JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

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J. Agric. Food Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jafc.7b04485 • Publication Date (Web): 29 Nov 2017

Just Accepted

ACS Publications

Journal of Agricultural and Food Chemistry is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Enhancing Fatty Acid Production of *Saccharomyces cerevisiae* as an Animal Feed Supplement

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ABSTRACT

Saccharomyces cerevisiae is used for edible purposes, such as human food or as an animal feed supplement. Fatty acids are also beneficial as feed supplements, but *S. cerevisiae* produces small amounts of fatty acids. In this study, we enhanced fatty acid production of *S. cerevisiae* by overexpressing acetyl-CoA carboxylase, thioesterase, and malic enzyme associated with fatty acid metabolism. The enhanced strain pAMT showed 2.4-fold higher fatty acids than the wild-type strain. To further increase the fatty acids, various nitrogen sources were analyzed and calcium nitrate was selected as an optimal nitrogen source for fatty acid production. By concentration optimization, 672 mg/L of fatty acids was produced, which was 4.7-fold higher than wild-type strain. These results complement the low level fatty acid production and make it possible to obtain the benefits of fatty acids as an animal feed supplement while, simultaneously, maintaining the advantages of *S. cerevisiae*.

KEYWORDS: fatty acids production, *Saccharomyces cerevisiae,* nitrogen optimization, cofactor balancing, animal feed supplement

INTRODUCTION

Fatty acids are common substances used by organisms to store energy for their metabolism. Fatty acids as food additives are required in the food industry in various ways to enhance or control the fatty acid composition of certain foods.[1] Also, advantages of using fatty acids as supplemental animal feed in agriculture have been reported.[2] Recent studies show that the use of fatty acids as feed for calves increases the synthesis of B vitamins in the intestines, increases feed efficiency, or increases the birth weight of calves.[3, 4]

Fatty acids have been studied and are obtained from three sources, including plant oils, animal fats, and microbial lipids. However as food prices continue to rise around the world, there is a continuing debate over the use of resources that can be used as human food or other uses.[5] Thus, studies using fatty acids derived from microorganisms have been attracting attention for many years.[6, 7] Compared to other microorganisms including *Escherichia coli*, *S. cerevisiae* shows pH robustness and a high tolerance to inhibitory compounds. Also, *S. cerevisiae* grows well in harsh environments and is easy to cultivate.[8] For this reason, *S. cerevisiae* is suitable as a strain to produce fatty acids. Furthermore, since it is a GRAS (Generally Recognized As Safe) microbe, it is suitable for use as an animal feed supplement in agriculture.[9, 10]

The synthesis of fatty acids in microorganisms is initiated by an enzyme called acetyl-CoA carboxylase (ACC). *S. cerevisiae* uses the type I fatty acid synthetase (FAS) system and only fatty acyl-CoA is released from the FAS complex. The fatty acyl-CoA chain lengths are maintained in equilibrium by a product synthesized by FAS, whereas in *S. cerevisiae*, most of the fatty acyl-CoA produced has C16 and C18 chain length.[11, 12] Long chain acyl-CoA, a form of C16 and C18 fatty acid precursors, affects several enzymes

involved in lipid production, in particular the feedback inhibition of ACC.[12] Native ACC from *S. cerevisiae* is strongly inhibited by feedback inhibition of acyl-ACP (acyl carrier protein). Thus, the overexpression of homologous ACC increases fatty acid production, but not significantly.[11] This inhibition is reduced by expressing heterologous ACC, which is not recognized by native acyl-ACP in the host strain.[13]

The fatty acid biosynthesis process is terminated by the conversion of fatty acyl-ACP or fatty acyl-CoA to fatty acids by an enzyme called thioesterase (TES). The TESs differ in their specificities and activities on the substrate depending on the characteristics of the host strain and the environments in which they live.[14] Due to these characteristics of TES, the production and composition of fatty acids is determined by the TES present in the cells. However, most studies report the TES in plants, and only some studies have overproduced fatty acids using microbial TES.[15, 16]

Malic enzyme (MAE) converts malate to pyruvate by generating NADH or NADPH and affects metabolic pathways such as photosynthesis and lipogenesis.[17, 18] Malic enzyme is found in most microorganisms and is assumed to be the rate-limiting step of lipid accumulation in oleaginous fungi.[19] Studies on the overexpression of MAE in microorganisms involved in the increment of fatty acid accumulation are reported in *E. coli* and the oleaginous yeast *Rhodotorula glutinis*.[17] In *S. cerevisiae*, NADPH is mainly produced by glucose-6-dehydrogenase, 6-phosphogluconate dehydrogenase, and isocitrate dehydrogenase of the pentose phosphate pathway.[20] In *S. cerevisiae*, there is only one malic enzyme located in mitochondria and its overexpression activates a metabolic pathway that requires NADPH.[21, 22] However, since the regenerative capacity of NADPH in *S. cerevisiae*

is poor, regulating the balance of the cofactor plays an important role in the metabolic pathway consuming NADPH.[22]

