in *Y. lipolytica* Po1h strain via expression of linoleic acid
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13 isomerase. Efforts towards effective carbon flow redistribution of lipid synthesis
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15 pathways in this promising yeast should continue to meet the increasing demand.
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22 A previous work has reported construction of a *de novo* GLA biosynthesis system
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24 in *Y. lipolytica* via expression of desaturases cloned from *Mortierella alpina* [18]. In
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26 the present study, we planned to improve GLA production in *Y. lipolytica* by
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28 expression of a codon-optimized $\Delta 6$ -desaturase from *M. alpina* under the control of
29
30 the hp4d promoter and to optimize fermentation conditions. Temperature is one of the
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32 principal factors that affect cell growth and fatty acid synthesis. However, little
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34 attention has been paid up to now to define the effects of temperature on GLA
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36 fermentation by *Y. lipolytica* and to study a two-stage process that uses different
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38 temperatures to optimize cell growth and GLA fermentation. In an attempt to enhance
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40 GLA production, fermentation conditions were modified using a temperature-shift
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42 strategy to redistribute carbon flux from saturated to unsaturated fatty acids in *Y.*
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44 *lipolytica*. By combining metabolic engineering with a temperature-shift strategy,
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46 GLA production was achieved in *Y. lipolytica* at levels of up to 6.1% of total fatty
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48 acids and 18.55 g/L DCW. To our best knowledge, this work represents the first effort
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1 to utilize a temperature-shift strategy for the production of GLA in this yeast and may
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3 provide alternative routes to lower production costs in future industrial applications.
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6 **2. Materials and Methods**

7 **2.1 Strains, plasmids and culture conditions**

8 *E. coli* DH5 α was grown in Luria-Bertani broth containing ampicillin (100
9 μ g/mL). The auxotrophic strain *Y. lipolytica* Po1f (ATCC # MYA-2613, *MatA*,
10 *leu2-270*, *ura3-302*, *xpr2-322*, *axpl-2/Ura⁻*, Leu⁻) and the hp4d-based expression
11 vector p0, carrying *URA3* selection marker, have been described previously [19, 20].
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21 Peptone dextrose culture medium (YPD) containing 20 g/L peptone, 10 g/L yeast
22 extract and 20 g/L glucose was used for normal growth. The synthetic minimal
23 medium, minus uracil (YSU-Ura), plates for the selection of transformed *Y. lipolytica*
24 were made with 6.7 g/L yeast nitrogen base (without amino acids), 0.77 g/L
25 CSM-Ura (Complete Supplement Mixture minus uracil), 20 g/L glucose and 20 g/L
26 agar. The shake flask fermentation experiments were carried out in a medium
27 comprising 8 g/L yeast nitrogen base (without amino acids), 9 g/L yeast extract, 80
28 g/L glucose and 10 μ g/L biotin. From an agar plate, a single colony of yeast was
29 inoculated into YPD medium (100 mL in 500 mL flask, 200 rpm, 28 °C, 48 h) and
30 grown to an optical density (OD₆₀₀) of 4.0-5.0. The pre-cultures were allowed to grow
31 in fermentation medium with an inoculum size of 10% (v/v), for a total duration of 7
32 days, during which aliquots were taken and biomass, lipid content and fatty acid
33 compositions analyzed.
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56 **2.2 Plasmid construction and transformation**

57 Standard molecular genetic techniques were used throughout this study [21].
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1 Recombinant vectors that express the gene of interest under the control of the hp4d
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3 promoter were generated using the ClonExpress^{TM} MultiS one step cloning kit
4
5
6 (Vazyme, China), according to the manufacturer's instructions.
7

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9 The expression vector for the $\Delta 6$ -fatty acid desaturase gene was constructed as
10
11 follows. The $\Delta 6$ -fatty acid desaturase gene from *Mortierella alpine* was codon
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13 optimized for expression in *Y. lipolytica*. The sequences for $\Delta 6$ -fatty acid desaturase
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15 and codon-optimized $\Delta 6$ -fatty acid desaturase were listed in Supplementary Table 1.
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18 The codon-optimized $\Delta 6$ -fatty acid desaturase gene was then amplified using the
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20 following primers:
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25 D6-F (5'-CAGTGGCCGGATCCGAAATTCATGGCCGCCGCCCCCTCCGT-3')

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28 D6-R (5'-GCGGCCGCCATATGGAAATTCTTACTGAGCCTTACCCATCT-3')

