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Overexpression of *ACC* gene from oleaginous yeast *Lipomyces starkeyi* enhanced the lipid accumulation in *Saccharomyces cerevisiae* with increased levels of glycerol 3-phosphate substrates

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The conversion of acetyl-CoA to malonyl-CoA by acetyl-CoA carboxylase (ACC) is the rate-limiting step in fatty acid biosynthesis. In this study, a gene coding for ACC was isolated and characterized from an oleaginous yeast, *Lipomyces starkeyi*. Real-time quantitative PCR (qPCR) analysis of *L. starkeyi* acetyl-CoA carboxylase gene (*LsACC1*) showed that the expression levels were upregulated with the fast accumulation of lipids. The *LsACC1* was co-overexpressed with the glycerol 3-phosphate dehydrogenase gene (*GPD1*), which regulates lipids biosynthesis by supplying another substrates glycerol 3-phosphate for storage lipid assembly, in the non-oleaginous yeast *Saccharomyces cerevisiae*. Further, the *S. cerevisiae* acetyl-CoA carboxylase (*ScACC1*) was transferred with *GPD1* and its function was analyzed in comparison with *LsACC1*. The results showed that overexpressed *LsACC1* and *GPD1* resulted in a 63% increase in *S. cerevisiae*. This study gives new data in understanding of the molecular mechanisms underlying the regulation of fatty acids and lipid biosynthesis in yeasts.

Key words: *Lipomyces starkeyi*; acetyl-CoA carboxylase; glycerol 3-phosphate dehydrogenase; fatty acid biosynthesis; *Saccharomyces cerevisiae*

Rising crude oil prices, unsustainable supply, and concerns over climate change have become increasingly important to search for renewable substitutes. Biodiesel is one of such alternative fuels, but using plant oils for producing biodiesel is a costly and unsustainable path.[1)] Microbial oils, as feedstock for producing biodiesel, have great advantages over that of plant oils, such as adaptability to diverse feedstocks, reduced land requirements, short life cycle, and easier scale-up.[2)] Using microbial oils as feedstock for producing biodiesel has attracted great interests in industry.

Some oleaginous yeast species, such as *Lipomyces* sp., *Rhodosporidium* sp., and *Yarrowia* sp., can accumulate intracellular lipids as high as 50% of their dry cell weight (DCW).[3)] In particular, *Lipomyces starkeyi*, originally isolated from soil, accumulates lipids up to 60% of its dry weight and the fatty acid composition highly similar to vegetable oils.[2,4,5)] Furthermore, *L. starkeyi* is capable of utilizing some low-cost raw materials to produce microbial oils, such as sewage sludge, fishmeal wastewater, sunflower meal hydrolysate, potato starch wastewater, and willow wood sawdust hydrolysate.[4,6–9)] Also, the complete genome information of *L. starkeyi* has been released and available to the public (http://genome.jgi.doe.gov/Lipst1_1/Lipst1_1.home.html), and the gene transformation systems for *L. starkeyi* have been recently established.[10,11)] These studies provide great opportunities to further engineer its genetic background for producing targeted strains for scale-up in industry. Although microbial oleaginity is known for decades, knowledge on the mechanisms controlling lipid accumulation in oleaginous yeasts remains limited. Hence, there is an immediate need for investigating the genetic mechanisms controlling lipid accumulation in oleaginous yeasts, specifically, identifying the key rate-limiting enzymes responsible for lipid biosynthesis and characterizing the functions of genes encoding these rate-limiting enzymes.

The biosynthesis process of microbial oils (in the form of triacylglycerols, TAGs) involves in *de novo*

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Abbreviations: ACC, acetyl-CoA carboxylase; TAG, triacylglycerol; G3P, glycerol 3-phosphate; GPD, glycerol 3-phosphate dehydrogenase; DCW, dry cell weight; DHAP, dihydroxyacetone phosphate; GPP, glycerol 3-phosphatase.