The ability of cells to accumulate lipids is influenced by N, P, Zn, Fe, Mn or Mg in the medium provided externally.[23] Among these various medium sources, increased lipid production in the nitrogen starvation state has been reported in several studies.[24, 25] Nitrogen starvation conditions are reported to increase lipid production in algae.[26] In the process of culturing *Chlorella vulgaris* for lipid production, the timing and degree of the application of nitrogen limitation conditions or the utilization of various nitrogen sources was analyzed.[27, 28] In a study analyzing the effect of nitrogen limitation conditions on lipid production in yeast, it was reported that the C/N ratio was adjusted to increase lipid production.[25] In culture conditions limiting the nitrogen source, the yeast induces the carbon metabolic flux to the fatty acid production pathway.[29]

In this study, we developed a recombinant strain that regulates the metabolic pathway of *S. cerevisiae* to increase fatty acid production. Two subunits of ACC and three putative TES from *C. glutamicum* were introduced into *S. cerevisiae* to strengthen the metabolic carbon flux to fatty acid production. MAE was introduced to solve the cofactor imbalance caused by the consumption of NADPH during fatty acid production. Also, we increased fatty acid production by analyzing the effect of various nitrogen sources on fatty acid production and by optimizing the concentration of the nitrogen source.

MATERIALS AND METHODS

Construction of plasmids and recombinant strains

The pESC-TRP vector (Stratagene, San Diego, USA), one type of yeast expression vector containing the Gal1 and Gal10 promoters and terminator sequences, was used as the backbone for construction of the target genes. In addition, the vector contains an ampicillin resistance gene and a TRP gene which can be used as markers for selecting transformants in *E. coli* DH5α and *S. cerevisiae* YPH499. Genomic DNA of *C. glutamicum* and *S. cerevisiae* was isolated using the wizard genomic DNA purification kit (Promega, Madison, USA). Primers containing the restriction enzyme sites were used for amplification and cloning of the *accBC*, *accD1*, *maeI, cgl0091, cgl1664, cgl2451* and *tesA* genes, which are listed in Table 1. The recombinant vectors containing both *accBC* and *accD1* (*pAcc*), *maeI* (*pMae*), *cgl1664* (*pcgl1664*), *cgl2451* (*pcgl2451*), *cgl0091* (*pcgl0091*), and *tesA* (*ptesA*) were constructed by inserting the multiple cloning site 1 and site 2 from the pESC vector. The genes *maeI* and *cgl1664* were used to assemble the cassette and were introduced into *pAcc* to construct *pAM* and *pAMT*. The resulting strain, pAMT, was constructed by introducing the cassette, which contained the *cgl1664,* into *pAM*. The expression vector was introduced into *S. cerevisiae* YPH499 using the LiAc/SS carrier DNA/PEG method. The LiAc/SS carrier DNA/PEG method was performed as previously described.[30] Yeast transformants were cultured for 3-4 days to obtain single colonies.

Cultivation conditions

E. coli DH5α was cultured at 37 °C in LB (Luria-Bertani) media. The recombinant vectors were transformed into *E. coli* DH5α by a heat shock at 42 °C for 45 s. The transformed cells were screened on LB plates containing 50 μg/mL ampicillin. *S. cerevisiae* YPH499 (Clontech Laboratories Inc., Mountain view, CA, USA) was cultured using YPD

130 medium, with 10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose. The strains with
131 enhanced fatty acid biosynthesis were selected in synthetic media containing 0.67 g/L yeast
132 nitrogen base, 0.13 g/L tryptophan dropout amino acid supplements, 20 g/L glucose and 20
133 g/L agar. For fatty acid production, recombinant yeast was cultured with synthetic media
134 containing 0.67 g/L yeast nitrogen base, 0.13 g/L tryptophan dropout amino acid
135 supplements and 20 g/L galactose. All yeast strains were aerobically cultured at 30 °C.
136 Fatty acid production through fermentation was carried out for 72 h.
137
138 **Thioesterase activity assay**
139 The activity of three putative thioesterases (Cgl1664, Cgl2451, and Cgl0091) and
140 acyl-CoA thioesterase TesA (EC 3.1.2.2) was measured spectrophotometrically using 5,5'-
141 dithiobis (2-nitrobenzoic acid, DTNB).[31] Cell extracts were mixed at a final concentration
142 of 10 μL with 0.3 mM DTNB, 10 mM HEPES, 50 mM KCl, 4 μL of 5 mg/mL palmitoyl-
143 CoA, and distilled water up to 200 μL as a total volume. Absorbance readings at 412 nm
144 (A_{412}) were taken at 15 min at room temperature. For the analysis of relative thioesterase
145 activity, the activities were determined by changing of the absorbance at 412 nm and each
146 activity was normalized by protein concentration. pTesA was used as a control group and
147 each thioesterase activities were expressed as relative percent values. All experiments were
148 conducted in duplicate.
149
150 **Malic enzyme assay**
151 The malic enzyme (EC 1.1.1.40) assay was performed at 30 °C and was measured at
152 a wavelength of 340 nm in a spectrophotometer. Assay mixture was composed of 100 mM

