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Metabolic engineering for enhanced fatty acids synthesis in *Saccharomyces cerevisiae*

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ABSTRACT

Microbial production of biofuel has attracted significant attention in recent years. The fatty acids are important precursors for the production of fuels and chemicals, and its biosynthesis is initiated by the conversion of acetyl-CoA to malonyl-CoA which requires acetyl-CoA as key substrate. Herein, the yeast *Saccharomyces cerevisiae* was proposed to be metabolically engineered for cytosol acetyl-CoA enhancement for fatty acid synthesis. By gene disruption strategy, *idh1* and *idh2* genes involved in citrate turnover in tricarboxylic acid cycle (TCA cycle) were disrupted and the citrate production level was increased to 4- and 5-times in mutant yeast strains. In order to convert accumulated citrate to cytosol acetyl-CoA, a heterologous ATP-citrate lyase (ACL) was overexpressed in yeast wild type and *idh1,2* disrupted strains. The wild type strain expressing *acl* mainly accumulated saturated fatty acids: C14:0, C16:0 and C18:0 at levels about 20%, 14% and 27%, respectively. Additionally, the *idh1,2* disrupted strains expressing *acl* mainly accumulated unsaturated fatty acids. Specifically in Δ*idh1* strain expressing *acl*, 80% increase in C16:1 and 60% increase in C18:1 was detected. In Δ*idh2* strain expressing *acl*, 60% increase in C16:1 and 45% increase in C18:1 was detected. In Δ*idh1/2* strain expressing *acl*, there was 92% increase in C16:1 and 77% increase in C18:1, respectively. The increased fatty acids from our study may well be potential substrates for the production of hydrocarbon molecules as potential biofuels.

1. Introduction

The biofuel production is playing a more and more important role in modern society since it can rectify the problems resulting from fossil oil production: its competition with food, questionable land-use practices, higher prices, and increased environmental concerns (Meher et al., 2006; Thornley et al., 2008). Fatty acids are logical precursors for biofuels (Lennen et al., 2010; Lu et al., 2008) and there have been various studies focusing on microbial production of fuels and chemicals from fatty acids (Zhang et al., 2008; Steen et al., 2010; Handke et al., 2011). Compared with conventional practices, modern biotechnology techniques including metabolic engineering and genetic engineering provide a more effective approach to generate biofuels. The yeast *Saccharomyces cerevisiae* offers significant advantages for biofuel production due to its established genetic tools, high accessibility of molecular, short generation time, ease of cultivation and high tolerance to organic solvent. Based on clear understanding of its metabolism process, the *S. cerevisiae* is an ideal role in metabolic engineering pathway for fatty acid production (Nielsen, 2009; Raab et al., 2010).

Fatty acids biosynthesis is initiated by the conversion of acetyl-CoA to malonyl-CoA via the enzyme of acetyl-CoA carboxylase in cytosol, which requires acetyl-CoA, together with NADPH and ATP as key substrates and cofactors (Tehlivets et al., 2007). In yeast cells, there exist two different pathways for cytosol acetyl-CoA generation. For non-oleaginous yeasts such as *S. cerevisiae* and *Candida albicans*, the acetyl-CoA is produced from glycolysis of fermentable sugars through pyruvate-acetaldehyde-acetate pathway via the activity of cytoplasmic acetyl-CoA synthases (ACS) while for oleaginous fungi, another source of cytosol acetyl-CoA production is from excess of citrate, which is exported to cytosol via citrate transport protein (Anoop et al., 2003) and then cleaved by ATP-citrate lyase (ACL), an enzyme present in all oleaginous microorganism studied so far (Hynes and Murray, 2010; Fatland et al., 2002).

Herein, the *S. cerevisiae* is proposed to be a metabolic engineering platform for fatty acids synthesis. To accumulate citrate in TCA cycle and then transport it out to generate cytosol acetyl-CoA can be a promising pathway to provide precursor for fatty acid biosynthesis (Ratledge et al., 1997). The mitochondrial enzyme, isocitrate dehydrogenase, which responsible for catalyzing the isocitrate to form alpha-ketoglutarate, are encoded by two

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distinct genes, *idh1* and *idh2*. They participate in the TCA cycle, solely (Wang et al., 2010), and are known as enzyme down-regulate of citrate catabolism. Citrate consumption can be reduced by decreasing this enzyme's activity. ACL is required for the production of cytosolic acetyl-CoA, since it can cleave citrate into acetyl-CoA and oxaloacetate. This enzyme exists in human cells, animal cells, plant cells and in yeasts (Hynes and Murray, 2010). However, as it was mentioned previously, in yeast, the ATP-citrate lyase is widely present in fungi, but is absent in members of *Saccharomycotina* (Hynes and Murray, 2010). The primary ACL enzyme sequences contain two conserved domains: both for CoA and ATP binding. In green plants, fungi and a few of prokaryotes species, the enzyme is composed of ACLA and ACLB two subunits, and requires an ammonium ion for its activation. In animal and human cells, this enzyme is homomeric, which presumably results from an evolutionary fusion of ACLA and ACLB (Hynes and Murray, 2010; Beopoulos et al., 2011). There are researches related to the effects of the gene *acl* on the cytoplasmic acetyl-CoA generation or fatty acid accumulation. Both the evidences that small interfering RNA-mediated silencing of *acl* expression in adipocytes affects the patterns of lipid accumulation and histone acetylation and targeting a heterologous *acl* into plastids of tobacco significantly leads to fatty acid synthesis enhancement strongly suggested the relationship between ACL activity and acetyl-CoA precursor production for fatty acids synthesis (Wellen et al., 2009; Rangasamy and Ratledge, 2000).

The whole work flow in this study is to increase the amount of biofuel precursor by modifying the normal yeast cellular machinery. The gene disruption method is used to block the citrate turnover and accumulate the citrate level in yeast strains. The gene cloning and expression method will be used to introduce the enzymes which catalyze the accumulated citrate to acetyl-CoA in cytosol for fatty acid synthesis (Fig. 1).