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fatty acid synthesis and TAG assembly through Kennedy pathway at the endoplasmic reticulum and lipid bodies.[12] The TAG assembly consumes acyl-CoAs using substrate glycerol 3-phosphate (G3P) with diverse enzymes. There, G3P is formed from sugars by the reduction of dihydroxyacetone phosphate (DHAP) concomitant with NADH oxidation catalyzed by cytosolic glycerol 3-phosphate dehydrogenase (GPD) (E.C.1.1.1.8).[13] The subsequent dephosphorylation of G3P leads to the formation of glycerol by glycerol 3-phosphatase (GPP) (E.C. 3.1.3.21) (Fig. 1).[14]

The process of *de novo* fatty acid synthesis produces acyl-CoAs in cytosol. Acetyl-CoA carboxylase (ACC) catalyzes the carboxylation of acetyl-CoA to form malonyl-CoA which is the first committed step in fatty acyl-CoAs biosynthesis and has been postulated to be a rate-limiting step in the process of cytosolic TAG synthesis, as shown in Fig. 1. Previous studies have described the successful genetic improvement in lipid accumulation by modifying the activity of ACC.[15–18] The genes coding for the ACC enzyme (EC 6.4.1.2) have been authenticated from several organisms.[19,20] In comparison with prokaryotes and plastids of most plants, mammals, fungi, and yeasts contain multifunctional ACC enzymes, which possess three functional components biotin carboxylase (BC), biotin carboxyl carrier protein (BCCP), and carboxyl transferase (CT) in a single polypeptide. Furthermore, there are two isoforms of the ACC: cytosolic and mitochondrial enzymes.[21,22] However, little is known about the function of ACC in regulating lipid biosynthesis from oleaginous and non-oleaginous yeasts.

In this study, we cloned the full-length *L. starkeyi* acetyl-CoA carboxylase gene (*LsACC1*) from oleaginous *L. starkeyi* and characterized its sequence structure. In particular, we performed the functional analysis of *LsACC1* by transferring it into non-oleaginous *Saccharomyces cerevisiae* with the overexpression of the endogenous *S. cerevisiae* glycerol 3-phosphate dehydrogenase gene (*GPD1*). Also, we compared the functions of *LsACC1* and the endogenous *S. cerevisiae* acetyl-CoA carboxylase gene (*ScACC1*) in *S. cerevisiae*. The current study provides new data in understanding of the molecular mechanisms underlying the regulation of fatty acids and lipid biosynthesis in yeasts.

Materials and methods

Organisms, growth conditions, and plasmids. *L. starkeyi* AS 2.1560 obtained from the China General Microbiological Culture Collection Center (CGMCC) was used as a genetic resource and was maintained as described previously.[3] It was maintained at 4 °C on YPD agar slants containing 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose, and 1.5% (w/v) agar and subcultured twice a month. The nutrient-rich medium used for the preculture was YPD medium. The nitrogen-deficient medium used for lipid production contained: KH_2PO_4 (12.5 g/L), Na_2HPO_4 (1 g/L), $(NH_4)_2SO_4$ (0.1 g/L), $MgSO_4{\cdot}7H_2O$ (2.5 g/L), $CaCl_2{\cdot}2H_2O$ (0.25 g/L), yeast extract (0.9 g/L), glucose (36 g/L), and 0.625 mL trace element solution. The trace element solution contained (g/L): $FeSO_4{\cdot}7H_2O$ 16, $MnSO_4{\cdot}H_2O$ 4, $Al_2(SO_4)_3{\cdot}18H_2O$ 5.52, $CoCl_2{\cdot}6H_2O$ 2.92, $ZnSO_4{\cdot}7H_2O$ 0.8, $Na_2MoO_4{\cdot}2H_2O$ 0.8, $CuCl_2{\cdot}2H_2O$ 0.4, H_3BO_3 0.2 and KI 1.6 in 5 N hydrochloric acid. The *S. cerevisiae* YA103 (MATa *gpp1*::*kanMX4 gpp2*::*HIS3*)[23] was used as the recipient for expression of the cloned gene *LsACC1*. The yeast was grown in YPD medium at 30 °C. *Escherichia coli Trans*1-T1 (TransGen, Beijing, China) was used for plasmid propagation and was grown at 37 °C in a Luria–Bertani medium (LB) supplemented with 100 μg/mL of ampicillin when necessary. The pX2-GAL vector contains the ampicillin resistance gene, *GAL1* promoter for inducing expression, and the *LEU2* selectable marker. The pWXY1.0 contains the ampicillin resistance gene, *GAL1* promoter for inducing expression, and the *TRP1* selectable marker.[24] The two vectors were used for gene expression in yeast. The p*EASY*™-T5 Zero Cloning Vector (TransGen, Beijing, China) was used for DNA sequencing. All media were autoclaved at 121 °C for 15 min.

