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Role of pyruvate carboxylase in accumulation of intracellular lipid of the oleaginous yeast *Yarrowia lipolytica* ACA-DC 50109

Guang-Yuan Wang · Yan Zhang · Zhe Chi · Guang-Lei Liu · Zhi-Peng Wang · Zhen-Ming Chi

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Abstract *Yarrowia lipolytica* ACA-DC 50109 is an oleaginous yeast. In order to know the function of pyruvate carboxylase (PYC) in lipid biosynthesis, the *PYC* gene cloned from *Pichia guilliermondii* Pcla22 was overexpressed in the oleaginous yeast. The lipid contents in the wild-type strain ACA-DC 50109 and the transformants P4, P7, and P103 were 30.2 % (*w*/*w*) 36.5 % (*w*/*w*), 38.2 % (*w*/*w*), and 37.9 % (*w*/*w*). However, the amount of the secreted citric acids by strains ACA-DC 50109, P4, P77, and P103 were 0.5, 10.1, 11.5, and 9.4 g/L. In order to reduce the amount of the secreted citric acid, the *PYC* gene and endogenous *ACL1* gene encoding ATP citrate lyase (ACL1) were simultaneously overexpressed in the oleaginous yeast. The lipid contents of the transformants PA19, PA56, PA124 were 44.4 % (*w*/*w*), 45.3 % (*w*/*w*), and 43.7 % (*w*/*w*). At the same time, the amount of the secreted citric acid by the transformants PA19, PA56, and PA124 was reduced to 5.4, 6.2, and 6.3 g/L. The PYC and ACL1 activities and their gene transcriptional levels in all the transformants were greatly enhanced compared to those in their wild-type strain ACA-DC 50109. During 10-L fermentation, lipid content in the transformant PA56 was 49.6 % (*w*/*w*) and the amount of secreted citric acid was 2.9 g/L. This meant that PYC and ACL1 can play an important role in accumulation of intracellular lipid of the oleaginous yeast *Y. lipolytica* ACA-DC 50109.

Keywords Lipid · Pyruvate carboxylase (PYC) · ATP citrate lyase (ACL1) · Oleaginous yeast · *Y. lipolytica*

Introduction

It has been well known that microbial oils, especially yeast oils, can be used for production of biodiesel and specific lipid derivatives such as lubricants, adhesives, and plastics (Beopoulos et al. 2011). Therefore, it is very important to enhance oil biosynthesis by the oleaginous yeasts which contain more than 20 % of oils in their cells. Many strategies have been developed for enhancement of lipid biosynthesis in yeast cells. A high C/N ratio (an excess of carbon source and limitation of nitrogen) is a most effective way to promote lipid biosynthesis (Li et al. 2010). According to the pathway of lipid biosynthesis (Fig. 1) (Ratledge 2004), overexpression of malic enzyme (ME) in order to regenerate the $NADPH^+$ cofactor supply for fatty acid synthesis; ATP citrate lyase (ACL) to increase the acetyl-CoA, the main precursor of fatty acid synthesis; and acetyl-CoA carboxylase (ACC) to catalyze the first and rate-limiting step in de novo lipid synthesis have been found to be able to enhance lipid biosynthesis. Deletion of the *MIG1* encoding the main effector of glucose repression, the glycerol-3-phosphate dehydrogenase gene (*GUT2*) and the six *POX* genes encoding acyl-CoA oxidases, abolishing β-oxidation results in a significant increase in lipid accumulation (Beopoulos et al. 2011; Wang et al. 2013).

Pyruvate carboxylase (PYC) which is a key enzyme in the cytosolic reductive TCA pathway is a biotin-dependent tetrameric enzyme that catalyzes the carboxylation of pyruvic acid to oxaloacetic acid (Fig. 1). All the microbial PYC enzymes have been found to be biotin dependent, existing either as an

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G.-Y. Wang · Y. Zhang · Z. Chi · G.-L. Liu · Z.-P. Wang · Z.-M. Chi (✉)
Unesco Chinese Center of Marine Biotechnology, Ocean University of China, Yushan Road, No. 5, Qingdao, China
e-mail: zhenming@sdu.edu.cn

G.-Y. Wang
Shandong Provincial Key Laboratory of Applied Mycology, Qingdao Agricultural University, Changcheng Road, No.700, Qingdao, China

α4 or an α4β4 multimeric complex. The α4 PYC enzymes are allosteric, requiring acetyl-CoA for their activation, and are inhibited by aspartate, while α4β4 PYC enzymes are not subjected to allosteric control (Lietzan and Maurice 2013). When yeasts are grown on acetate, PYC-catalyzed oxaloacetate formation is repressed. So far, it has been well known that PYC can play an important role in biosynthesis of malic acid, polymalate, succinic acid, fumaric acid, citric acid, α-ketoglutaric acid and all the kinds of amino acids among the carboxylic acids produced by microorganisms (Brown et al. 2013; Ma et al. 2013; Yin et al. 2012; Xu et al. 2012; Gokarn et al. 1998; Guo et al. 2013). Because it catalyzes the carboxylation of pyruvic acid to oxaloacetic acid, a precursor for citrate and fatty acids biosynthesis (Fig. 1), it may also play an important role in biosynthesis of fatty acid and triglycerides. In order confirm this, the *PYC* gene cloned from *Pichia guilliermondii* Pcla22 which can produce malic acid and the endogenous *ACL1* gene from *Yarrowia lipolytica* were simultaneously overexpressed in the oleaginous yeast *Y. lipolytica* ACA-DC 50109. Then, lipid production in the genetically engineered yeasts was examined.