2. Materials and methods

2.1. Medium, culture conditions and chemicals

The wild type *S. cerevisiae* BY4741 strains used in this study were grown at 30 °C, 250 rpm in YPD medium (1% yeast extract, 2% peptone, 2% dextrose). The selective medium for gene disrupted strains were YPD medium with appropriate antibiotics: 200 μg/ml Geneticin (G418) for single gene disruption, 7.5 μg/ml Phleomycin (Phleo) for double gene disruption. The selective medium for gene insertion strains was YNBD-URA synthetic minimal medium (0.67% yeast nitrogen base, containing 2% glucose and supplemented with URA dropout amino acid mixture). Oligonucleotide primers were synthesized at 1st BASE Pte Ltd. The restriction enzymes were from NEB; T4 ligase was purchased from Fermentas. Taq DNA polymerase was from Promega. Phleomycin, antibiotics and other chemicals used in this study were purchased from Sigma. All of the chemicals were of reagent grade.

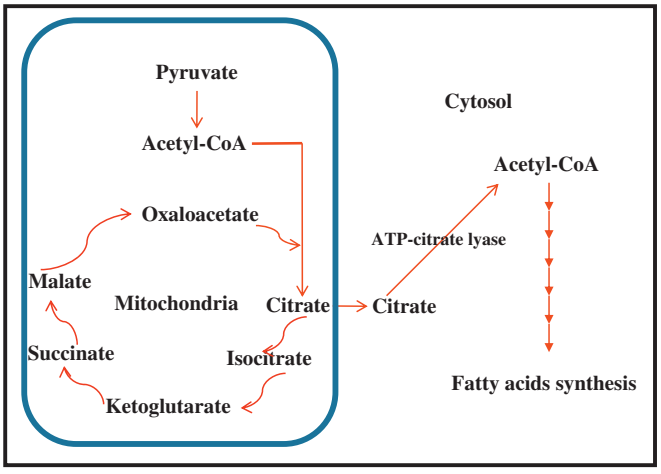

Fig. 1. The workflow of metabolic engineering pathway in *S. cerevisiae* connecting with citrate accumulation and cytosol acetyl-CoA generation via ATP-citrate lyase, which is benefit for final fatty acid synthesis.

2.2. Targeted gene disruption of idh1, idh2 and idh1/2 in Saccharomyces cerevisiae

The pUG plasmid series carrying gene disruption cassettes consisting of kan^r, ble heterologous marker genes with *loxP* sites were selected for single or double disruption strategy. The *idh1* and *idh2* genes of *S. cerevisiae* participated in the TCA cycle were selected for the targeted disrupted genes. The sequences flanking the target gene in the genome were added to the 5′ end of OL5′ (upstream of the Ptef resp. of the T elements in the pUG plasmid) and OL3′ (downstream of the Ptef resp. of the P elements in the pUG plasmid) sequences: 40 nucleotide stretches that are homologous to sequences upstream of the ATG start codon and downstream of the stop codon of targeted gene, respectively. The detail primer sequences were shown in Table 1 and the gene disruption approach was shown in Fig. 2A.

The single gene disruption was carried out by pUG 6 plasmid with kan^r marker and the double gene disruption was carried out by pUG66 plasmid with ble marker under the basis of single gene disruption. The *S. cerevisiae* BY4741 strain was transformed with the *idh1*::kan, *idh2*::kan fusions by PEG-LiAc method and selected on YPD+G418 antibiotic-contained plates, to yield Δ*idh1* and Δ*idh2* strains, respectively. The Δ*idh1* single gene disrupted strain was transformed with *idh2*::ble fusion, to yield Δ*idh1/2* strain. The double deletion strains were selected on YPD+Phleo antibiotic-contained plated. The results were determined by yeast colony PCR.

2.3. Molecular cloning of acl

The *acl* from mus musculus was synthesized by GENEART. The PCR was produced according to the primers with *NheI* and *BamHI* restriction enzyme (RE) site introduced. Under the *Taq* DNA polymerase, the PCR was performed in total of 30 cycles. Each cycle included three steps: 95 °C for 45 s, 56 °C for 45 s, 72 °C for 3.5 min. The PCR products were purified and digested by the restriction enzymes of *NheI* and *BamHI*. The products after double digestion was ligased with pVTU260 vector which was also digested by these two restriction enzymes and then transformed into Top10 compenent cells (Fig. 2B). The positive colony was confirmed by both bacteria colony PCR and double digestion results.

The recombinant plasmid was then transformed into both *S. cerevisiae* wild type and Δ*idh1,2* strains by PEG-LiAc yeast transformation method, to form wt-*acl*, Δ*idh1-acl*, Δ*idh2-acl* and Δ*idh1/2-acl* engineered strains. The positive colony was selected on YNBD –URA plates. An empty plasmid was also transformed into wild type to form wt-empty strain. For single gene disrupted strains with recombinant vector, 200 μg/ml G418 was added and for double gene disrupted strains with recombinant vector, 7.5 μg/ml Phleo was added. The results were confirmed by yeast colony PCR and the protein expression was confirmed by Western blot technologies.

Table 1
Primers, plasmids and strains used in this study.