Genetics techniques. All basic DNA manipulation procedures were performed according to Sambrook and Russell.[25] Primers used in this study synthesized by Generay Corporation (Shanghai, China) are listed in Table 1. All sequencing was performed at the Beijing Genome Institute (Shenzhen, China), and all plasmids were prepared by High-purify Plasmid DNA Miniprep Kit (Bioteke, China).

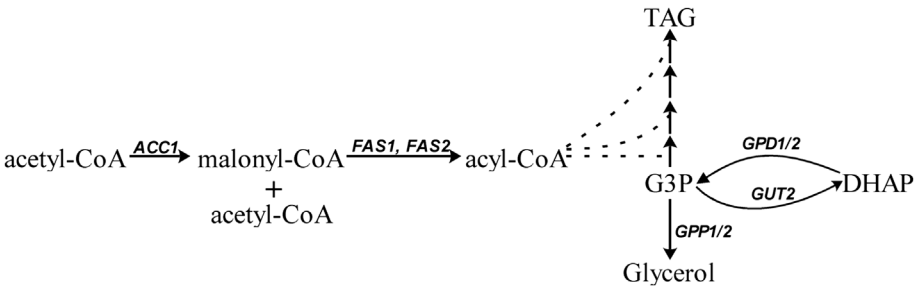

Fig. 1. Scheme of metabolic pathways and enzymes of *S. cerevisiae*.

Abbreviations: DHAP, dihydroxyacetone phosphate; G3P, glycerol 3-phosphate; TAG, triacylglycerol; *ACC1*, acetyl-CoA carboxylase; *FAS1*, *FAS2*, fatty acid synthase; *GPD1/2*, glycerol 3-phosphate dehydrogenase; *GPP1/2*, glycerol 3-phosphatase; *GUT2*, mitochondrial FAD-dependent glycerol 3-phosphate dehydrogenase.

Table 1. Primers used in this study.