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30 with the underlined nucleotides representing added *Eco*RI restriction sites.
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34 The PCR-amplified sequences were digested using *Eco*RI and one-step cloned
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36 into the *Eco*RI-digested p0 backbone to generate the recombinant vector p0-6-D. The
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38 newly constructed plasmid was screened by *Eco*RI digestion and PCR with the
39
40 primers D6-F and D6-R, and confirmed by DNA sequencing.
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45 Subsequently, the plasmid was linearized by digestion and integrated into Po1f
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47 genome, at the *XPR2* terminator locus, using the lithium acetate transformation
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49 method [22]. Transformants of the resulting strain Po1f-6-D were selected on
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51 YSC-Ura plates. To verify the transformants for correct integration, genomic DNA
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53 was extracted and used as template for PCR analysis. Verified transformants were
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55 stored for long term at -80 °C in glycerol solution and for short-term at 4 °C on
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YSC-Ura plates.

2.3 Analytical methods

The concentration of extracellular glucose was measured by a biosensor equipped with a glucose oxidase electrode (SBA-40C, Institute of Biology, Shandong Academy of Sciences, China). OD₆₀₀ were acquired by monitoring the optical density of the cultures at 600 nm using a UV–visible spectrophotometer (Lambda-25, Perkin-Elmer, USA) with appropriate sample dilution. DCW determination was performed by harvesting the culture broth, after which the cells were separated by centrifugation at 5000 rpm for 6 min and washed with distilled water. Subsequently, the supernatant was discarded and the cell mass was dried at 65 °C to constant weight.

2.4 Lipid extraction and fatty acid analysis

Lipids from an appropriate weighted aliquot of dried cells were extracted using the procedure by Folch et al [23]. A measured quantity of dried cells (roughly 0.5 g) was homogenized in 5 mL of 4 M HCl at room temperature for 30 min, resuspended in a mixture of 10 mL chloroform and 5 mL methanol and vortexed for 30 min. The lipids were separated by evaporating the chloroform.

The extracted total lipids were methylated to generate fatty acid methyl esters (FAMES) as follows: quantitative lipid extracts (roughly 5 mg) were methylated by adding 1 mL hexane and 100 µL methanol, after which the samples were vortexed for 10 min and centrifuged for 3 min at 3000 g. An aliquot comprising 800 µL of the hexane layer was then transferred into a clean 1.5 mL microcentrifuge tube for Gas

Chromatography-Flame Ionization Detector (GC-FID) analysis. Samples of 1 μ L were injected into a GC-FID (Agilent 6890 GC), equipped with an Agilent HP-5 column, with detector temperature set to 250 °C and N₂ flow at 1 mL/min. Fatty acid compositions were determined by comparing peak retention times to those of known standard substances. The amount of fatty acids was quantified by comparing the peak area of the analyte to the peak of the standard substance at known concentrations.

3. Results

3.1 Construction of the recombinant GLA producing strain

The DNA sequence of the Δ 6-fatty acid desaturase from *M. alpina* was redesigned using codons favored by *Y. lipolytica*, and the GC content was adjusted to that of the host. The codon adaption index (CAI) was improved from 0.83 (natural sequence) to 0.91 (synthetic codon gene). The single-copy expression vector p0, which places the gene of interest under the control of the strong quasi-constitutive hybrid hp4d promoter [19], was used to develop the recombinant strain. To produce GLA in *Y. lipolytica*, the codon-optimized Δ 6-desaturase gene from *M. alpina* was used to construct the recombinant vector p0-6-D bearing the desaturase expression cassette. The entire process of constructing the recombinant strain Po1f-6-D and verification of GLA production in the recombinant strain is shown in Fig. 2.

Based on the plasmid p0 (*URA3*), the recombinant plasmid p0-6-D was constructed as shown in Fig. 2A. The recombinant plasmid was digested with *AvrII* and used to transform *Y. lipolytica* Po1f strain (Fig. 2B). The integration of a single copy of p0-6-D at the *XPR2* terminator genomic site of Po1f allowed cell growth of