Materials and methods

Strains, media, and plasmids

The oleaginous yeast used in this study was *Y. lipolytica* ACA-DC 50109 (wild-type strain) (collection number 2E00680 at the Marine Microorganisms Culture Collection of China) which was kindly offered by Dr. Seraphim Papanikolaou from Laboratory of Food Microbiology and Biotechnology, Department of Food Science and Technology, Greece (Papanikolaou and Aggelis 2003). A uracil mutant (collection number 2E00681 at MCCC) was isolated from *Y. lipolytica* ACA-DC 50109 using 5′-fluororotic acid (5′-FOA) (Wang et al. 2009) and was grown in the medium containing 1.7 g/L of YNB (yeast nitrogen base without amino acids and ammonium sulfate), 5.0 g/L of $(NH_4)_2SO_4$, 10.0 g/L of glucose, 25.0 g/L of agar and 0.01 g/L of uracil. *P. guilliermondii* Pcla22 (collection number 2E00691 at MCCC) is an oleaginous yeast and a producer of malate (Wang et al. 2012). Yeast strains were grown in yeast peptone dextrose (YPD medium) (10.0 g/L yeast extract, 20.0 g/L bacto peptone, 20.0 g/L glucose). The yeast transformants were selected on YNB-N5000 medium (1.7 g/L YNB, 10.0 g/L glucose, 5.0 g/L ammonium sulfate). The *Escherichia coli* strain used in this study for plasmid recovery and cloning experiments was DH5α [F^- *endA1 hsdR17* (*rK_/mK*$^+$) *supE44 thi*$^-$ λ^-*recA1gyr96DlacU169* (*j80lacZDM15*)] and was grown in Luria–Bertani broth (LB). The *E. coli* transformants were grown in LB medium with 100.0 μg/mL of ampicillin. The compositions of the medium for lipid production were 40.0 g/L glucose, 20.0 g/L $CaCO_3$, 7.0 g/L KH_2PO_4, 2.5 g/L Na_2HPO_4, 1.5 g/L $MgSO_4 \cdot 7H_2O$, 0.15 g/L $CaCl_2$, 0.15 g/L $FeCl_3 \cdot 6H_2O$, 0.02 g/L $ZnSO_4 \cdot 7H_2O$, 0.06 g/L $MnSO_4 \cdot H_2O$, and 0.5 g/L yeast extract (Papanikolaou et al. 2002).

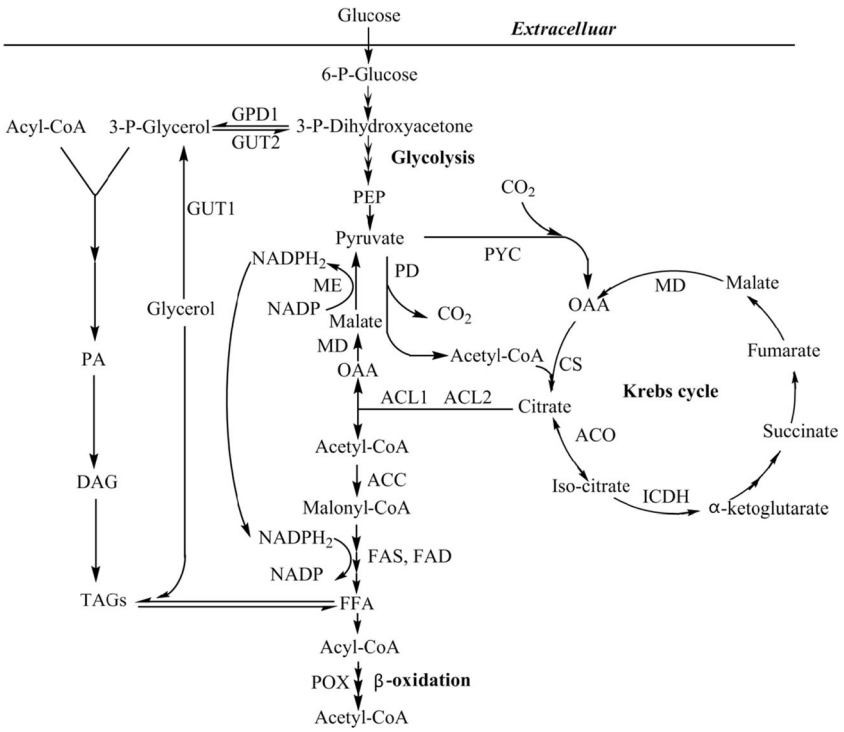

Fig. 1 The pathway for fatty acids and triglycerides (TAGs) biosynthesis in oleaginous yeasts. *PYC* pyruvate carboxylase, *ME* malic enzyme, *MD* malate dehydrogenase, *ACC* acetyl-CoA carboxylase, *FAS* fatty acid synthetase, *FAD* fatty acid dehydrogenase, *ACL* ATP citrate lyase, *CS* citrate synthetas, *ICDH* iso-citrate dehydrogenase, *ACO* aconitase, *GPD* glycerol-3-phosphate dehydrogenase, *GUT1* glycerol kinase, *GUT2* glycerol-3-phosphate dehydrogenase, *POX* acyl-CoA oxidase, *PA* phosphatidic acid, *DAG* diacylglycerol, *TAGs* triacylglycerols, *FFA* free fatty acids, *PEP* phosphoenolpyruvate, *OAA* oxaloacetic acid

Plasmids

The expression vector piNA1312-GY was constructed in this laboratory and pMD19-T simple for amplification of the target genes in *E. coli* was purchased from TaKaRa (Japan).