153 Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.4 mM $NADP^+$, 100 mM L-malate (pH 7.5), and cell
154 extracts[21]. The reaction was initiated by the addition of cell extracts into the assay mixture.
155 One unit of the enzyme was defined as the amount of enzyme that catalyzed the production
156 of 1 μmol of NADPH per min. The total protein concentration in the cell extracts was
157 determined using the Bradford protein assay, with bovine serum albumin used as a standard
158 sample.
159
160 **Fatty acid sampling for gas chromatography analysis**
161 Fatty acid analysis in this study was performed as previously described.[32] The total
162 fatty acids were determined by gas chromatography (GC) with flame ionization detector
163 (FID) analysis. Briefly, 2 mL of the samples as prepared and supplemented with 0.5 mg of
164 pentadecanoic acid as the internal standard and was partitioned with 2 mL of $CHCl_3$-
165 CH_3OH (2:1, *v*/*v*). The cell suspension was mixed well and shaken for 20 s with a vortex
166 mixer. The mixtures were stored for 1 h at 4 °C. The lower phase (chloroform) was
167 collected into a glass tube. The collected lower phase solvents were evaporated until
168 dryness by nitrogen gas. Fatty acids were transesterified and determined as fatty acid
169 methyl ester (FAME) forms. Transesterification was performed by adding 2 mL of boron
170 trifluoride-methanol complex solution (14%) from Sigma-Aldrich. The samples were
171 incubated in water bath at 100 °C for 1 h. FAMEs were re-extracted by adding 2 mL of
172 hexane and 1 mL of distilled water. The hexane phase was evaporated by nitrogen and was
173 resuspended by adding 300 μL of hexane.
174
175 **GC analysis conditions**

FAMEs of each sample were analyzed using a GC 7890 from Agilent with FID. The HP-5MS column (30 m, 25 mm i.d., 0.25 μm film) was used to analyze the FAMEs. The GC elution conditions were as follows. The starting temperature was 100 °C for 5 min, followed by a 15 min ramp to 250 °C with an increase in the temperature of 10 °C/min and a hold at 250 °C for 10 min. The result was normalized with pentadecanoic acid as an internal standard.

Optimization of nitrogen sources

To analyze the effects of various nitrogen sources, an inorganic nitrogen source containing ammonium ions or nitrate ions and organic nitrogen sources such as tris (hydroxymethyl) aminomethane and urea were used. The addition of the nitrogen source was calculated based on the molar concentration of nitrogen contained in each molecule. The concentration of the nitrogen source was calculated based on 8 mM to deriving nitrogen limited condition. It was calculated so as to add the same nitrogen concentration. In order to analyze the production of fatty acids by the concentration of calcium nitrate selected as the nitrogen source, 5, 10, 20 and 40 mM calcium nitrate was added to the culture media.

RESULTS AND DISCUSSION

Enhancing carbon flux into the fatty acid metabolic pathway to increase fatty acid production

TES is the enzyme that is directly connected to fatty acid production. The specific activity of TES differs depending on the origin of the enzyme.[14, 33] We compared four TESs,

199 namely Cgl0091, Cgl1664, and Cgl2451 from *C. glutamicum*[34] and one TES, TesA, from
200 *E. coli*. All of the TESs was analyzed by the DTNB method.[31] Most of the fatty acids
201 produced by *S. cerevisiae* are palmitic acid and stearic acid. Thus, we used palmitoyl-CoA
202 as a substrate to analyze the enzyme activity. Enzyme activity was measured by a
203 spectrophotometer and was shown as the relative enzyme activities (Figure 1A). TesA is a
204 TES whose activity is reported to be introduced into the metabolic pathway of *S.*
205 *cerevisiae*.[35] The relative TES activity was analyzed using pTesA as a control group.
206 Comparatively, both the recombinant strains pCgl1664 and pCgl2451 showed 4.6-fold and
207 4.5-fold higher activities, respectively, compared with that of the control group pTesA
208 (Figure 1B). However, strain pCgl0091 did not show an additional increment in
209 thioesterase activity to palmitoyl-CoA. It was confirmed that cgl0091 was found to have
210 acyl transferase activity as a putative thioesterase in the previous article[1], but it was
211 classified as EC 3.3.2.9 because it has activity of hydrolase belonging to Alpha/beta
212 hydrolase superfamily when gene homology is confirmed. This information may explain
213 the fact that cgl0091 has lower activity and fatty acid production than that of cgl1664 or
214 cgl2451. As a result of analysis of fatty acid production using pCgl1664, which is the most
215 active TES for palmitoyl-CoA, the production of fatty acid was 222 mg/L, which was about
216 1.6-fold higher than that of the wild-type strain, and 1.4-fold higher than that of pTesA.
217 These results indicate that pCgl1664 has good activity on long chain fatty acids and is an
218 enzyme that is suitable for use in *S. cerevisiae* to enhance the production of fatty acids.
219 ACC initiates the synthesis of fatty acids by converting acetyl-CoA to malonyl-CoA,
220 which consists of three functional components, including biotin carboxylase (BC), biotin
221 carboxyl carrier protein (BCCP), and carboxyl transferase (CT).[36] Unlike general ACC, the

ACC in *C. glutamicum* only has two subunits, including biotin carboxylase (*accBC*) and carboxyl transferase (*accD1*).[37] We introduced the *accBC* and *accD1* genes into the pESC-Trp vector to designate *pAcc*. The *pAcc* introduced strain was named pACC. The heterologous expression of ACC resulted in a fatty acid production of about 220 mg/L, which was 1.6-fold higher than that of the wild-type strain (Figure 1B).