1 the corresponding transformants on Uracil-deficient plates after 2-3 days of
2 incubation. Subsequently, correct plasmid integration was verified by PCR, as
3 described in the Materials and Methods section (Fig. 2C). To determine whether
4 codon-optimized $\Delta 6$ -desaturase could be expressed effectively in *Y. lipolytica*, the
5 engineered strain Po1f-6-D was incubated in fermentation medium (Fig. 2D). When
6 lipids were extracted and fatty acid profiles were analyzed by GC, it was observed
7 that GLA represented 4.6% of total fatty acids in the engineered strain Po1f-6-D,
8 when it was not detected in the control strain Po1f (Fig. 2E). These results indicate an
9 efficient conversion of the endogenous linoleic acid to GLA, catalyzed by the
10 heterologous $\Delta 6$ -desaturase.
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28 The fermentation performance of the engineered GLA-producing strain Po1f-6-D
29 was comparable to (and even slightly better than) that of the control strain Po1f, as
30 shown in Fig. 3. The value of DCW increased in the engineered strain compared to
31 the control, reaching a maximum of 14.84 g/L (Fig. 3A). Consistently with the
32 increased cell growth, the glucose uptake rate was also higher in the engineered strain
33 (Fig. 3B). The lipid yield, which attained 6.55% in the engineered strain, was not
34 significantly different from that of the control strain at 6.23%. The final fatty acid
35 profiles of the two strains are shown in Fig. 3C. Concomitantly with the appearance
36 of GLA in the engineered strain, there was a slight decrease in linoleic acid and oleic
37 acid production. No other significant difference in the final lipid profiles of the two
38 strains was documented, with palmitic acid, stearic acid and oleic acid being the most
39 abundant fatty acid species and accounting for roughly 38%, 23% and 18%,
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respectively.

3.2 Optimization of GLA synthesis by using a temperature-shift strategy

The engineered strain *Y. lipolytica* Po1f-6-D, obtained by expressing a codon-optimized $\Delta 6$ -desaturase, produced a certain amount of GLA (4.6% of total fatty acid) under standard growth conditions at 28 °C. Starting from this result, efforts were continued to improve the production of GLA by Po1f-6-D, which can be achieved by enhancing either DCW or intracellular GLA proportion. It is known that GLA formation entails a complex combination of different reactions, and both cell growth and glucose utilization are important for GLA production. It has been reported that the maximal growth temperature of *Y. lipolytica* does not exceed 32-34 °C [24]. In the present study, cell growth was severely inhibited above 35 °C (data not shown). *Y. lipolytica* Po1f-6-D was consequently cultivated at 15 °C, 20 °C and 28 °C. As illustrated in Fig. 4A, cultivation at 28 °C led to the highest value of DCW, namely 14.84 g/L, compared to 13.34 g/L at 20 °C and 11.57 g/L at 15 °C. In accordance with increasing cell growth at higher temperature, the residual glucose level decreased between 15 °C and 28 °C (Fig. 4B). There was almost 30 g/L glucose left in the culture broth at 15 °C, while the initial glucose concentration was 80 g/L. A comparison of GLA concentrations at different temperatures is shown in Fig. 4C: it was generally higher at lower temperatures. The maximum GLA ratio was 7.99% of total fatty acids at 15 °C and a relatively high GLA ratio of 6.91% was achieved at 20 °C (Table 2), whereas the maximum value of GLA concentration (57.82 mg/L) was achieved at 20 °C (Fig. 4C) due to the obvious cell growth inhibition at 15 °C.

1 These results are in line with the situation in other eukaryotic microorganisms, such
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3 as *M. alpina*, in which a relatively high temperature enhanced cell growth, and a
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5 lower temperature was more favorable for PUFA synthesis [25].
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9 Although a lower temperature is more favorable for increased GLA synthesis, it
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11 is disadvantageous for practical reasons, since it results in low biomass concentration.
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13 As shown in Fig. 5A, controlled temperature-shift processes were developed, to take
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15 into account the differences of temperatures suitable for cell growth or GLA synthesis:
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17 a temperature of 28 °C at an earlier fermentation stage was favorable to cell growth,
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19 and shifts to 20 °C or 15 °C were done after 1 or 2 days of growth at 28 °C. The
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21 effects of these temperature-shift strategies on GLA concentration, glucose
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23 consumption and DCW are shown in Fig. 5B to 5E. The GLA concentration and
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25 DCW of cultures grown in the four temperature-shift experiments were all higher
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27 than in the control at 28 °C. The improvement in biomass concentration was
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29 accompanied by increased glucose consumption. The highest GLA content of 6.1%
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31 and the highest DCW of 18.78 g/L were achieved with the shift to 20 °C after 1 day.
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33 An interesting phenomenon was also observed when the shift was done at 2 days, in
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35 the early stationary phase: although a longer growth phase at optimal temperature
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37 would have been expected to be beneficial, the accumulation of GLA and biomass
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39 concentration were in fact lower than when the shift was done at day 1, during the log
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41 phase (Fig. 5). A comparison of the fermentation data obtained using the different
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43 temperature control strategies is summarized in Table 1. The best temperature-shift
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45 strategy (shift from 28 to 20 °C after 1 day) considerably improved the DCW, total
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lipids and GLA concentration, which were increased by 25.7%, 19.6% and 60.9%, respectively compared to standard cultivation at 28 °C. Table 2 and 3 also showed some interesting variations of fatty acid profiles of *po1f-6-D* and *po1f* in cells under different temperature control strategies. Here, the best result in term of GLA ratio, namely 6.17%, obtained by using temperature shifting strategies, does not have a competitive edge over the result of 7.99% previously obtained at 15 °C. However, in order to develop a viable process, we should not take into account only the GLA percentage, but also the DCW and total lipid amount.