Name	Description	Reference
Primers for gene disruption		
idh1-F	5′-CTCATTCTTCTCCCTTTTCCTCCATAATTGTAAGAGAAAACAGCTGAAGCT TCG TAC GC-3′	This study
idh1-R	5′-CACTTAAGTTGCAGAACAAAAAAAAGGGGAATTGTTTTCAGCATAGGCCACTAGTGGATCTG-3′	This study
idh2-F	5′-ACAGATCGGGAACGAACAACAATTATAATATTTTTTAATACAGCTGAAGCTTCGTACGC-3′	This study
Idh2-R	5′-CAAGGCGTGAAAAAACCTGAGAGGGAAAAGAATAGGACTTGCATAGGCCACTAGTGGATTG-3′	This study
Primers for acl insertion		
acl-F	5′-(CGGCTAGC)ATGTCAGCCAAGGCAATTTCAGAGC-3′	This study
acl-R	5′-(CGGGATCC)TTACATGCTCATGTGTTCTGGAAGA-3′	This study
Plasmids		
pUG6	*loxP-kanMX-loxP* disruption module plasmid	*Dr. Johannes H. Hegemann.*
pUG 66	*loxP*- Phleo -*loxP* disruption module plasmid	*Dr. Johannes H. Hegemann.*
pVTU260	Expression vector with ADH1 promoter and terminator, URA maker	Euroscarf
Strains		
S. cerevisiae BY4741	Δ*MAT, his*3Δ, *leu*2Δ, *met*15Δ, *ura*3Δ	ATCC

Notes: The enzyme sites for gene insertion are underlined.

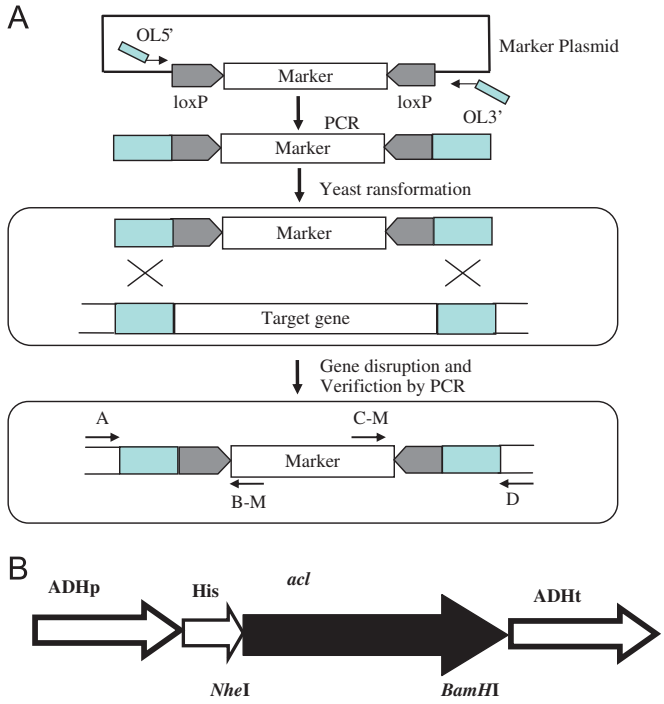

Fig. 2. (A) Outline of the one-step gene disruption approach for generation of Δ*idh1*, Δ*idh2* and Δ*idh1/2* mutant strains. (B) Schematic diagram of the pVTU260-*acl* construct.

2.4. Protein extraction, SDS-PAGE, and Western blot analysis

The crude protein from yeast were extracted using a lysis buffer (50 mM HEPES, 5% glycerol, 1 mM DTT, 1 mM PMSF, 1 mM EDTA) with equal volume of glass beads (425–600 μm; Sigma). After vortexing, the cleared supernatant was collected by centrifugation. Protein concentration was determined via 2D-quant kit. The protein extract was loaded on a 6% SDS-PAGE and run at 20 mA. Later for Western blot analysis, the SDS-PAGE was electrophoretically transferred to a PVDF membrane at 21 V for 1 h. The membrane was block with 5% fat-free milk in a TBS buffer with 0.5% Tween-20 (TBST) for 1 h at room temperature, washed with 2 times and later incubated overnight at 4 °C with anti-ACL specific antibody (Cell signal), diluted to 1:1000, in the same TBST buffer with 5% fat-free milk. The membrane was then washed with 3 times (with 10 min every time) with TBST buffer and the anti-rabbit antibody diluted to 1:2000 was added as second antibody and incubated at room temperature for 1 h. After washing with three times in the same condition, the ECL Western blot detection reagent was used for signal visualization.

2.5. GC–MS sample preparation

Same amounts of yeast cell cultures (10 OD) were collected by centrifugation at growth phase and stationary phase (determined from growth curve) and wash twice with distilled water. The cell pellets were then dissolved in a mixture of methanol/water extraction solvent in ratio of 5:2 (v/v). 10 μl ribitol (2 mg/ml) and 10 μl heptadecanoic acid (2 mg/ml) was added as internal standard in the extracted solvent to correct for mechanism loss during sample preparation. The samples were put into liquid nitrogen for 2 min and then throw into ice bath untra-sonicator for another 2 min with total for 6 cycles and then added chloroform to form a mix of chloroform/methanol/water in ratio of 2:5:2 (v/v/v) and centrifuged. The supernatants of water phase and chloroform phase were collected separately. The citrate was included in water phase and the lipids were included in the chloroform phase. For extracellular citrate analysis, the supernatant were collected from centrifuge of 10 OD yeast cell cultures and passed through a 0.45 μm filter and washed with methanol. All of these were evaporated to complete dryness.

The derivatization was performed prior to GC–MS analysis. For citrate derivatization, 50 μl 20 mg/ml of methoxyamine hydrochloride in pyridine solution was added and kept at 37 °C for 1 h. Silylation was then carried out by adding 99 μl MSTFA and 1 μl TMCS to each sample at 70 °C for 30 min. The samples were then shaken for 1 h at room temperature after incubation then transferred to vials for GC–MS.

For lipid derivatization, 500 μl boron trifluoride-methanol solution was added to each of the sample and incubated at 90 °C for 30 min, immediately add 50 μl saturated sodium chloride solution to stop the reaction. In this condition, lipid was mainly converted to fatty acid methyl ester by transesterification reaction and the calculated products in the following results section reflected the results of total fatty acids from lipid in yeast cells. Later, 300 μl hexane was added for extraction of fatty acids esters.