Overexpression of ACC alone does not significantly change fatty acid production because of the feedback inhibition of long chain fatty acyl-ACP.[11] An other study on the effect of heterologous expression of ACC on the increased yields showed that overexpression of *C. glutamicum* ACC in *E. coli* resulted in a significant increase in malonyl-CoA and the final products.[13] Therefore, it is essential to reduce the feedback inhibition of ACC in order to increase the fatty acid production, and it is effective to convert long chain fatty acyl-ACP to long chain fatty acid.

Increased fatty acid production by balancing the consumption of the cofactor NADPH in fatty acid elongation

There are various enzymes involved in the anaplerotic reaction in microorganisms. One of these reactions is performed by MAE, in which MAE converts malate to pyruvate, with the regeneration of NADPH. Almost all microbes have this enzyme, and it is suggested to be an important enzyme related to lipid accumulation.[19, 38] Some studies report that MAE generates NADPH and affects fatty acid production, but some strains are not affected by MAE.[39, 40] In this study, endogenous MAE was overexpressed in *S. cerevisiae* to develop a recombinant strain called pMAE. By measuring the production of NADPH by malic enzyme, we confirmed the activity of MAE in the recombinant strain. The cells of the

recombinant strain were disrupted to analyze the enzyme activity using the MAE present in the supernatant (Table 2). It was confirmed that the activity of the enzyme relative to the wild-type strain increased to about 1.5-fold in the recombinant strain. pMAE was cultured for 72 h with the wild-type strain in order to confirm that the productivity of fatty acids improved. The effect of MAE on the production of fatty acids was shown by the increased production of fatty acids compared to that of the wild-type strain (Figure 2).

Since NADPH, which is directly consumed in fatty acid synthesis, is regenerated by MAE, there was a continuous difference in fatty acid production from 24 h to 72 h. Fatty acid production increased from 24 h to 48 h, and as NADPH continued to increase by MAE, fatty acid production continued to increase after 48 h to 72 h. Interestingly, the enzyme activity increased by 1.5-fold, but the fatty acid production increased by about 1.2-fold. These results show that NADPH in *S. cerevisiae* is a significant component of fatty acid production, and subsequent studies will show that a balanced intracellular supply of NADPH leads to a further increase in fatty acid production.

Increased fatty acid production by simultaneously enhancing fatty acid metabolism and NADPH regeneration in *S. cerevisiae*

Studies to increase the production of fatty acids were conducted for multiple purposes because of their various availabilities.[15-17, 33, 41] The strain pAM was developed by introducing MAE into pACC. The selected TES, Cgl1664, was introduced into pAM to produce a pAMT strain as a final strain. When MAE, ACC, and Cgl1664 were overexpressed, the fatty acid production of pAM and pAMT was about 296 mg/L and 341 mg/L, respectively, which is about 2.1- and 2.4-fold higher, than that of wild-type (Figure

3). When various enzymes involved in fatty acid metabolism were introduced, the strain was able to observe almost similar growth. However, in the case of pAM, which is MAE and ACC introduced strain, its growth was unstable compared to a slightly different strains. This instability can be caused by a balance of carbon flow and cofactor. NADPH is not only used to produce fatty acids but also as a cofactor for the reaction of other enzymes. Therefore, it is not helpful that there is a large amount of NADPH inside the cell and NADPH and NADP+ should be well balanced.[39] For this reason this unstability was not observed in pAMT. Therefore, it is considered that the combination of three enzymes in the fatty acid production process is more effective for the growth of the strain than in the case of using two enzymes. Our study showed that the metabolism of carbon flux leads to the fatty acid metabolic pathway, which leads to an increased production of fatty acids, and at the same time, the regeneration of NADPH, which supports the production of fatty acids. In other words, the highest amounts of fatty acids were produced in strains that simultaneously improved the initiation, elongation and termination of fatty acids.

In a preceding study, the production of lipid increased to 340 mg/L by overexpressing heterologous ACC from oleaginous yeast *Lipomyces starkeyi* as a method of increasing lipid production.[41] Also, modifying the ACC gene to improve the yield of fatty acids resulted in 343 mg/L of total fatty acids.[42] Overexpression of *E. coli* acyl-ACP TES with long chain fatty acid ligase 1 (FAA1) and long chain fatty acid ligase 4 (FAA4) disruption in *S. cerevisiae* yielded 207 mg/L fatty acids, and when combined with overexpression of the fatty acid synthase (FAS) and acetyl-CoA carboxylase, it resulted in the production of up to 400 mg/L fatty acid.[43] In another study, disruption of the *β*-oxidation pathway and acyl-CoA synthetases, overexpression of different TESs, and

enhancement of the supply of acetyl-CoA resulted in the production of more than 120 mg/L fatty acid.[8] However, as shown in most studies, the production of fatty acids by *S. cerevisiae* tends to be no more than a certain amount of production, as the limit of the strain itself is fixed. Thus, we optimized the nitrogen source after regulating the metabolic pathway.