4. Discussion

The aim of this study was to construct a *de novo* GLA biosynthesis pathway by transforming the yeast *Y. lipolytica* with a codon-optimized $\Delta 6$ -desaturase from *M. alpina*. The bulk of the isolated fatty acids were palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid and arachidic acid in the control strain *Y. lipolytica* *Po1f*. We choose to produce GLA in *Y. lipolytica* instead of using the model microorganism *Saccharomyces cerevisiae*, because *Y. lipolytica* is an oleaginous yeast with the innate ability to accumulate linoleic acid, a precursor of GLA. To date, several groups have been able to produce low amounts of GLA in *S. cerevisiae* via heterologous expression of $\Delta 6$ -desaturases, but supplementation with linoleic acid as exogenous substrate was necessary [26, 27].

Chuang et al. [18] previously used a single-copy integrative vector with *hp4d* promoter to express a $\Delta 6$ -desaturase, cloned from *M. alpine*, in *Y. lipolytica*. In our study, a codon-optimized $\Delta 6$ -desaturase was expressed under the control of the same *hp4d* promoter. When the codon-optimized $\Delta 6$ -desaturase integrant was cultivated on

1 glucose in shake flasks at 28 °C, GLA was synthesized in the engineered strain in the
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3 absence of exogenously added fatty acid substrates and represented 4.6% of total
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5 lipids, a level similar to the 4.9% found in the work of Chuang et al. [18]. Therefore,
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7 it seems that the fact of using in our study a synthetic gene, adapted to *Y. lipolytica*
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9 codon bias and GC content, the two expression systems being otherwise similar, is
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11 not particularly beneficial.
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17 During the time course of GLA fermentation, the engineered strain Po1f-6-D
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19 completed exponential growth by hour 48, after which DCW remained relatively
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21 constant. In addition to GLA synthesis, the biomass from the engineered strain was
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23 significantly higher than in the control and glucose was consumed more rapidly,
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25 suggesting that the expression of $\Delta 6$ -desaturase elevated the glucose uptake rate and
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27 somewhat favored the growth of *Y. lipolytica*. This result was different from a
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29 previous work in which overexpression of acetyl-CoA carboxylase (ACC1) and
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31 diacylglycerol acyltransferase (DGA1), allowing to improve lipid content from 8.7%
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33 to 41.4%, impaired growth in *Y. lipolytica* [28]. Thus, expression of ACC1 and DGA1
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35 impaired cell growth, while our Po1f-6-D engineered strain consumed glucose more
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37 rapidly than the control, because it diverted more cellular resources toward oil
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39 synthesis instead of synthesizing non-lipid biomass. The integration of $\Delta 6$ -desaturase
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41 also resulted in a higher glucose uptake than in control strain. However, more cellular
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43 resources were diverted toward non-lipid biomass instead of lipogenesis when overall
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45 lipid content did not change. This might be the reason why Po1f-6-D engineered
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47 strain reached a higher biomass concentration than the control strain.
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1   The GLA yield obtained initially from Polf-6-D was not very high (4.6% of total
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3 fatty acids). In order to optimize GLA synthesis, we took advantage of the high
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5 sensitivity of fatty acid desaturases to temperature change and higher activity of the
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7 enzymes at lower growth temperatures [29]. A relatively high temperature was
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9 favorable for glucose consumption and biomass accumulation, but a lower
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11 temperature was beneficial for GLA synthesis. This may be due to distinct
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13 rate-limiting reactions which rely on temperature, resulting in a better utilization of
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15 carbon and/or energy at the suitable temperature and in increased availability of vital
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17 coenzymes/cofactors (e.g. ATP, NADPH) for either cell reproduction or production of
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19 metabolites [30]. Our results illustrate that a low culture temperature favored
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21 unsaturated fatty acid accumulation during GLA fermentation (Table 2 and 3). In
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23 order to maintain a proper membrane lipid fluidity and function, the proportions of
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25 unsaturated fatty acids, including GLA, increase in response to low cultivation
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27 temperature [31]. On the other hand, it is also known that dissolved oxygen (DO)
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29 level is lower at higher temperatures. Thus, at lower temperature a greater amount of
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31 molecular oxygen is available that allows the oxygen-dependent enzymes to catalyze
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33 fatty acid desaturation [32]. In the present study, the changes in GLA yield at
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35 different temperatures might be due to a combination of all of the factors above.