Isolation of DNA, restriction digestions, and transformation

The genomic DNAs were isolated from the yeasts *P. guilliermondii* Pcla22 and *Y. lipolytica* ACA-DC 50109 and DNA manipulations were carried out using standard methods (Sambrook et al. 1989). Bacterial plasmid DNA was purified using TIANprep Mini Plasmid Kit (TIANGEN). Restriction endonuclease digestions and DNA ligations were performed according to the manufacturer's recommendations. *E. coli* was transformed with plasmid DNA according to Sambrook et al. (1989). *Y. lipolytica* was transformed according to the methods described by Xuan et al. (1988).

Construction of the vectors for expression of the *PYC* gene and *ACL1* gene in *Y. lipolytica*

To express the *PYC* gene from *P. guilliermondii* Pcla22 in *Y. lipolytica* ACA-DC 50109, the primers for amplification of the *PYC* gene were designed according to the sequence of the gene (accession no. XM_001484326.1). The forward primer was P1 and the reverse primer was P2 (Table S1 in the supplementary file and Fig. 2). The genomic DNA of *P. guilliermondii* Pcla22 was used as the template for PCR. Meanwhile, in order to increase the copy numbers of the endogenous *ACL1* gene in *Y. lipolytica* ACA-DC 50109, the primers (A1 and A2) for amplification of the *ACL1* gene were designed according to the sequence of the gene (accession no. XM_504787.1). The forward primer was A1 and the reverse primer was A2 (Table S1 and Fig. 2). The genomic DNA of *Y. lipolytica* ACA-DC 50109 was used as the template for PCR. The PCR reaction system was 50.0 μL containing 1.0 μL La Taq, 5.0 μL 10× La PCR buffer II (Mg^{2+} Plus), 8.0 μL 2.5 mM dNTPs, 1.0 μL 20.0 μM each primer, 1.0 μL of 10 ng/mL the genomic DNA, sterile deionized water up to 50.0 μL. The conditions for the PCR amplification were initial denaturation at 94 °C for 10 min, denaturation at 94 °C for 1 min, annealing temperature at 55 °C for 1 min, extension at 72 °C for 4 min, final extension at 72 °C for 10 min. PCR was run for 30 cycles. The PCR products (3.6 kb) were separated by agarose gel electrophoresis and ligated into the plasmid pMD19-T simple vector. The recombinant vector was transformed into *E. coli* DH5α. The recombinant vectors carrying the PCR products were extracted from the *E. coli* transformants and purified. The purified recombinant vectors carrying the *PYC* gene or the *ACL1* gene were digested with *Sac*II and *Spe*I, and the digests were ligated into pNA1312-GY digested with the same enzymes, respectively (data not shown). The resulting plasmid carrying the *PYC* gene was designated as pINA1312-GY-*PYC* and the resulting plasmid carrying the *ACL1* gene was designated as pINA1312-GY-*ACL1* (data not shown).

Transformation and selection

The plasmids pINA1312-GY-*PYC* and pINA1312-GY-*ACL1* obtained above were digested with the enzyme *Not*I. The linear fragments carrying the *PYC* gene or *ACL1* gene were separated in agarose gel and recovered using TaKaRa Agarose Gel DNA Purification Kit Ver.3.0. The recovered linear fragments (about 9 kb) carrying the *PYC* gene or *ACL1* gene were transformed into the cells of the uracil mutant of *Y. lipolytica* ACA-DC 50109 mentioned above by lithium acetate methods (Xuan et al. 1988). The transformants obtained were spread on YNB-N5000 plates without uracil. The positive transformants were grown in the lipid production medium at 28 °C for 96 h and lipid contents in the cells of the different positive transformants were determined as described below, respectively, and *Y. lipolytica* ACA-DC 50109 was used as a control. After determination of lipid contents in the cells of over 200 positive transformants, it was found that lipid contents in the cells of the transformants P4, P77, and P103 among them only carrying the *PYC* gene were the highest while lipid contents in the cells of the transformants PA19, PA56, and PA124 among them carrying both the *PYC* gene and *ACL1* gene were the highest. Therefore, the transformants P4, P77, P103, PA19, PA56, and PA124 were used as the lipid producers, subsequently.

Confirmation of the integrated *PYC* gene and *ACL1* gene

The genomic DNAs in the transformants mentioned above and *Y. lipolytica* ACA-DC 50109 were extracted as described above and used as the templates for PCR. The DNA fragments (the *PYC* gene) were PCR amplified using the primers P1 and P2 and the DNA fragments (the *ACL1* gene) were PCR amplified using the primers A1 and A2 (Table S1). The sizes of the PCR products were estimated using the Automated Documentation and Analysis System (Gene-Genius, USA). The PCR products were sequenced by Nanjing Genscript Company (Zhao et al. 2010). The integrated *PYC* gene and *ACL1* gene were also confirmed by Southern blotting as described by Watanabe et al. (2008).