Enhanced production of fatty acids by reinforcing fatty acid production metabolism through optimization of the nitrogen source

Nitrogen limited conditions have been studied primarily to increase lipid production in oleaginous yeast *Yarrowia lipolytica*.[24, 29] Previous studies show that when nitrogen starvation occurs in microorganisms, adenosine monophosphate deaminase (AMPD) converts adenosine monophosphate (AMP). At this time, AMP is a cofactor of isocitrate dehydrogenase (IDH), an essential enzyme in the TCA cycle, resulting in the interruption of the TCA cycle.[44] When the TCA cycle is stopped, the carbon flux from acetyl-CoA to the fatty acid metabolic pathway is increased, resulting in an increase in fatty acid production.[29] Thus, to the best of our knowledge, only an increase of fatty acid production by yeast using a concentration of ammonium sulfate as a nitrogen source has been reported thus far.

In this study, various nitrogen sources were used to analyze the production of fatty acids. Fatty acid production using inorganic nitrogen sources and organic nitrogen sources was analyzed. Ammonium sulfate, which is mainly used as a source of nitrogen in yeast culture, was increased by about 26% compared to the control. The addition of potassium nitrate and urea increased fatty acid production by 50% and 41%, respectively, and the

addition of calcium nitrate increased the fatty acid production by 64% (Figure 4). Since the limitation of nitrogen in the medium inhibits the TCA cycle, an appropriate nitrogen source concentration is an important factor for the production and growth of bacteria. Therefore, calcium nitrate, which has the highest fatty acid production capacity, was selected and the change of fatty acid production by concentration was analyzed (Figure 4).

The nitrogen limitation condition was set to 5 mM, and the nitrogen abundance condition was set to 40 mM. At a concentration of 10 mM, the maximum fatty acid production was 672 mg/L. In the control group, without optimizing the nitrogen source, this result was about 4.7-fold higher than that of the wild-type strain and 2.0-fold higher than that of the recombinant strain (Figure 5). To the best of our knowledge, this is the first study of the optimization of fatty acid production using another nitrogen source in yeast, other than ammonium sulfate, and the increased production by optimization of the nitrogen source is also known to be the highest. As a result, the fatty acid metabolic pathway was enhanced and the increase of fatty acid production by optimizing the nitrogen source was applied to *S. cerevisiae*, thereby efficiently increasing the fatty acid production.

As shown in the results (Figure 4 and Figure 5), the nitrogen source was as an important factor, just as carbon sources, in the production of fatty acids in microorganisms. Thus, other study also report improvements in lipid production by optimizing ammonium sulfate as a nitrogen source in oleagenious yeast *Candida* sp. 107, which improved by 37% in the nitrogen limitation condition[29], and *Yarrowia lipolytica* showed 11% increased ptoduction.[24] Recent studies have shown that nitrogen source optimization has been performed to produce fatty acids from microalgae *Trachydiscus minutus*. In this article, the addition of various nitrogen sources was analyzed. Urea was added as an optimal nitrogen

source and cultured for 24 days to obtain lipid of about 2.5 g/L.[45] There is also a possibility that calcium ion may have influenced the production of fatty acids, but this has not yet been revealed in yeast. In the microalgae, metal ions transfer the signal to increase the accumulation of lipids. In future studies, the effects of metal ions on fatty acid production from yeast should be studied.[46, 47]

Recent studies have shown that fatty acids have many advantages as an animal feed supplement.[2, 4] Since *S. cerevisiae* is a safe microorganism for food, the purpose of this study was to enhance the fatty acid metabolism pathway to produce *S. cerevisiae* containing excessive fatty acids.[48, 49] As a result of this study, the fatty acid content in pAMT was improved to 4.7-fold (672 mg/L) compared with the wild-type strain. These results suggested potential strategies with *S. cerevisiae* containing excessive fatty acids for agriculture and animal feed supplement in a generally recognized as safe yeast. It is possible to obtain advantages by simultaneously using yeast and fatty acids as feed supplement.

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Funding

This work was supported by the National Research Council of Science & Technology (NST) grant by the Korea government (MSIP) (No. CAP-11-04-KIST).

Notes

The authors do not have any conflicts of interest.

365 **Supporting Information**

366 The Supporting Information is available free of charge on the ACS Publications
367 website at DOI: XXXXXXXXXXXXXXXXXXX.
368 Schematic diagrams for enhancing fatty acid production, Construction diagrams of
369 expression vector, Relative fatty acid production data, Growth curve for recombinant
370 strains (PDF).

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FIGURE CAPTIONS

Figure 1. (A) Comparison of relative TES activities in enhanced *S. cerevisiae* strains. pTesA was the control, and the result was the comparison of pCgl1664, pCgl2451, and pCgl0091. (B) Production of C16 and C18 fatty acids from enhanced strains that pACC and pCgl1664.

Figure 2. Analysis of fatty acid production over time with pMAE. The wild-type strain and pMAE were each used to measure the production of fatty acids per 24 h, and the samples were analyzed.