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38   It has been reported that intracellular lipid accumulation increases following
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40 depletion of nitrogen from the medium (when DCW reaches its maximum value and
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42 remains relatively constant), and the biochemical mechanisms leading to fatty acid
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44 biosynthesis after nitrogen exhaustion have been clarified [33, 34]. Thus, lipogenesis
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continues throughout the fermentation process, despite cessation of biomass accumulation within 2 days. When metabolite production is not dependent on cell growth but is affected by culture temperature, a novel two-stage temperature cultivation strategy could be applied to maximize the level of the target metabolite. The idea was to achieve optimal cell reproduction and GLA production by the sequential use of separately optimized temperatures. Thus, we developed a temperature-shift strategy for the production of GLA in *Y. lipolytica* and determined the optimal time-shift point by lowering the temperature (from 28 °C to 15 °C or 20 °C) during the log phase (shift after day 1) or in the early stationary phase (shift after day 2). Interestingly, the GLA ratio and biomass concentration were higher with a shift after day 1, suggesting that cells have to be shifted to lower temperature at a certain point in the log phase to produce a high level of GLA. This result is in good agreement with previous studies which have shown that cells shifted to a lower temperature during the log phase have the potential to produce a higher level of ricinoleic acid [35]. Zheng et al. [36] demonstrated specific cell growth rate are higher in the log phase than that of in the stationary phase. Thus, the cells shifted to lower temperature in the log phase were competent to produce a high level of GLA as described in the work of R. Holic et al. [35]. The highest total lipid and GLA concentrations of 1.16 g/L and 71.6 mg/L were obtained from cells shifted to 20 °C after day 1. However, the GLA portions of total fatty acids obtained with the temperature-shifts remain lower than those from corresponding single temperature cultures at 20 °C or 15 °C (Table 2), which infers that increased biomass

1 is probably a main contributor to increased GLA concentration in the
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3 temperature-shift experiments.
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6 In the present study, the optimal biomass concentration, total lipids and GLA
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8 concentration were 18.55 g/L, 1.16g/L and 71.6 mg/L under the conditions of 28 °C
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10 for 1 day followed by 20 °C for 6 days, which represents increases of 25.7%, 19.6%
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12 and 60.9%, respectively, compared to standard cultivation at 28 °C. Moreover, this
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14 optimal GLA yield still represents an increase of 24.7% when compared to the best
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16 result obtained in an experiment at a single temperature, namely 20 °C, at which GLA
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18 production was favored despite low cell growth. It appears that cell growth and GLA
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20 accumulation could be optimized simultaneously by applying this temperature-shift
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22 strategy, as opposed to the single temperature controls in which lipid accumulation
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24 was in conflict with biomass concentration. Higher dissolved oxygen levels at lower
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26 temperatures were maybe also able to mitigate cell autolysis at later stages of
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28 fermentation [30]. This might be the reason why the biomass concentration did not
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30 decrease but actually increased when the culture temperature was shifted to a lower
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32 value after 24 or 48 h of growth.
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44 5. Conclusions

45 In conclusion, a *de novo* GLA biosynthesis system was successfully constructed
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47 by transforming *Y. lipolytica* with a codon-optimized $\Delta 6$ -desaturase gene from *M.*
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49 *alpina*. The GLA concentration in the engineered strain was further increased by
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51 applying a temperature-shift cultivation strategy and reached 6.1% from total fatty
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53 acids. Similarly, the two-stage culture strategy proposed here, based on *Y. lipolytica*
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55 fermentation characteristics, could be applied to improving production of many other
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1 metabolites.