Single cell oil production at flask level

The cells of the transformants mentioned above and its wild-type strain ACA-DC 50109 were transferred to 50.0 mL of the medium (YPD medium) for the seed culture and cultivated at the shaking speed of 180 rpm and 28 °C for 24 h, respectively. Two milliliters of the culture (2.5×10^8 cells/mL) were

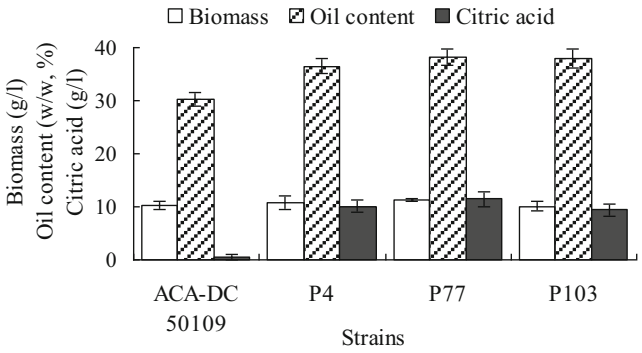

Fig. 2 Cell growth, oil contents and citric acid productions by the different *PYC* transformants and their wild-type strain ACA-DC 50109. Data are given as means±SD, n=3

transferred to 50.0 mL of the lipid production medium and the yeast cells were cultivated at the shaking speed of 180 rpm and 28 °C for 72 h. The measurements of the cell mass, lipid contents, and secreted citric acid concentration were performed as described below.

Determination of lipid contents

The total lipids in the cells (1.0 g) were extracted according to Folch et al. (1957) and Zhao et al. (2010). The extracted lipids were weighed and oil content per 100 g of cell dry weight was calculated.

Preparation of cell-free extracts

The cell-free extracts of the transformants mentioned above and *Y. lipolytica* ACA-DC 50109 were prepared as described by Zhang et al. (2013). The disrupted cells were centrifuged at 12,000×*g* for 20 min at 4 °C and the supernatants obtained were used for determination of PYC and ACL1 activities. Total protein quantity in the supernatants was determined using Coomassie brilliant blue assay (Bradford 1976). The crude intracellular enzymes heated at 100 °C for 5 min were used as the inactivated intracellular enzymes.

Measurement of pyruvate carboxylase and ATP citrate lyase activities

Pyruvate carboxylase (PYC) activity was measured according to the methods described by Chávez-Cabrera et al. (2010). A unit of PYC activity was defined as the amount of enzyme that catalyzed the oxidization of one micromole of NADH per minute. ATP citrate lyase activity (ACL1) was measured using the hydroxamate assay (Lipmann and Tuttle 1945).

Fluorescent real-time PCR

Fluorescent real-time PCR was performed according to the methods described by Liu et al. (2011). All the primers used for fluorescent real-time PCR were designed according to the corresponding gene sequences of *P. guilliermondii* Pcla22 and *Y. lipolytica* ACA-DC 50109 (Table S1). The primers P3 and P4 were designed according to the *PYC* gene sequence (GenBank accession no. XM_001484326.1) in *P. guilliermondii*, the primers A3, A4, A5, and A6 were designed according to the *ACL1* gene sequence (GenBank accession no. accession no. XM_504787.1) and the *ACL2* gene sequence (GenBank accession no. XM_503231.1), respectively, in *Y. lipolytica* ACA-DC 50109 and the primers 26s1 and 26s2 were designed according to 26S rRNA gene sequence (GenBank accession no. JQ690257.1) in *Y. lipolytica* ACA-DC 50109 (Table). The transcriptional levels of the *PYC* gene, *ACL1* gene, and *ACL2* gene in *Y. lipolytica* ACA-DC 50109 were regarded as 100 %.

Lipid production by batch fermentation

Lipid production by batch fermentation was carried out in a 10-L fermentor [BIOQ-6005-6010B, Huihetang Bio-Engineering Equipment (Shanghai) Co., Ltd]. The seed culture of the transformant PA56 grown in YPD medium at the shaking speed of 180 rpm and 28 °C for 24 h was prepared as described above. A volume of 700.0 mL of the seed culture (OD_{600nm}=18.0) was transferred to 7.0 L of the lipid production medium with initial 40.0 g/L glucose. The fermentation was performed under the conditions of an agitation speed of 200 rpm, an aeration rate of 7 L/min, a temperature of 28 °C and a fermentation period of 288 h. Only 10.0 mL of the culture was collected in the interval of 12 h and was centrifuged at 5000×*g* and 4 °C for 5 min. The lipid contents, cell mass, reducing sugar, and citric acid in the supernatant obtained were determined as described above and below. The

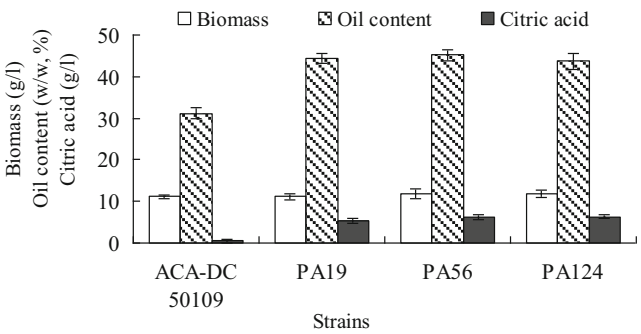

Fig. 3 Cell growth, oil contents, and citric acid productions by the different transformants and their wild-type strain ACA-DC 50109. Data are given as means±SD, n=3

cell dry weight in 5.0 mL of the culture during the 10-L fermentation was also measured.