Primers	Sequences 5′ to 3′
LsACC1-F1	TGGGCNGGNTGGGGNCAYGC
LsACC1-R1	GCRAADATRTGNCCRAAYTG
LsACC1-F2	TCYTTYGGNCCNCARGARGA
LsACC1-R2	GCRTACATYTCCATYTGRTC
LsACC1-F3	CACGCCGCTGTTCGACTT
LsACC1-R3	GAGATAAGCACCAATACCAACCG
LsACC1	ATCTCATCGGACACGGTGACG
LsACC2	TGCGTGCTGGGCAACTATGGT
LsACC3	TTTGGGTTTTCGGAGGCGTGAC
3SP1	GAAATGGTCGTCCAGGAAGCC
3SP2	TCTGGCGGTCAGCGTGATATGTA
3SP3	ATTTTCGTCTACATTCCGCCGCAT
AD1	NTCGASTWTSGWGTT
AD2	NGTCGASWGANAWGAA
AD3	WGTGNAGWANCANAGA
AD4	TGWGNAGWANCASAGA
AD5	AGWGNAGWANCAWAGG
AD6	CAWCGICNGAIASGA
LsACC1-1	CAGGAGACCCTTAGCGGCT
LsACC1-2	CGTTGATACCAGCCTCAGCAT
LsactinF	ACCGTGAGAAGATGACCCAGAT
LsactinR	CACCATCACCAGAATCAAGCAC
GPD1-1	gattctagaactagtggatcccccATGTCTGCTGCTGCTGATAGAT
GPD1-2	cttgatatcgaattcctgcagcccCTAATCTTCATGTAGATCTAATTCTTC
LsACC1-3	aaggagaaaaaaccccggattctagATGTCTGCTGCGGCCAGT
LsACC1-4	agcgtgacataactaattacatgacTTAATTAGAAGCCTGCTTGAAA
ACC1-1	aaggagaaaaaaccccggattctagATGAGCGAAGAAAGCTTATTC
ACC1-2	agcgtgacataactaattacatgacTTATTTCAAAGTCTTCAACAATTT
GPD1-3	GCTCAAGAACACTGGTCTGAAAC
GPD1-4	AAGTAAGGTCTGTGGAACAAGGC
ACC1-3	AGGAACTGGAATGGACCGAG
ACC1-4	TTGATGCTTCGCCTACCTGAT
actinF	CACCATGTTCCCAGGTATTGC
actinR	TGGACCACTTTCGTCGTATTCT

Notes: Codes for degenerate bases in primers: R (A or G), W (A or T), S (C or G), Y (C or T), D (G, A, or T), N (A, C, G, or T), I is hypoxanthine. The lowercase letter showed overlapping sequence used in *E. coli* recombination.

Culture conditions of L. starkeyi. Preculture of *L. starkeyi* AS 2.1560 was made in YPD medium at 30 °C for 72 h. Lipid production was performed in 250-mL Erlenmeyer flasks containing 50 mL of the nitrogen-deficient medium. The cultures were initiated with $OD_{600} = 0.4$ and incubated in a rotary shaker at 200 rpm, 30 °C. Samples (2 mL each) were withdrawn at 12 h (sample "12-h"), 48 h (sample "48-h") and 96 h (sample "96-h"). The collected samples were flash frozen in liquid nitrogen and stored at −80 °C until total RNA extraction. Parallel samples were also collected for biomass and lipid content determination as described below.

DNA extraction. *L. starkeyi* AS 2.1560 was grown in 50 mL YPD media at 30 °C on a rotary shaker until OD_{600} reached 5.0. A sample (2 mL) of cells was harvested by centrifugation (5000 × *g*, 5 min) and was suspended in 500 µL extraction buffer (2% Triton X-100, 100 mM NaCl, 10 mM Tris–HCl, 1 mM EDTA, 1% SDS, 0.1% β-mercaptoethanol, pH 8.0). Lysis of yeast cells was achieved by addition of acid-washed 0.4–0.6 mm diameter glass beads, and continuous vortexing for 10 min at the highest speed utilizing a vortex MM400 (Retsch, Germany). The suspension of disrupted cells was purified by adding equal volumes of phenol–chloroform–isoamylalcohol (25:24:1), followed by isopropanol precipitation. Subsequently, the pellet was washed by 70% ethanol and dissolved in 50 µL TE containing 50 µg/mL RNaseA. The DNA concentration was measured using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA).