2.6. GC–MS analysis

The GC–MS analysis was performed with a Shimadzu QP2010Plus system (From Shimadzu, Kyoto, Japan), equipped with a 30 m × 250 μm i.d. and 0.25 μm thickness DB-5 capillary column. Total of 1 μl samples after derivation was injected into the system. Helium was used as carrier gas and the flow rate was 1.1 ml/min. The cutoff

time of solvent was 3.5 min. The injection temperature was kept at 280 °C and the ion source temperature was kept at 200 °C. The oven temperature program was according to follows: 100 °C for 4 min and a ramp to 320 °C by increased at 4 °C/min. The detection was performed in an electron impact ionization mode at 70 eV and the mass spectrum was recorded from 35 to 600 m/z, with a 0.3 s of scan time. The Shimadzu GC–MS solution software was applied to acquire the chromatogram and identify mass spectra. Prior to the peak area integration, the noise reduction and baseline correction were operated. The quantification of fatty acids was achieved by integrated peaks through comparing with the added internal fatty acids standards and the components were identified from the standard and the metabolite mass spectra database. All of the prepared derivation samples were run within 24 h.

2.7. ATP and NADPH assay

The detection of ATP and NADPH in yeast cells were performed according to ATP colorimetric/fluorometric assay kit and NADP+/NADPH quantification kit (both provided by BioVision, USA). Since the specific amounts of these components were hard to control in different culture batches, herein, we use comparative ratio to reflect the changes between yeast wild type and gene disruption strains.

3. Results

3.1. Citrate level description between wild type and idh1,2 disrupted strains

According to the genetic strategy described in methods part, two strains with a single gene disruption, Δ*idh1* and Δ*idh2*, and one with both disruptions Δ*idh1/2* were obtained. When they grew in YPD medium, no significant growth deficiency was observed. The citrate level was expected to be higher in Δ*idh1,2* strains compared to the wild type strain, since the *idh1,2* genes, responsible for the down regulation of citrate metabolism, were inactivated. From the results, it could be seen easily that both single and double gene disruptions led to a significant increase in cellular citrate levels with 4- to 5-fold. The double mutants of Δ*idh1/2* showed more citrate accumulation compared with the single mutants and the maximum concentration of intracellular citrate reached 22 nmol/10^8 cells (Fig. 3A). The increased citrate pool in Δ*idh1,2* strains also led to increase citrate secretion. The results of citrate concentration in culture supernatant showed the citrate levels in Δ*idh1,2* strains were about 2–3 times higher than wild type strain (Fig. 3B). Since our focus was about cellular citrate metabolism, more attention was placed on the changes of intracellular citrate in yeast strains.

3.2. ATP and NADPH level description between wild type and idh1,2 disrupted strains

NADPH is the key source of reducing equivalents for fatty acids synthesis. Since the genes disrupted here encode the enzyme of isocitrate dehydrogenase which is partially responsible for NADPH production in yeast mitochondria, and there are concerns about that the accumulation of citrate is at the cost of NADPH reduction. Herein, we examined the intracellular NADPH level (Fig. 4) and NADPH/$NADP^+$ ratio (Table 2) of both the wild type and Δ*idh1,2* strains using the NADP+/NADPH assay kit. The NADPH/$NADP^+$ ratio and NADPH production levels in all strains were much higher in growth phase than those of stationary phase. This could be regarded as a result of glucose depletion in stationary phase which disturbed the redox balance. However, there were no significant decreases of NADPH levels in Δ*idh1,2* strains compared to that in wild type strain.

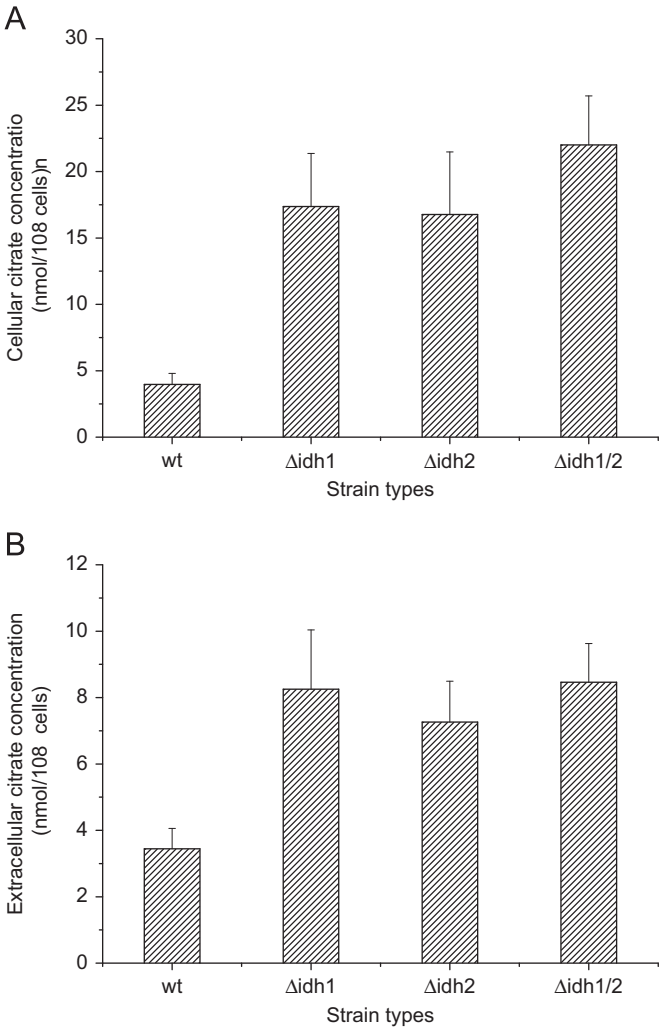

Fig. 3. (A) Differential production levels of cellular citrate between *S. cerevisiae* wild type and Δ*idh1,2* strains. (B) Differential production levels of extracellular citrate between *S. cerevisiae* wild type and Δ*idh1,2* strains.