Determination of citric acid

Citric acid (CA) was estimated by the methods described by Camp and Farmer (1967).

Determination of reducing sugar in the fermented media

Reducing sugar in the fermented media was determined by the Nelson–Somogyi method (Spiro 1966).

Measurement of cell dry weight

Cell dry weight was measured according to the methods described by Chi et al. (2001).

Results

Effects of the *PYC* gene expression on cell growth, oil contents, and citric acid production

Y. lipolytica ACA-DC 50109 has been reported to be an oleaginous yeast and significant quantities of lipids were accumulated inside the yeast cells (Papanikolaou et al. 2002). It has been reported that *P. guilliermondii* Pcla22 is an oleaginous yeast and a producer of malate whose biosynthesis is associated high pyruvate carboxylase activity (Brown et al. 2013; Wang et al. 2012). In order to know if pyruvate carboxylase can play a role in lipid biosynthesis and further enhance lipid production in this oleaginous yeast. The *PYC* gene from *P. guilliermondii* Pcla22 was integrated into the genomic DNA of the uracil mutant of *Y. lipolytica* ACA-DC 50109 as described in the "Materials and methods" section. After determination of cell mass, oil contents and the secreted citric acid by the different *PYC* transformants and their wild-type strain ACA-DC 50109, the results in Fig. 2 indicated that the lipid contents of the wild-type strain ACA-DC 50109 and the transformants P4, P7, and P103 were 30.2 % (*w*/*w*) 36.5 % (*w*/*w*), 38.2 % (*w*/*w*) and 37.9 % (*w*/*w*) while the amount of the secreted citric acids by wild-type strain ACA-DC 50109 and the transformants P4, P77, and P103 were 0.5, 10.1, 11.5, and 9.4 g/L (Fig. 2). However, their cell growth was not significantly affected (Fig. 2). The data clearly showed that after expression of the heterologous *PYC* gene, lipid biosynthesis and citric acid biosynthesis by the transformants were indeed greatly enhanced.

Effects of simultaneous expression of the *PYC* gene and *ACL1* gene on cell growth, oil contents, and citric acid production

As shown in Fig. 2, after expression of the heterologous *PYC* gene, lipid contents of the transformants P4, P7, and P103 were increased. However, the secreted citrate was also greatly increased. It can be seen from the pathway of fatty acid biosynthesis in Fig. 1 that after citric acid is transformed into acetyl-CoA and oxaloacetic acid by ACL1, the acetyl-CoA formed is the precursor for biosynthesis of fatty acids and the oxaloacetic acid formed can be further metabolized to offer $NADPH_2$ for fatty acid biosynthesis. This meant that it was very important to overexpress the *ACL1* gene in the

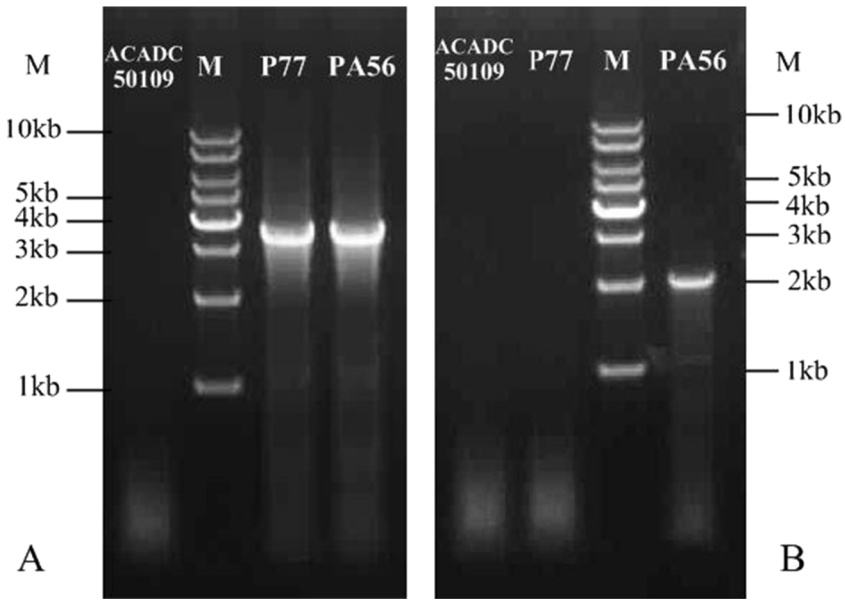

Fig. 4 PCR products from the genomic DNAs of the transformants P77, PA56, and their wild-type strain ACA-DC 50109. *M* 1 kb DNA marker

transformants in order to reduce the formed citric acid. Therefore, the *PYC* gene and *ACL1* gene were simultaneously expressed in *Y. lipolytica* ACA-DC 50109 as described in the "Materials and methods" section. It can be clearly observed from the data in Fig. 3 that compared to lipid contents (30.2 % *w*/*w*) in the cells of the wild-type strain ACA-DC 50109, those in the transformants PA19, PA56, and PA124 were 44.4 % (*w*/*w*), 45.3 % (*w*/*w*) and 43.7 % (*w*/*w*). At the same time, compared to the amount of the secreted citric acid in Fig. 2, the amount of the secreted citric acid by the transformants PA19, PA56, and PA124 was reduced to 5.4, 6.2, and 6.3 g/L, respectively (Fig. 3). However, their cell growth of was not significantly affected (Fig. 3). This demonstrated that after expression of the *ACL1* gene, lipid contents in the transformants were further increased due to the decrease in citric acid.