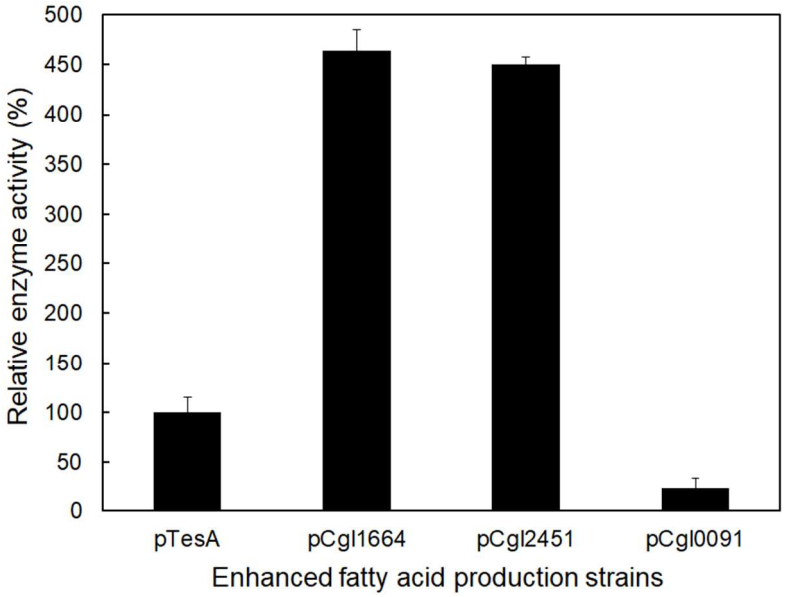
Figure 3. Fatty acid production from the enhanced fatty acid production strains with pMAE, pAM, and pAMT. Each sample was incubated for 72 h before sampling and analyzing.

Figure 4. Effect of various types of nitrogen sources on fatty acid production. As inorganic nitrogen source, harboring ammonium ions or nitrate ions, and organic nitrogen sources were used. We adjusted the concentration of nitrogen source to 8 mM according to the molar ratio of nitrogen atoms in the added nitrogen source.

Figure 5. Analysis of fatty acid production by nitrogen source concentration. Calcium nitrate was selected as the nitrogen source, and 5, 10, 20 and 40 mM calcium nitrate were added to the culture media.

Figure 1

A


Relative enzyme activity (%)
500
450
400
350
300
250
200
150
100
50
0
pTesA
pCgl1664
pCgl2451
pCgl0091
Enhanced fatty acid production strains

B

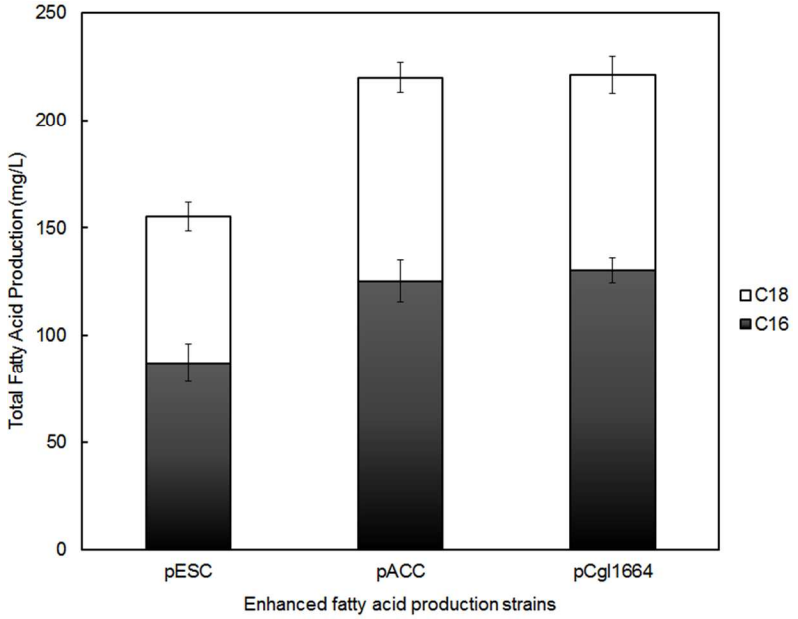

Total Fatty Acid Production (mg/L)
250
200
150
100
50
0
C18
C16
pESC
pACC
pCgl1664
Enhanced fatty acid production strains