Cloning of the LsACC1 gene and sequencing. The part sequences of 5′ and 3′ terminal of the *LsACC1* gene were amplified by PCR with two pairs of degenerated primers (LsACC1-F1, LsACC1-R1, and LsACC1-F2, LsACC1-R2 in Table 1), which were designed based on conserved amino acid sequences WAGWGHA, QFGHIFA and SFGPQED, DQMEMYA, respectively. After getting the partial sequence of *LsACC1*, the unknown sequences between 5′ and 3′ terminal were amplified by the primers LsACC1-F3 and LsACC1-R3. To clone the full-length *LsACC1* gene, the other unknown flanking sequences were amplified by TAIL-PCR.[26] The specific primers for the 5′ and 3′ terminal sequences were LsACC1, LsACC2, LsACC3 and 3SP1, 3SP2, 3SP3, respectively. The arbitrary degenerate (AD) primers were AD1, AD2, AD3, AD4, AD5, and AD6 (Table 1), and the PCR cycle conditions were the same as those described by Liu and Chen.[26] Amplified flanking sequences were cloned into p*EASY*™-T5 Zero Cloning Vector and validated by

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sequencing. The full-length sequence of *LsACC1* was obtained through all assembly sequences. The sequence of the *LsACC1* gene has been deposited in the GenBank database under the accession no. KJ948118.

Sequence analyses and real-time quantitative PCR (qPCR). Nucleotide sequences and amino acid sequences were primarily analyzed using Vector NTI advance 11.0 software (Invitrogen, USA). Multiple sequence alignment and construction of the phylogenetic tree were performed using the neighbor-joining criteria in MEGA5 with 1000 bootstrap replicates.[27)]

The collected samples of 12 h, 48 h, and 96 h were disrupted with glass beads, and total RNA was extracted following the standard protocol of TRIZOL reagent (Invitrogen, USA). The RNA samples were quantified by NanoDrop 2000 spectrophotometer. QPCR was performed to validate the transcription levels of *LsACC1*. Total RNA (1 μg) was reverse transcribed into cDNA using PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Dalian, China). Relative expression levels of *LsACC1* were quantified using SYBR® *Premix Ex Taq*™ (Tli RNaseH Plus) (TaKaRa, Dalian, China) with a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). *L. starkeyi actin* gene (*LsACT1*) as the endogenous reference gene was used to normalize the amount of the total mRNA in all samples.

Plasmid construction and genes expression in yeast. To express the *GPD1*, the open reading frame (ORF) of *GPD1* was amplified from *S. cerevisiae* genomic DNA using primers GPD1-1 and GPD1-2 (Table 1). The *GPD1* was ligated to the SmaI site of pX2-GAL to yield pX2-GPD1 by homologous recombination in *E. coli Trans*1-T1.[28)] To express the *LsACC1* and *ScACC1*, the coding sequences (CDSs) of the genes were amplified from the cDNA of *L. starkeyi* AS 2.1560 and *S. cerevisiae* genomic DNA using primers LsACC1-3, LsACC1-4 and ACC1-1, ACC1-2, respectively (Table 1). The *LsACC1* and *ScACC1* were ligated to the XhoI/KpnI site of pWXY1.0 to yield pLSA1.0 and pSCA1.0 by homologous recombination in *E. coli Trans*1-T1, respectively.[28)]

The plasmid was transformed into YA103 via the lithium acetate method as described previously.[29)] The transformed cells were selected on SC-L, SC-T, or SC-TL agar plates (0.67% bacto-yeast nitrogen base without amino acids, 2% glucose and a mixture of appropriate nucleotide bases and amino acids without leucine, tryptophan or tryptophan and leucine, 2% agar) at 30 °C for 3–4 days. Expression of recombinant genes was induced by cultivation of the transformants in nitrogen-limited SD-L, SD-T, or SD-TL medium containing 0.17% bacto-yeast nitrogen base without amino acids and ammonium sulfate, 2% galactose and a mixture of appropriate nucleotide bases and amino acids without leucine, tryptophan or tryptophan and leucine, 1 g/L ammonium sulfate.[30)] To test whether overexpression of the *GPD1* gene can accumulate lipid in yeast, the transgenic yeasts with pX2-GAL or pX2-GPD1 were fed on oleic acid (C18:1), respectively. Oleic acid was added into the SD-L media to a final concentration 2 mg/mL with 2 mg/mL Tween-20 for making oleic acid dissolve completely. The cultures were grown to the stationary phase at 30 °C.