Confirmation of the integrated *PYC* and *ACL1* genes in the transformants

In order to know if the *PYC* gene and the *ACL1* gene have been integrated into the genomic DNA in the transformants, the PCR products from the genomic DNAs of the transformants P77 and PA56 and their wild-type strain ACA-DC 50109 were amplified. The results in Fig. 4a revealed that the PCR products (around 3.6 kb) carrying the

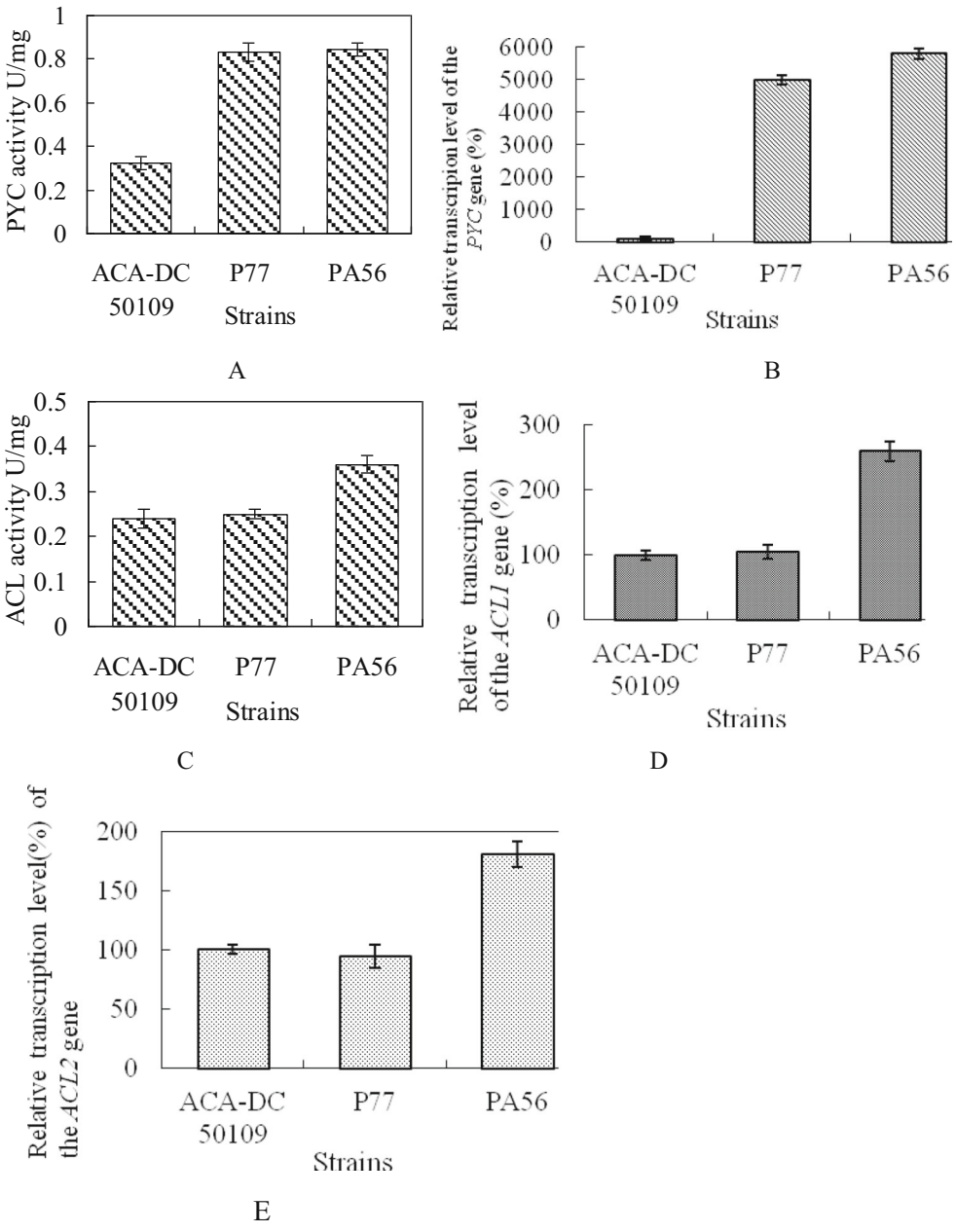

Fig. 5 PYC (**a**) and ACL (**c**) activities and transcriptional levels of the *PYC* gene (**b**), *ACL1* gene (**d**) and *ACL2* gene (**e**) in the different yeast strains. Data are given as means±SD, $n=3$

whole sequence of the *PYC* gene were PCR amplified from the genomic DNAs of both the transformants P77 and PA56 using the primers P1 and P2 (Table S1). However, no such PCR products were PCR amplified from the genomic DNAs of their wild-type strain ACA-DC 50109. At the same time, the results in Fig. 4b indicated that the PCR products (around 2.06 kb) carrying the sequence of the *ACL1* gene were PCR amplified from only the genomic DNA of the transformant PA56 using the primers A1 and C1 (Table S1). However, no such PCR products were PCR amplified from the genomic DNAs of the transformant P77 and their wild-type strain ACA-DC 50109 using the same primers. The correct integrations of the replacement constructs were also verified by PCR and Southern blot analysis (data not shown). The results demonstrated that both the *PYC* gene and the *ACL1* gene were indeed integrated into the genomic DNAs of both the transformant PA56 and the *PYC* gene was indeed only integrated into the genomic DNA of the transformant PA56 because both the expression vectors piNA1312-GY-PYC and piNA1312-GY-ACL1 contain the zeta-elements (Madzak et al. 2004).