2 3 **Acknowledgements**

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6 This work was financially supported by the National Science Foundation for
7
8 Distinguished Young Scholars of China (No. 21225626), the National Natural
9
10 Science Foundation of China (Nos. 21376002 and 21476111), Jiangsu Province
11
12 Natural Science Foundation of China (No. BK20131405), the National High-Tech
13
14 R&D Program of China (No. 2014AA021703), and the Priority Academic Program
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17 Development of Jiangsu Higher Education Institutions.
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Fig. 1. Synthetic pathways of fatty acids in *Yarrowia lipolytica*. The native fatty acid pathway is indicated in solid lines and the engineered pathway is indicated in dotted lines. TCA cycle, tricarboxylic acid cycle; FAS, fatty acid synthase; mCT, mitochondrial membrane citrate transporter; cCT, cytomembrane citrate transporter; PA, palmitic acid; SA, stearic acid; OA, oleic acid; LA, linoleic acid; GLA, γ -linolenic acid; ALA, α -linolenic acid; STA, stearidonic acid; EDA, eicosadienoic acid; ETrA, eicosatrienoic acid; ETA, eicosatetraenoic acid; DGLA, dihomo- γ -linolenic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentenoic acid; DHA, docosaheptaenoic acid.

Fig. 2. Overview of the construction of recombinant *Yarrowia lipolytica* strain Po1f-6-D. (A) The recombinant plasmid p0-6-D was constructed by one step cloning. (B) p0-6-D was used to transform Po1f and the transformants were selected on YSC-Ura plates. (C) The recombinant strain was confirmed by PCR; Lane 1: the PCR fragment amplified from the control strain Po1f genomic DNA; Lane M: Marker DL 10000; Lane 2: the PCR fragment amplified from the genomic DNA of engineered strain Po1f-6-D. (D) Flask fermentation of the confirmed transformant cultured in triplicate. (E) Gas chromatographic analysis of fatty acid composition in the engineered strain Po1f-6-D and the control strain Po1f. C16:0, palmitic acid; C16:1, palmitoleic acid; C18:0, stearic acid; C18:1, oleic acid; C18:2, linoleic acid; C18:3, γ -linolenic acid; C20:0, arachidic acid.

Fig. 3. Fermentation performance of the control strain *Yarrowia lipolytica* Po1f and the engineered strain *Yarrowia lipolytica* Po1f-6-D in shake flask cultures. Each strain

1 was cultured in triplicate. (A) Time course of dry cell weight (DCW). (B) Time
2
3 course of glucose consumption. (C) Final fatty acid distribution profiles at the time
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5 cells were harvested for DCW determination. C16:0, palmitic acid; C16:1,
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7 palmitoleic acid; C18:0, stearic acid; C18:1, oleic acid; C18:2, linoleic acid; C18:3, γ -
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9 linolenic acid; C20:0, arachidic acid.
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14 Fig. 4. Effect of culture temperature on dry cell weight (DCW) (A), glucose (B) and
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16 γ -linolenic acid (GLA) concentration (C) during GLA fermentation by the engineered
17
18 *Yarrowia lipolytica* strain Po1f-6-D.
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23 Fig. 5. Effect of a temperature shift from 28 °C to 20 °C or 15 °C of the engineered
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25 *Yarrowia lipolytica* strain Po1f-6-D at log and stationary phases. (A) Schematic
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27 diagram of temperature-shift experiments performed with the engineered strain
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29 Po1f-6-D. (B to E) Time course of glucose consumption, dry cell weight (DCW) and
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31 γ -linolenic acid (GLA) concentration of the engineered strain Po1f-6-D with
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33 temperature shift from 28 °C to 15 °C after day 1 (B), 28 °C to 20 °C after day 1 (C),
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Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

A

Fig. 5.

Table 1 Comparison of data obtained at the end (day 7) of the engineered *Yarrowia lipolytica* strain Polf-6-D fermentations at different temperatures.

Temperature (°C)	DCW (g/L)	Lipid (g/L)	GLA(mg/L)	GLA/DCW(%)
15	11.5	0.68	54.3	0.47
20	13.3	0.83	57.4	0.43
28	14.8	0.97	44.5	0.3
28 (0-24h) 15 (after 24h)	16.7	1.1	56.1	0.34
28 (0-24h) 20 (after 24h)	18.6	1.16	71.6	0.39
28 (0-48h) 15 (after 48h)	15.5	1.02	51.5	0.33
28 (0-48h) 20 (after 48h)	15.7	1.05	51.7	0.33
Increase ^a	(+25.7%)	(+19.6%)	(+60.9%)	/

^a Increase obtained by a shift to 20 °C after 24 h, compared to the result obtained in standard cultivation conditions single temperature control experiment at 28 °C.