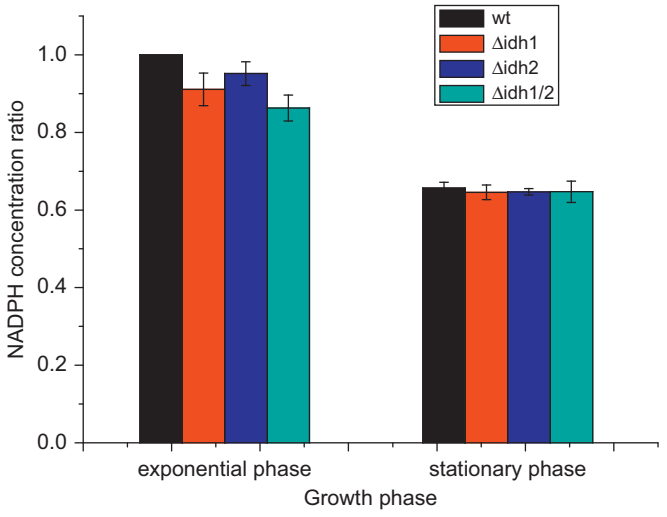

Fig. 4. NADPH production levels comparison between *S. cerevisiae* wild type and Δ*idh1,2* strains.

ATP is important cellular energy transfer which transports chemical energy within cells of cellular metabolism. Together with acetyl-CoA and NADPH, these three comprises most important

substrates and cofactors required for fatty acids synthesis. Herein, the ATP level of wild type and Δ*idh1,2* strains were examined in both growth phase and stationary phase (Fig. 5). At the exponential phase, the ATP level was much lower in Δ*idh1,2* strains, only about 50–60% of that in wild type strain, which was in accordance with the situation: The blockage of TCA cycle to accumulate citrate was at the expense of ATP production to a certain extent. Once the yeast strains enter into stationary phase, lots of ATP has been consumed for cellular metabolism and its level reduced to only about 20% of that in wild type strain in growth phase.

3.3. Expression of acl in wild type and idh1,2 disrupted strains

The expression of *acl* in wild type and Δ*idh1,2* strains was examined using protein samples of yeast cells collected from stationary phase in shake flasks with 50 ml YNBD-URA medium supplemented with antibiotics, accordingly to the gene disruption strategy. The *acl* encoding the ATP-citrate lyase was successfully cloned and expressed in both the *S. cerevisiae* wild type and Δ*idh1,2* strains. The results were confirmed by both the yeast colony PCR and Western blot methods (Fig. 6). The wild type strain with empty pVTU260 plasmid was added as control. The PCR fragment size of recombinant strains colony PCR on DNA gel was about 3.3 kb and no band was observed from the control strains. The protein size from Western blot result was about 120 kDa; similarly, there were no protein bands from the control strains. These results matched the real situation that the cloned *acl* fragment from mus musculus was 3342 bp and the protein molecular weight was 119.7 kDa. Same amount of protein samples was loaded on the SDS-page gel and the Western blot results reflected the fact that the expression level of *acl* was almost equal in Δ*idh1,2* strains compared to the wild type strain.

Table 2
NADPH/NADP+ ratio between engineered *S. cerevisiae* and wild type strains.

	Wild type strain	Δ*idh1* strain	Δ*idh2* strain	Δ*idh1/2* strain
Exponential phase	9.45 ± 1.06	8.95 ± 1.12	8.95 ± 1.31	8.57 ± 1.24
Stationary phase	3.16 ± 0.48	2.18 ± 0.32	2.02 ± 0.47	2.05 ± 0.41

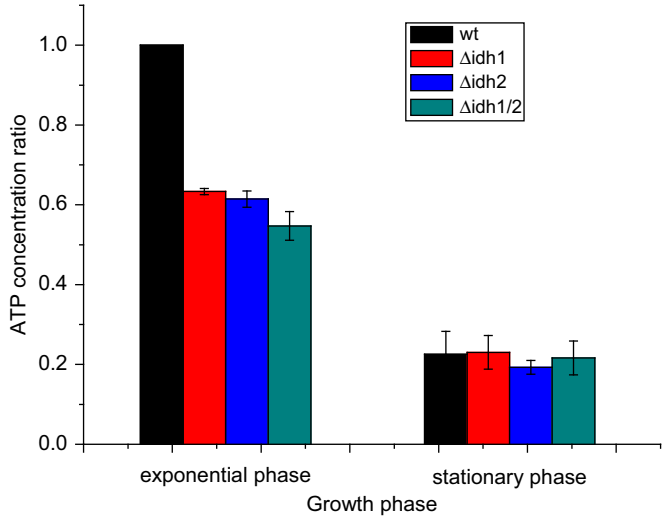

Fig. 5. ATP production levels comparison between *S. cerevisiae* wild type and Δ*idh1,2* strains.

3.4. Growth rate depicted between wild type and engineered strains

The expression plasmid here was pVTU260 which contains constitutive promoter *ADH*1, and no need to be induced for the

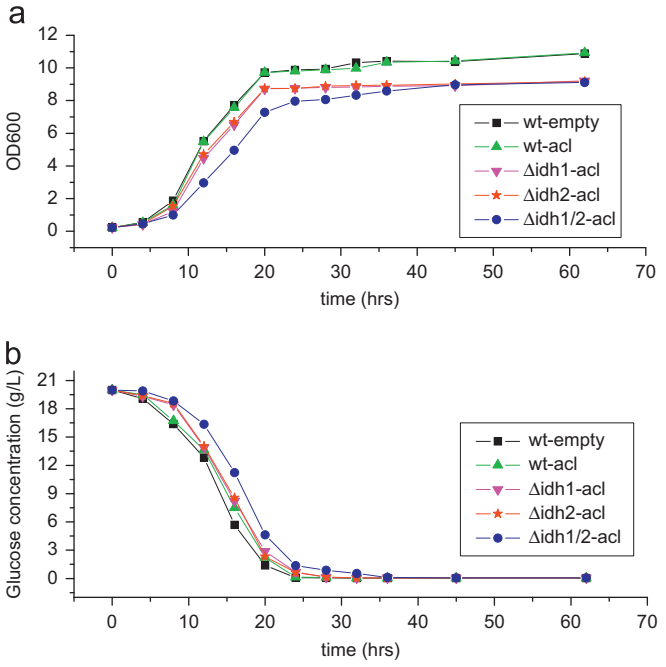

Fig. 7. Growth curve and glucose consumption curve of wild type and engineered strains.