Pyruvate carboxylase and ATP citrate lyase activities and transcriptional levels of their genes

After determination of pyruvate carboxylase activity and its gene transcriptional levels in the transformants P77, PA56, and their wild-type strain ACA-DC 50109, the results in Fig. 5a showed that PYC activities of the wild-type strain ACA-DC 50109 and the transformants (P77 and PA56) were 0.32, 0.83, and 0.84 U/mg and the results in Fig. 5b showed that the transcriptional levels of the *PYC* gene in the transformants P77 and PA56 were much higher (more than three-fold) than those of the *PYC* gene in their wild-type strain ACA-DC 50109. These results confirmed that the *PYC* gene was indeed overexpressed and pyruvate carboxylase activity was greatly enhanced in the transformants P77 and PA56. Meanwhile, the results in Fig. 5c showed that ACL activity of the transformant PA56 was 0.36 U/mg while ACL activities of the transformant P77 and the wild-type strain ACA-DC 50109 were 0.23 and 0.25 U/mg. The results in Fig. 5d indicated that the transcriptional levels of the *ACL1* gene in the transformant PA56 were much higher than those of the *ACL1* gene in the transformant P77 and their wild-type strain ACA-DC 50109. It was interesting to note that the transcription of the *ACL2* gene could be enhanced by the overexpression of *ACL1* in the yeast of *Y. lipolytica* and the relative transcriptional levels of *the gene ACL2* in the strain ACA-DC 50109, P77, and PA56 were 100, 94.3, and 180.5 %, respectively (Fig. 5e). These results also evidenced that the *ACL1* gene was indeed overexpressed and ATP citrate lyase activity was greatly enhanced in the transformant PA56.

Lipid production by the transformant PA56 during the batch fermentation

During the time where transformant PA56 was grown in the 10-L fermenter, oil content, cell growth, glucose concentration, and citric acid concentration were monitored. It can be seen from the data in Fig. 6 that oil content in the cells of the transformant PA56 and cell growth reached the highest (49.6 % *w*/*w* and 12.2 g/L) within 72 h of the fermentation, while the amount of the secreted citric acid reached the highest (2.9 g/L) within 36 h of the fermentation. Therefore, the titer of lipid was 6.21 g/L, the productivity of lipids was 0.084 g/L/h and the yield of the lipid was 0.16*g*/*g* of glucose. The results in Fig. 6 also indicated that only 1.7 g/L of reducing sugar was left in the fermented medium, suggesting that 95.75 % of the added glucose was transformed into cells, lipid, and citric acid. After the data in Figs. 3 and 6 were compared, we can see that lipid biosynthesis in the transformant PA56 was increased from 45.3 (*w*/*w*) to 49.6 % (*w*/*w*) and the amount of secreted citric acid was decreased from 6.2 to 2.9 g/L when the cell cultivation at flask level was changed to that in fermenter. This may be due to the fact that the cultivation conditions in the fermentor were better than those in the flask, leading to more citric acid which was transformed into lipid in the cells.

Discussion

In order to know the function of pyruvate carboxylase in lipid biosynthesis in *Y. lipolytica* and offer the precursor oxaloacetic acid for citric acid and lipid biosynthesis according to the pathway for fatty acids and triglycerides (TAGs) biosynthesis in oleaginous yeasts (Fig. 1), the heterologous *PYC* gene was overexpressed in the oleaginous yeast *Y. lipolytica* ACA-DC 50109 (data not shown). It was found that lipid contents in the

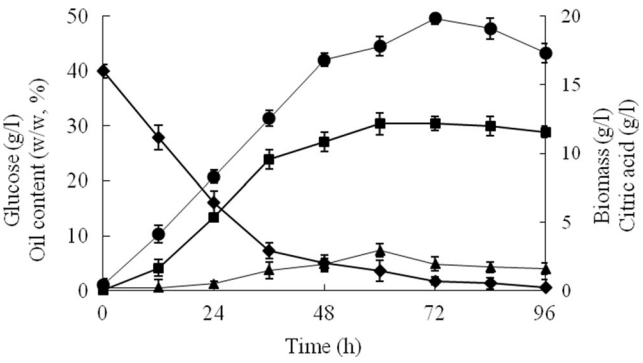

Fig. 6 The time course of oil content (*filled circle*), cell growth (*filled square*), glucose concentration (*filled diamond*), citric acid concentration (*filled upright triangle*) during the batch fermentation. Data are given as means±SD, $n=3$

genetically engineered yeast strains were enhanced (Fig. 2), However, the amount of the secreted citric acid was also increased (Fig. 2). Yin et al. (2012) showed that after overexpression of heterologous pyruvate carboxylase genes in *Y. lipolytica* WSH-Z06, α-ketoglutaric acid (KGA) production (62.5 g/L) by the genetically engineered *Y. lipolytica*-RoPYC2 was enhanced. It also has been reported that concentrations of the by-products fumarate (FA), malate (MA), succinic acid (SA), and pyruvic acid (PA) decreased significantly by overproduction of fumarase (FUM) and increased by overproduction of PYC and also of FUM and PYC simultaneously in the recombinant *Y. lipolytica* strains (Yin et al. 2012).