DCW: dry cell weight; GLA: γ -linolenic acid.

Table 2 Fatty acid profiles of the engineered *Yarrowia lipolytica* strain Po1f-6-D under various temperature control strategies.

Temperature (°C)	Fatty acid composition (%)						
	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:0
15	28.47	5.01	17.82	21.51	15.11	7.99	4.07
20	32.93	4.14	19.28	19.41	14.16	6.92	3.74
28	39.64	3.60	23.19	17.12	8.87	4.55	3.03
28 (0-24h) 20 (after 24h)	30.03	5.37	19.41	20.49	14.85	6.17	2.40
28 (0-48h) 20 (after 48h)	33.41	4.06	20.32	19.98	13.58	4.92	3.65
28 (0-24h) 15 (after 24h)	32.70	2.73	20.99	22.12	11.15	5.10	5.20
28 (0-48h) 15 (after 48h)	34.99	3.60	20.53	21.02	13.47	5.09	2.29

C16:0, palmitic acid; C16:1, palmitoleic acid; C18:0, stearic acid; C18:1, oleic acid; C18:2, linoleic acid; C18:3, γ -linolenic acid; C20:0, arachidic acid.

Table 3 Fatty acid profiles of the control strain *Yarrowia lipolytica* Po1f under various temperature control strategies.

Temperature (°C)	Fatty acid composition (%)					
	C16:0	C16:1	C18:0	C18:1	C18:2	C20:0
15	29.12	6.23	17.65	24.10	19.23	3.67
20	34.02	5.03	18.25	22.54	17.19	3.05
28	40.62	4.12	23.27	18.39	11.23	2.56
28 (0-24h) 20 (after 24h)	31.21	6.02	19.99	24.51	16.46	1.81
28 (0-48h) 20 (after 48h)	34.25	4.83	22.46	21.95	13.62	2.89
28 (0-24h) 15 (after 24h)	33.12	4.67	21.45	23.89	14.19	2.68
28 (0-48h) 15 (after 48h)	35.31	3.89	20.96	23.25	13.17	3.42

Supporting information

Engineering *Yarrowia lipolytica* for efficient γ -linolenic acid production

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Supplementary Table 1

The sequence of codon-optimized $\Delta 6$ -fatty acid desaturase and $\Delta 6$ -fatty acid desaturase