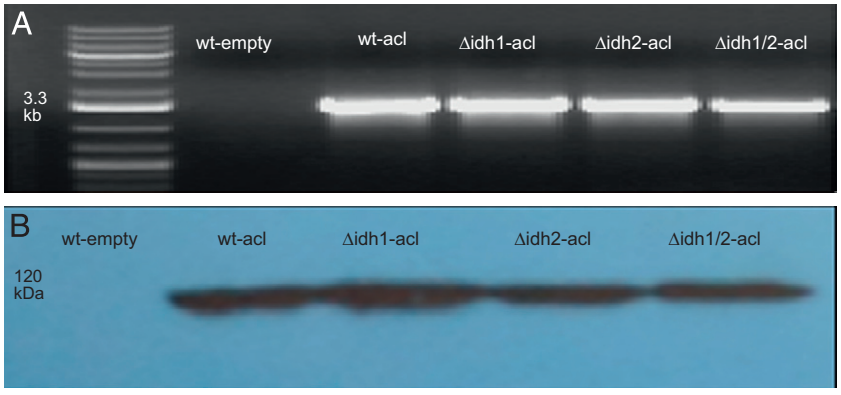

Fig. 6. (A) Yeast colony PCR confirmation of the recombinant vector pVTU260-*acl* in *S. cerevisiae* wild type and Δ*idh1, 2* strains. (B) Over-expression of *acl* in *S. cerevisiae* wild type and Δ*idh1, 2* strains.

expression. Therefore, the culture of engineered strains could be performed in medium without inducible reagent. However, compared with wild type strain cultured in YNBD-URA medium, the gene disrupted strains with *acl* gene grew a little slower. The growth curve and glucose consumption details were shown in Fig. 7. The results showed that at the beginning of growth, the Δ*idh1/2-acl* strain showed 20% less amount of biomass than wild type strain, 10% less than the Δ*idh1-acl* and Δ*idh2-acl* strains. In the late stationary phase, the density of Δ*idh1/2-acl* strain reached the same with the Δ*idh1-acl* and Δ*idh2-acl*. However, all of gene disrupted strains showed a little lower cell density compared to the wild type when they reached to the stationary phase. Similarly, the glucose consumption situations of different strains were consisted with the growth curve. The Δ*idh1/2-acl* strain consumed glucose slower than the wild type strain and Δ*idh1-acl* and Δ*idh2-acl* strains. After about 24 h, the glucose was consumed out and all the cells entered into the stationary phase.

3.5. Fatty acids synthesis description in engineered strains

The designed metabolic engineered pathway of *S. cerevisiae* strains in this project aims at providing more precursor acetyl-CoA for fatty acids synthesis. Herein, GC–MS detection system was applied for metabolites analysis in yeast strains. The total fatty acids levels from lipid were shown in Fig. 8. Compared with growth phase, the maximum production amount was in stationary phase, which reached to 120–150 μg/10^8 cells. The strains with *acl* gene expression showed a 10%–20% increase compared to wild type: 15.4% increased in wt-*acl* strain, 15.5% increased in Δ*idh-acl* strain, 11.2% increased in Δ*idh2-acl* strain and 21.1% increased in increased in Δ*idh1/2-acl* strain. However, in growth phase, no such significant increase was observed.

In the *S. cerevisiae* strains studied here, the major compositions of fatty acid were tridecanoic acid (C14:0), hexadecanoic acid (C16:0), 9-hexadecanoic acid (C16:1), octadecenoic acid (C18:0) and 9-octadecenoic acid (C18:1). Calculated from standards, it was found that saturated fatty acid C14:0 accounted for about 30% of total fatty acids. The unsaturated fatty acids C16:1 and C18:1 accounted for about 10–20% and 3–5% individually and others were saturated fatty acids C16:0 and C18:0. No C16:2 and C18:2 fatty acids were detected (Fig. 9B).

Compared with wild type strain with empty plasmid, the fatty acids compositions changes described in engineered strains were as follows: the wt-*acl* strain showed higher level of saturated fatty acids. 20% increased in C14:0, 14% increased in C16:0 and 27% increased in C18:0, but no significant changes were observed in C16:1 and C18:1. On the contrary, the gene disrupted strains with *acl* gene showed much higher level in unsaturated fatty acids. For Δ*idh1-acl*, 80% increased in C16:1 and 60% increased in C18:1; for Δ*idh2-acl*, 60% increased in C16:1 and 45% increased in C18:1; for Δ*idh1/2-acl*, 92% increased in C16:1 and 77% increased in C18:1. However, no significant changes were observed in saturated fatty acids (Table 3).

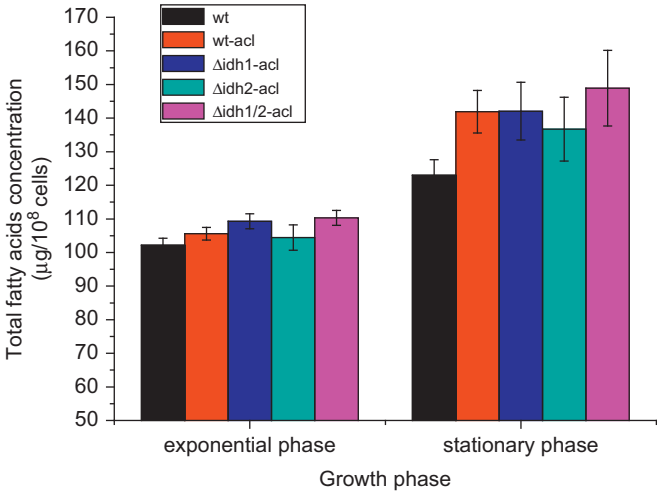

Fig. 8. Total fatty acids production levels in wild type and engineered strains.