Figure 2

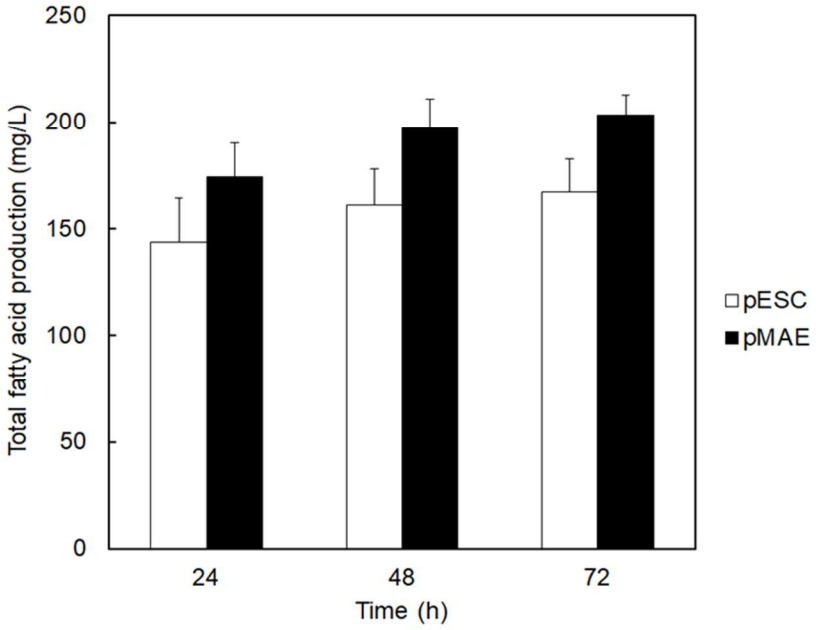


Figure 3

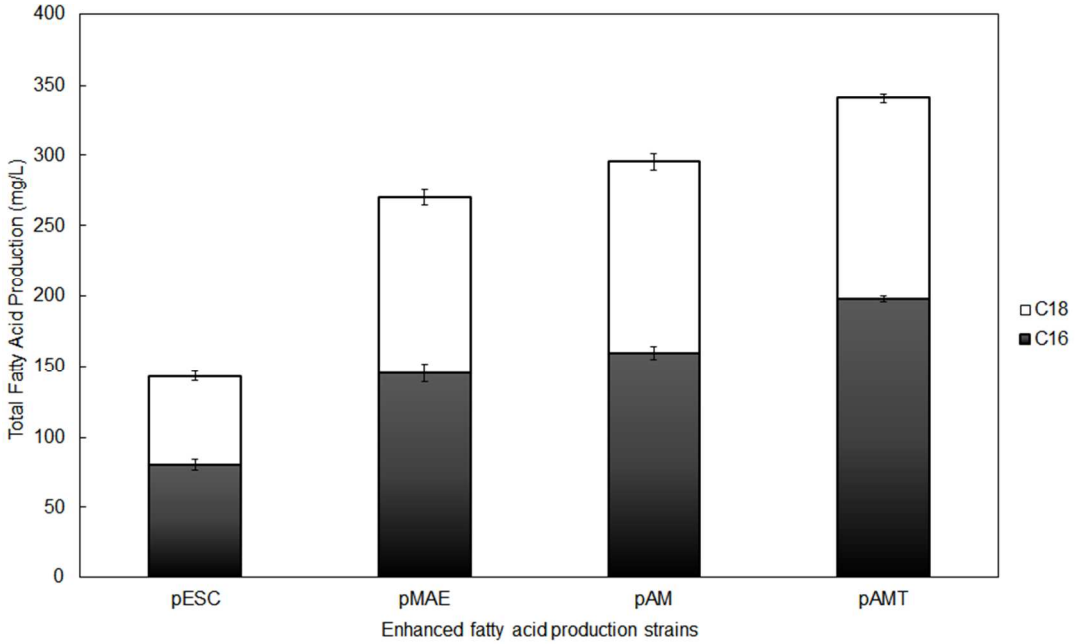


Figure 4

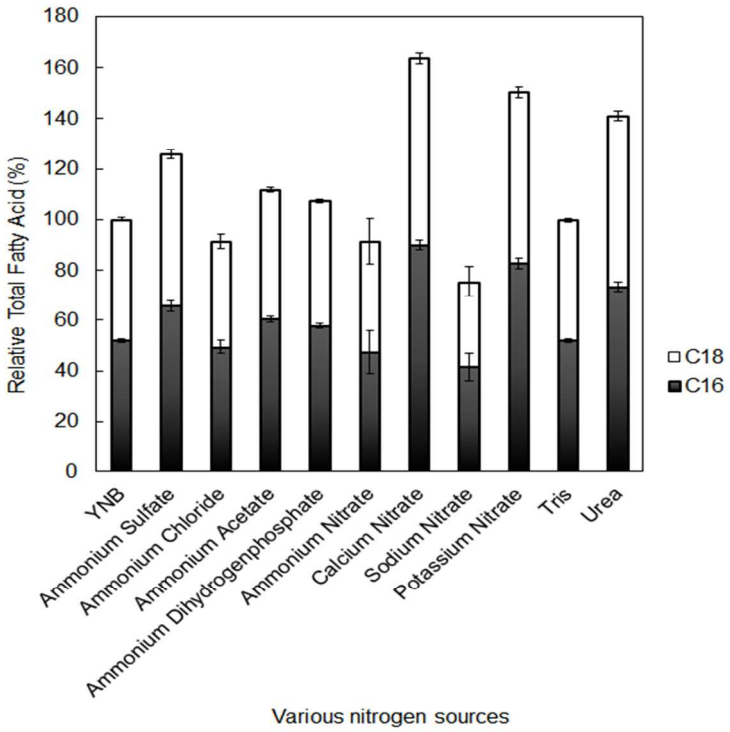


Figure 5

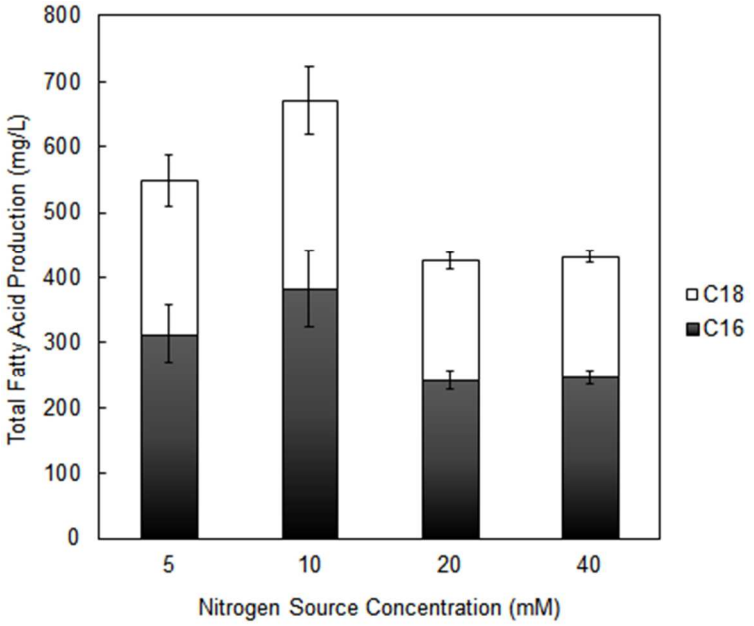


Table 1. Bacterial strains, yeast strains and plasmids used in this study.

Strain or plasmid	Description	Source, reference, or target
Strains		
E. coli		
DH5α	F⁻ *ϕ*80*dlacΔ*(*lacZ*)M15 *Δ*(*lacZYA-argF*)*U169 endA1 recA1 hsdR17*(r_K^-, m_K^+) *deoR thi-1 phoA supE44* λ^- *gyrA96 relA1*	Invitrogen[a]
pESC	*E. coli* DH5α, pESC-*trp*	Stratagene[b]
pACC	*E. coli* DH5α, pESC-*trp::pAcc*	This study
pMAE	*E. coli* DH5α, pESC-*trp::pMae*	This study
pCgl1664	*E. coli* DH5α, pESC-*trp::pcgl1664*	This study
pCgl2451	*E. coli* DH5α, pESC-*trp::pcgl2451*	This study
pCgl0091	*E. coli* DH5α, pESC-*trp::pcgl0091*	This study
pTesA	*E. coli* DH5α, pESC-*trp::ptesA*	This study
pAM	*E. coli* DH5α, pESC-*trp::pAM*	This study
pAMT	*E. coli* DH5α, pESC-*trp::pAMT*	This study
S. cerevisiae		
YPH499	*MATa ura3-52 lys2-801 amber ade2-101 ochre trp1-Δ63 his3-Δ200 leu2-Δ1*	Clontech[c]
pESC	*S. cerevisiae* YPH499, pESC-*trp*	This study
pACC	*S. cerevisiae* YPH499, pESC-*trp::pAcc*	This study
pMAE	*S. cerevisiae* YPH499, pESC-*trp::pMae*	This study
pCgl1664	*S. cerevisiae* YPH499, pESC-*trp::pcgl1664*	This study
pCgl2451	*S. cerevisiae* YPH499, pESC-*trp::pcgl2451*	This study