Gene	Sequence
codon- optimized $\Delta 6$ -fatty acid desaturase	ATGGCCGCCGCCCCCTCCGTCCGAACCTTCACCAGAGCCGAAATCCTGA ACGCTGAGGCCCTGAACGAGGGCAAGAAGGACGCCGAGGCCCTTTCC TCATGATCATCGACAACAAGGTGTACGACGTGCGAGAATTCGTGCCCCG ACCACCTGGTGGTTCGGTGATTCTGACCCATGTCGGTAAGGACGGCAC TGACGTCTTTGACACCTTCCACCCCGAGGCCGCTTGGGAAACCCTGGCT AATTTTTATGTTGGTGACATTCACGAGTCTGACCGAGACATCAAGAACG ACGACTTTGCTGCCGAAGTCCGAAAGCTGCGAACCCTGTTTCAGTCCCT GGGTTACTACGATTCTTCTAAGGCCTACTACGCCTTCAAGGTCTCCTTCA ACCTGTGCATCTGGGGCCTGTCCACCTTCGTGCTCGCCAAGTGGGGTCA AACCTCTACCCTCGCTAACGTTGTTTCTGCTGCTCTGCTGGGTCTGTTCT GGCAGCAGTGCGGTTGGCTCGCCACGATTTCTGCACCATCAGGTCTT CCAGGACCGATTCTGGGGCGACCTCTTTGGCGCTTTCCTCGGTGGCGTG TGTCAGGGTTTCTCCTCCTCCTGGTGGAAGGACAAGCACAATACCCACC ACGCCGCCCCAACGTGCATGGTGAGGACCCCGACATCGACACTACCC CCTCCTGACCTGGTCCGAGCACGCTCTGGAGATGTTCTCCGATGTCCCC GACGAGGAGCTCACCAGAATGTGGTCCAGATTCATGGTGCTGAACCAAG ACCTGGTTCTACTTCCCCATCCTGTCTTTCGCCGACTCTCCTGGTGCCTC CAGTCCATCCTCTTCGTATGCCAACGGTCAGGCTCATAAACCTTCTGG TGCCCGAGTGCCTATTTCCCTGGTGGAACAGCTGTCCCTCGCCATGCAC TGGACCTGGTATTTAGCCACCATGTTCTGTTCTGTAAGGACCCTATCA ACATGTTCTGTACTTCTGTTGTCTCAGGCCGTGTGTGGCAACCTGCT GGCTCTGGTCTTTTCTCTGAACCACAACGGCATGCCGTCATCTCCAAG GAGGAGGCCGTGACATGGATTTCTTCACCAAGCAGATCATCACCGGC CGAGATGTCCACCCTGGCCTGTTGCCAACTGGTTCACCGGCGGCCTGA ACTACCAGATCGAGCATCACCTGTTCCCCTCCATGCCCCGACACAATTTC TCCAAAATCCAGCCCGCCGTGGAGACCCTCTGTAAGAAGTACAACGTGC GATACCACACTACCGGTATGATCGAGGGCACCGCCGAGGTCTTCTCCCG ACTGAACGAGGTCTCTCGAGCCGCCTCTAAGATGGGTAAGGCTCAGTA A
$\Delta 6$ -fatty acid desaturase	ATGGCTGCTGCTCCAGTGTGAGGACGTTTACTCGGGCCGAGATTCTGA ATGCCGAGGCCCTGAATGAAGGCAAGAAGGATGCTGAGGCACCCTTCC TGATGATCATCGACAACAAGGTGTACGATGTCCGCGAGTTTGTCCCTGA TCATCCCGGTGGAAGTGTGATTCTCACGCACGTTGGCAAGGACGGCAC TGACGTCTTTGACACTTTCCACCCCGAGGCTGCTTGGGAGACTCTTGCC AACTTTTACGTTGGTGATATTCATGAGAGCGACCGCGACATCAAGAATG ATGACTTTGCGGCCGAGGTTTCGCAAGCTGCGTACCTTGTTCCAGTCTCTT GGCTACTACGATTCTTCCAAGGCATACTACGCCTTCAAGGTCTCGTTCAA

	CCTCTGCATCTGGGGCCTGTCGACATTCGTTGTTGCCAAGTGGGGCCAG ACCTCGACCCTCGCCAATGTAGTTTCGGCTGCGCTTTTGGGTCTTCTG GCAGCAGTGCGGATGGTTGGCGCACGACTTTTTGCATCACCAGGTCTTC CAGGATCGTTTCTGGGGCGATCTTTTCGGTGCCTTCTTGGGAGGTGTCT GCCAGGGTTTCTCATCCTCCTGGTGGAAGGACAAGCACAACACTCACCA CGCCGCTCCCAACGTCCACGGTGAGGATCCCGACATTGACACTCACCT CTGTTGACCTGGAGTGAGCATGCTCTGGAGATGTTCTCGGATGTCCCTG ACGAGGAGCTGACCCGCATGTGGTCGCGCTTCATGGTCCTCAACCAGA CCTGGTTCTACTTCCCCATTCTCTCGTTTGCCCGTCTCTCCTGGTGCTCC AGTCTATTCTCTTTGTTATGCCTAACGGTCAGGCCCAAGCCCTCGGGT GCGCGTGTGCCATTTCTTGGTGAGCAGCTGTCTCTCGCTATGCACT GGACCTGGTACCTCGCCACCATGTTCTGTTGTTGAAGGATCCCATCAA CATGTTTGTGTACTTTTTGGTATCGCAGGCTGTTTGCGGCAACTTGTTGG CGCTTGTGTTCTCACTCAACCACAACGGTATGCCTGTGATTTCCAAGGA GGAGGCAGTCGATATGGATTTCTTCACCAAGCAGATCATCACGGGTCGT GATGTCCACCCTGGTCTGTTTGCCAACTGGTTCACAGGTGGATTGAACT ACCAGATTGAGCATCACTTGTTCCCTTCGATGCCCCGCCACAACCTTTCA AAGATCCAGCCTGCTGTCGAGACCCTGTGCAAAAAGTACAATGTCCGAT ACCACACCACGGCATGATTGAGGGAACTGCAGAGGTCTTTAGCCGTCT GAACGAGGTCTCCAGGGCTGCCTCCAAGATGGGCAAGGCACAGTAA