4. Discussion

The citrate metabolism is important to provide cells with biosynthesis precursors and energy. Previous studies have proved that the activity of NAD^+-dependent isocitrate dehydrogenase (*idh*) was correlated with the onset of lipid accumulation in yeasts (Leroux et al., 2011; Papanikolaou and Aggelis, 2011). Under a nitrogen-limited condition, the *idh* activity in mitochondria is depressed due to the diminution of adenosine monophosphate (AMP), which is the allosteric activator of *idh*. In this situation, the citrate were accumulated, transported to cytosol and cleaved by ATP-citrate lyase to acetyl-CoA for fatty acids synthesis. The control of citrate metabolism by modifying *idh* activity can make a greater flux from carbon source to lipid. The ATP-citrate lyase, which exists in most oleaginous yeast cells but not in *S. cerevisiae* has been proved to have a correlation of lipid accumulation (Evans and Ratledge, 1985a, 1985b; Boulton and Ratledge,

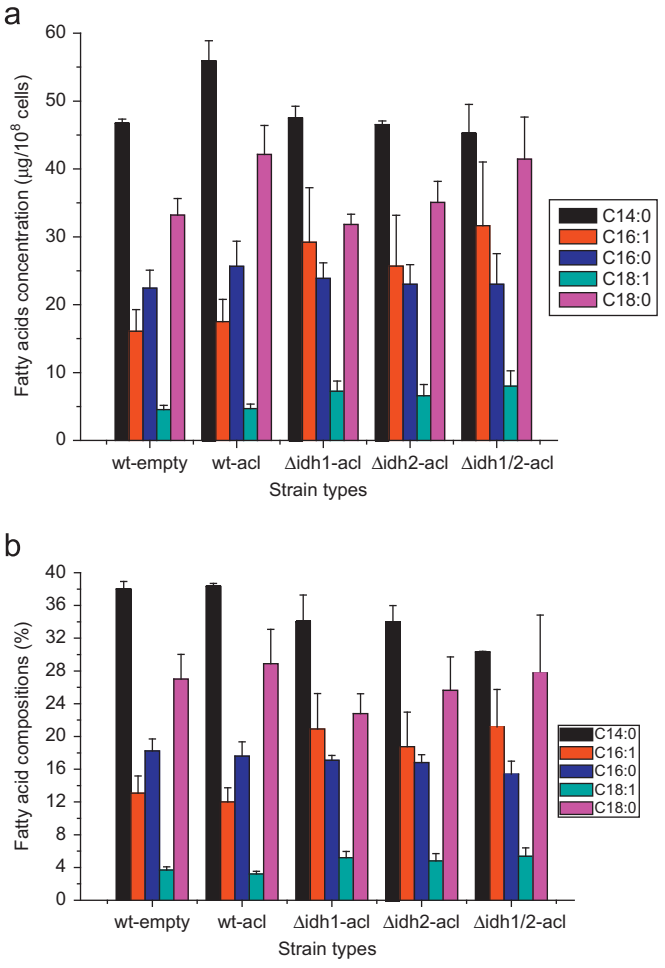

Fig. 9. (A) Differential production levels of individual types of fatty acids in wild type and engineered strains. (B) Fatty acids composition (% of total fatty acid) in wild type and engineered strains.

Table 3
Fatty acids composition changes between engineered *S. cerevisiae* and wild type strains.

	Tridecanoic acid (C14:0) (fold changes)	9-Hexadecanoic acid (C16:1) (fold changes)	Hexadecanoic acid (C16:0) (fold changes)	9-Octadecenoic acid (C18:1) (fold changes)	Octadecenoic acid (C18:0) (fold changes)
Wild type strain with *acl* gene	1.21 ± 0.0475	1.09 ± 0.0090	1.14 ± 0.0300	1.04 ± 0.0009	1.27 ± 0.0359
Δ*idh1* strain with *acl* gene	1.02 ± 0.0508	1.80 ± 0.2322	1.07 ± 0.0247	1.59 ± 0.1116	0.96 ± 0.0252
Δ*idh2* strain with *acl* gene	1.01 ± 0.0017	1.60 ± 0.2079	1.03 ± 0.0088	1.45 ± 0.1645	1.06 ± 0.0159
Δ*idh1/2* strain with *acl* gene	1.08 ± 0.0779	1.92 ± 0.1384	1.04 ± 0.0135	1.77 ± 0.2541	1.25 ± 0.0941

Notes: The data of fold changes for engineered strains herein were based on the comparison results with wild type strain with empty plasmid. The fatty acid amount of wild type strain was taken as control (100%), and others are a ration to the control.

1981): (1) its activity closely relates to the fatty acid synthesis rate; (2) the citrate, as the substrate for ATP-citrate lyase, physically accumulates in yeast cytosol and then in culture media during lipogenesis; (3) the ACC activity by citrate has been proved to ensure that the acetyl-CoA generated in the ACL reaction is directly utilized for fatty acid synthesis by a "feed-forward" form. These facts gave the clue to modify yeast cells in which the *idh* encoding genes were directly deleted. Upon regulating enzymes in TCA cycle, the citrate can be successfully accumulated. In this study, all of the Δ*idh1*, Δ*idh2* and Δ*idh1/2* mutant strains showed obviously higher level of citrate. The accumulated citrate was beneficial for the fatty-acid biosynthetic pathways. In this study, the ATP-citrate lyase was introduced into *S. cerevisiae* and the results showed formation of cytosol acetyl-CoA from excess citrate for fatty acid synthesis was realized. The fatty acids in various cells were detected and different engineered yeast cells showed increased amounts in different fatty acid types.