Determination of intracellular G3P in S. cerevisiae. To extract the intracellular G3P from *S. cerevisiae*, an aliquot of cultures containing approximately 1.5×10^9 cells was harvested by centrifugation ($5000 \times g$ for 5 min) and was suspended in 1 mL phosphate buffer (pH 7.0). Lysis of yeast cells was achieved by addition of acid-washed 0.4–0.6 mm diameter glass beads, and continuous vortexing for 10 min at the highest speed utilizing a vortex MM400. The resulting suspension was cleared by centrifugation at $13000 \times g$ for 10 min, and the G3P was measured using an ultrahigh performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) method. The samples were separated on a Waters Acquity UPLC type BEH C18 (100 mm × 2.1 mm, 1.7 μm) analytical column with a mobile phase of A (methanol) and B (5 mM formamide solution) (v/v = 10:90). The flow rate was set at 0.2 mL/min, and the injection volume was 1 μL. For operation in the MS/MS mode, the electrospray ion source was operated with negative ion modes in a single run. The ESI parameters were set as follows: source temperature, 150 °C; desolvation temperature, 250 °C; desolvation gas flow, 10 L/h. The optimized cone voltages were 20 V. The multiple reaction monitoring mode using specific precursor/product ion transitions was employed for quantification. The molecular ions of G3P were fragmented at collision energy of 15 eV using argon as collision gas. Ion detection was performed by monitoring the transition: m/z 170.95 → 96.96 → 78.97.

The standards G3P (35%, w/w, AKSci, USA) were dissolved in methanol to make stock solutions at a concentration of 437.5, 43.75, and 4.375 μg/mL, respectively. The working solutions were diluted by methanol to construct calibration curves at the designated concentration ranges, and then, the samples were analyzed by UPLC-MS/MS.

Determination of lipid content. Total lipids of cultured cells were extracted by the method of Zhao et al. [3)] Briefly, yeast cells were harvested from 20 mL culture broth and dried at 105 °C to constant weight to give the DCW (g/L). In parallel, yeast cells harvested from 20 mL culture medium were digested with 4 M HCl at 78 °C for 1 h, and then, the total lipids were extracted using the chloroform/methanol (1:1, v/v) method. The extracts were washed with 0.1% NaCl and distilled water, dried over anhydrous Na_2SO_4, and evaporated *in vacuo*, and the residue was dried at 105 °C overnight to give the total cellular lipid. The lipid content (%) was expressed as gram lipid per gram DCW.

RNA extraction and qPCR analysis. *S. cerevisiae* YA103 cells of stationary phase were harvested by

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centrifugation, disrupted with glass beads, and RNA was extracted by the method described in section "Sequence analyses and real-time quantitative PCR (qPCR)". QPCR was performed to validate the transcription levels of *GPD1* and *ScACC1*, respectively. Total RNA (1 μg) was reverse transcribed into cDNA using PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time). Relative expression levels of *GPD1* and *ScACC1* were quantified using SYBR® *Premix Ex Taq*™ (Tli RNaseH Plus) with a CFX96 Real-Time PCR Detection System, respectively. *ACT1* as the endogenous reference gene was used to normalize the amount of the total mRNA in all samples.

Results and discussion

Identification of the LsACC1 gene

The partial *LsACC1* gene was cloned using degenerate PCR amplification, and we got two fragments about 1094 and 1264 bp. The full-length sequence of *LsACC1* gene (7786 bp), including the complete ORF (from position 207 to 7138 bp, total 6932 bp) was obtained using TAIL-PCR approach as described in method section.