It has been confirmed that there is a strong correlation between the presence of ACL activity and the ability to accumulate lipid in yeasts (Ratledge 2004). It also has been known that ACL in *Y. lipolytica*, unlike some other organism, is encoded by two genes, *ACL1* (accession no. XM_504787 in NCBI) and *ACL2* (accession no. XM_503231 in NCBI) (Beopoulos et al. 2011; Liu et al. 2013). In order to transform the produced citric acid into acetyl-CoA and oxaloacetic acid for lipid biosynthesis according to the pathway for fatty acids and TAGs biosynthesis in oleaginous yeasts (Fig. 1), both the *PYC* and *ACL1* gene were simultaneously overexpressed in the oleaginous yeast *Y. lipolytica* ACA-DC 50109 (data not shown). In this case, the amount of the produced citric acid was lyzed to produce acetyl-CoA for malonyl-CoA and lipid biosynthesis according to the pathway for fatty acids and TAGs biosynthesis in oleaginous yeasts (Fig. 1). Thus, lipid contents were further increased in the genetically engineered yeast strains due to high PYC and ACL activities (Figs. 3 and 5). For example, lipid contents in the transformant PA56 was 45.3 % (*w*/*w*) and the amount of the secreted citric acid by the transformants PA56 was reduced to 6.2 g/L (Fig. 3). The results in Fig. 5e showed that after the expression of the *ACL1* gene, expression of the *ACL2* gene was also enhanced. It was possible that the enhanced *ACL2* gene expression in *Y. lipolytica* ACA-DC 50109 was due to coordinated regulation after the expression of the *ACL1* gene. This phenomenon will be elucidated in this laboratory. In contrast, in our previous studies (Liu et al. 2013), we found that after removal of some of the *ACL1* gene in citric acid-producing yeast *Y. lipolytica* SWJ-1b, lipid contents were decreased and the secreted citric acid was increased. Overexpression of the *ACL* gene in *Y. lipolytica* WSH-Z06 enhanced KGA production (increased from 36.3 to 46.7 g/L) (Zhou et al. 2012). After the *ACL* gene from *Mus musculus* was expressed in *Y. lipolytica* WSH-Z06, the ACL activity in the transformants was 11.6-fold increased and the amount of the secreted KGA was increased from 36.3 to 46.7 g/L (Zhou et al. 2012). After the simultaneous coexpression of *ACC1* gene encoding acetyl-CoA carboxylase and *DGA1* gene encoding diacylglycerol acyltransferase in the wild-type *Y. lipolytica* W29 strain (ATCC20460), lipid content in the ACC1+DGA1 transformant reached to 41.4 % (*w*/*w*), demonstrating synergistic effects of *ACC1+DGA1* coexpression (Tai and Stephanopoulos 2013). Furthermore, after lipogenesis capability in *Y. lipolytica* was drastically increased by multiplexing genomic engineering, the saturated cells containing upwards of 90 % lipid content and titers exceeding 25 g/L lipids were attained (Blazeck et al. 2014). The oleaginous yeast *Y. lipolytica* was also genetically engineered to produce eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Xue et al. 2013).

During 10-L fermentation, oil content in the cells of the transformant PA56 and cell growth reached the highest (49.6 % *w*/*w* and 12.2 g/L) within 72 h of the fermentation while the amount of the secreted citric acid reached the highest (2.9 g/L) within 36 h of the fermentation (Fig. 6). After the *MIG1* gene in the same oleaginous yeast *Y. lipolytica* ACA-DC 50109 was disrupted and the disruptant M25 obtained had more lipid bodies than its parent yeast strain and the disruptant M25 contained 48.7 % (*w*/*w*) of oil based on its cell weight while the parent yeast strain only contained 36.0 % (*w*/*w*) of oil (Wang et al. 2013). In a *Y. lipolytica* Po1d genetic background, *GPD1* (encoding the catabolic dehydrogenase) overexpression, *GUT2* (encoding the anabolic dehydrogenase) inactivation or both mutations together result in 1.5-, 2.9-, and 5.6-fold respective increases in the level of glycerol-3-phosphate (G3P), leading to an increase of TAG accumulation (Dulermo and Jean-Marc 2011). At the same time, deletion of *POX1-6* or *MFE1* genes (encoding β-oxidation pathway of fatty acids) increased TAG and free fatty acids content (Dulermo and Jean-Marc 2011). It has been thought that in order to increase the acyl-CoA support to regenerate the $NADPH^+$ cofactor supply and enhance malonyl-CoA offer, ATP citrate lyase (ACL1), malic enzyme (ME) and acyl-CoA carboxylase (ACC1/2) overexpression may increases lipid accumulation in oleaginous yeasts (Beopoulos et al. 2011). As mentioned above, the ACC1+DGA1 transformant grown in a 2-L bioreactor fermentation achieved 61.7 % lipid content after 120 h. The overall yield and productivity were 0.195 *g*/*g* and 0.143 g/L/h, respectively, during the lipid accumulation phase of the fermentation (Tai and Stephanopoulos 2013). Therefore, this is the first time to report that overexpression of both the *PYC* gene and *ACL1* gene in the oleaginous yeast *Y. lipolytica* ACA-DC 50109 can enhance lipid biosynthesis.

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