The source of NADPH for fatty acid synthesis in *S. cerevisiae* is mainly from: (1) pentose-phosphate pathway (PPP), under the enzymes of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase; (2) the reaction catalyzed by the $NADP^+$-dependent isocitrate dehydrogenase; (3) the reaction catalyzed by the $NADP^+$-dependent acetaldehyde dehydrogenase and (4) the reaction catalyzed by malic enzyme (Minard and McAlister-Henn, 2005; Bruinenberg et al., 1983; dos Santos et al., 2004). The PPP pathway is responsible for the main source of NADPH production. Under the activity of glucose-6-phosphate dehydrogenase, 12 mol of NADPH can be produced from 1 mol of glucose-6-phosphate (G-6-P), whereas, in TCA cycle, only 2 mol of NADPH can be produced from 1 mol of G-6-P under the activity of $NADP^+$-linked dehydrogenase (Bruinenberg et al., 1983). The little effect on NADPH production for fatty acid synthesis by the yeast engineered cells here no need to be taken into consideration. The detection of NADPH in our study matched this point well: there were no significant NADPH changes in gene disrupted strains compared to wild type strain, which ensured the supply of NADPH cofactor for fatty acid synthesis. However, the blockage of TCA cycle caused the reduction of ATP, which was the important energy source for fatty acid synthesis. It was inevitable in the gene disrupted strains and if this problem could be successfully solved, maybe more fatty acid could be expected to enhance by the engineered yeast strains. The maximal activity of ACL exhibited in stationary phase, which was reflected in our results: all of the strains accumulated more fatty acids in stationary phase than that in growth phase.

The Palmitic acid and Stearic acid are the main fatty acids synthesized by most yeast fatty acid synthase (Trotter, 2001). In our study, the Myristic acid was also found as the fatty acid with high amount in *S. cerevisiae*. There was an interesting phenomenon found in the fatty acid compositions changes: For the wt-*acl* strain, the increased levels of fatty acids mainly performed in saturated fatty acids: C14:0, C16:0 and C18:0. However, for the Δ*idh1,2-acl* strains, the increased levels of fatty acids mainly performed in unsaturated fatty acids: C16:1 and C18:1. However, no C16:2 or C18:2 fatty acids were detected. The increased fatty acids in engineered strain with *acl* demonstrated the effects of ATP-citrate lyase on fatty acid enhancement.

In *S. cerevisiae*, the unsaturated fatty acids composition consist almost exclusively of the mono-unsaturated fatty acids formed by the Δ-9 fatty acid desaturase, which introduces a double bond between carbons 9 and 10 of palmitoyl (C16:0)- or stearoyl (C18:0)-CoA to form palmitoleic (C16:1) or oleic (C18:1) acid (Bossie and Martin, 1989). The mono-unsaturated fatty acids are essential for membrane expansion in growing cells and are major components of storage lipids in stationary phase cells (McDonough et al., 1992). So most of the additional lipids studied in this paper were speculated to be stored in additional membrane. There were a lot of researches focus on the relationship between fatty acid compositions and environmental conditions (Alexandre et al., 1994; Beaven et al., 1982; Sajbidor et al., 1995). It has been found that *S. cerevisiae* cell grown in the presence of ethanol appears to increase the amount of mono-unsaturated fatty acids in cellular lipid (Sajbidor et al., 1995; You et al., 2003). The authors suggested that the increased proportions of mono-unsaturated fatty acids counteract the membrane-fluidizing effects of ethanol and confer ethanol tolerance by maintaining functional mitochondria during ethanol stress. Herein, the Δ*idh1,2* strains led to the blockage of TCA cycle; hence, the reasons for accumulated mono-unsaturated fatty acids in our study were boldly assumed: compared with wild type strains, more pyruvate from glucose-6-phosphate did not enter into TCA cycle but were used for ethanol production, the increased ethanol was accompanied by the increased amount of mono-unsaturated fatty acids to reduce the ethanol stress on membrane-fluidizing effect. Meanwhile, the saturated fatty acids would be partially consumed for desaturation to form these mono-unsaturated fatty acids. This was why the saturated fatty acids level in Δ*idh1,2-acl* strains showed relative lower than wt-*acl* strain. However, more evidence needs to be discovered to support this explanation and later studies on proteomic profiling analysis and metabolic profiling analysis, will be performed to understand deep metabolic mechanism of the engineered strains.

The fatty acids are important precursors for biofuels including biodiesel production and one of major problems associated with the application of biodiesel is poor cold flow properties, which was indicated by parameters such as pour points and cloud points (Knothe, 2005). Compared with saturated fatty acid methyl esters, the mono-unsaturated fatty acid methyl esters have significantly lower melting points and thus, biodiesel fuels derived from fatty acids with increased mono-unsaturated fatty acids will display

lower pour points and cloud points. Besides, the biodiesel fuels from mono-unsaturated fatty compounds giving a much lower viscosity than that from saturated fatty compounds. All of these effects can improve cold flow property of biodiesel.

In conclusion, the gene disruption methods and molecular cloning and expression strategy were successfully applied to accumulate citrate generated from TCA cycle, which in turn is used to produce cytosol acetyl-CoA under the enzyme activity of ATP-citrate lyase. The increased level of cytosolic acetyl-CoA should favor the fatty acid synthesis. The different types of metabolic engineered yeast cells showed enhancement in different fatty acids compositions. The increased fatty acids may be potential candidate substrates for biofuel production such as bioalkane. A global metabolomics and proteomic system for the engineered strains will be later established for better understanding of the metabolic mechanism, for further improvement of yield of intracellular biofuel precursors.

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Appendix A. Supplementary Information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2013.01.003.

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