Multiple alignment analysis showed that amino acids sequences of *LsACC1* exhibited a high homology BC, BCCP, and CT domains with other acetyl-CoA carboxylase gene (*ACC*) from several organisms tested, particularly in regions of the conserved amino acid sequences for the ATP-, biotin-, carboxybiotin-, and acetyl-CoA-binding sites (Fig. 2(A)). The CDS of *LsACC1* gene encoding a protein of 2234 amino acids spanned 6705 bp with a calculated molecular mass of 248 kDa and an isoelectric point (pI) of 6.078. The subsequent sequence analysis showed an ORF composed of 6932 bp containing four putative introns. These introns were 66, 57, 56, and 48 bp in length and were located at nucleotides +124 to +189, +2833 to +2889, +2972 to +3027, and +4522 to +4569, respectively, relative to the initiation codon (ATG). In the NCBI database, amino acid alignment of *L. starkeyi* acetyl-CoA carboxylase protein (LsACC1) with other proteins presented that it had high homology with other yeasts ACC proteins (ACCs) that correspond to the result of phylogenetic analysis (Fig. 2(B)). The amino acid sequence identities of the LsACC1 to the ACCs of yeasts, animals, and cytosol of plants are 65–70, 47, and 41–45%, respectively.

Three conserved catalytic domains BC, BCCP, and CT existing in the *LsACC1* were found using the NCBI conserved domain search[31] and similar to the previous reports for *ACC* from other eukaryotic organisms, such as animals and fungi in addition to *ACC* in the cytosol of plants.[32] Moreover, a common sequential arrangement of the domains, NH_2-BC-BCCP-CT-COOH, was also found in the LsACC1, in which the BC (residues 39–550) and BCCP (residues 614–749) domains were located at the N-terminus, whereas the CT domain was located in the C-terminal sequence from residues 1573 to 2124 (Fig. 2(A)). Within the BC domain, a putative binding site for ATP (residues 226–245) was found. In addition, the two proline residues located 24 and 32 residues upstream from the putative biotin-binding site may be involved in the formation of a hinge region that, in principle, promotes the movement of the carboxybiotin.[33] Furthermore, the highly conserved biotin-binding site, EVMKM, was located from residues 714 to 718 and represents the region of the BCCP

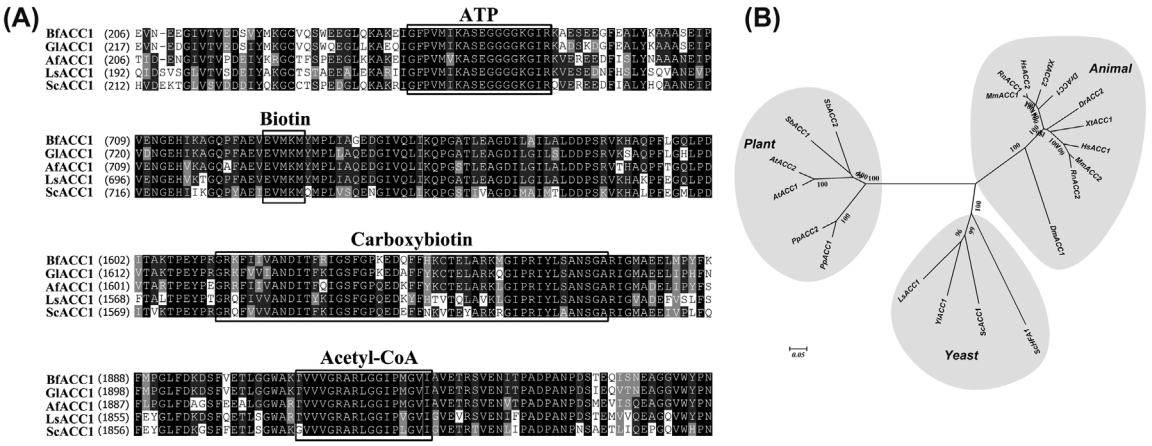

Fig. 2. The structural organization of the conserved domains in ACC and phylogenetic relationships of multi-domain ACC proteins (ACCs) from various organisms.