pCgl0091	*S. cerevisiae* YPH499, pESC-*trp::pcgl0091*	This study
pTesA	*S. cerevisiae* YPH499, pESC-*trp::ptesA*	This study

pAM	*E. coli* DH5*α*, pESC-*trp::pAM*	This study
pAMT	*E. coli* DH5*α*, pESC-*trp::pAMT*	This study
Plasmids		
pESC-*trp*	pUC plasmid origin, auxotrophic marker gene *TRP1,* GAL1 and GAL10 promoter, Amp^R, protein expression studies in *S. cerevisiae*	Stratagene[b]
pESC-*trp::pAcc*	pESC-*trp*, carrying *accBC and accD1* gene	This study
pMT-*tac::pMae*	pESC-*trp*, carrying *maeI* gene	This study
pMT-*tac::pcgl1664*	pESC-*trp*, carrying *cgl1664* gene	This study
pMT-*tac::pcgl2451*	pESC-*trp*, carrying *cgl2451* genes	This study
pMT-*tac::pcgl0091*	pESC-*trp*, carrying *cgl0091* genes	This study
pMT-*tac::ptesA*	pESC-*trp*, carrying *tesA* genes	This study
pMT-*tac::pAM*	pESC-*trp*, carrying *accBC, accD1, and maeI* genes	This study
pMT-*tac::pAMT*	pESC-*trp*, carrying *accBC, accD1, maeI, and cgl1664* genes	This study

[a] Invitrogen Corporation, Carlsbad, California, USA.
[b] Stratagene Corporation, San Diego, USA.
[c] Clontech Laboratories Incorporated, Mountain View, CA, USA.

Table 2. Malic enzyme activity assay.

Strains	Enzyme activity (nmol/min/mg of protein)[a]
pESC	7.52 ± 0.41
pMAE	11.30 ± 0.32

[a] One unit of the enzyme was defined as the amount of enzyme that catalyzed the production of 1 μmol of NADPH per min

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