Notes: (A) The deduced amino acid sequence of the ATP-, biotin-, carboxybiotin-, and acetyl-CoA-binding sites of LsACC1 (*L. starkeyi* acetyl-CoA carboxylase) was aligned with those from BfACC1 (*Botryotinia fuckeliana* acetyl-CoA carboxylase), GlACC1 (*Glarea lozoyensis* acetyl-CoA carboxylase), AfACC1 (*Aspergillus fumigatus* acetyl-CoA carboxylase), and ScACC1 (*S. cerevisiae* acetyl-CoA carboxylase) in box. Identical amino acid residues are shaded in black, while conserved residues are shaded in gray. (B) The plant, animal, and yeast ACCs are in the same branch, respectively. AtACC1, *Arabidopsis thaliana* NP_001185143; AtACC2, *A. thaliana* NP_174850; DmACC1, *Drosophila melanogaster* NP_001097277; DrACC1, *Danio rerio* XP_005165611; DrACC2, *D. rerio* XP_005165252; HsACC1, *Homo sapiens* NP_942131; HsACC2, *H. sapiens* O00763; LsACC1, *L. starkeyi* KJ948118; MmACC1, *Mus musculus* Q5SWU9; MmACC2, *M. musculus* NP_598665.2; PpACC1, *Physcomitrella patens* XP_001754424; PpACC2, *P. patens* XP_001773073; RnACC1, *Rattus norvegicus* NP_579938; RnACC2, *R. norvegicus* CBU93243; SbACC1, *Sorghum bicolor* XP_002446178; SbACC2, *S. bicolor* XP_002442242; ScACC1, *Saccharomyces cerevisiae* NP_014413; ScHFA1, *S. cerevisiae* NP_013934; XtACC1, *Xenopus tropicalis* NP_001131086; XtACC2, *X. tropicalis* XP_004911956; YlACC1, *Yarrowia lipolytica* XP_501721.

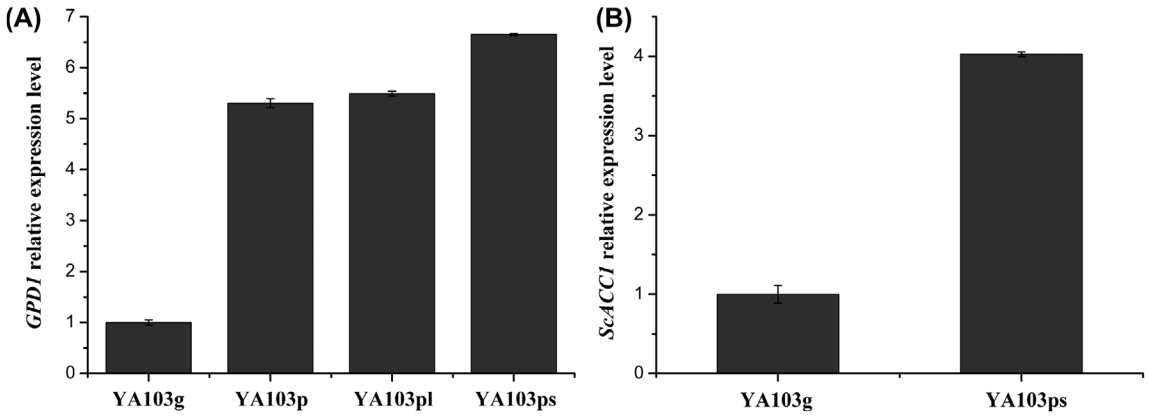

Fig. 3. Relative expression levels of *GPD1* and *ScACC1* in transgenic yeasts.
Notes: (A) The estimation of relative expression levels was based on $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T = (C_T$ gene of interest—C_T internal control) overexpressing *GPD1* gene of YA103p, YA103pl and YA103ps—(C_T gene of interest—C_T internal control) control YA103 g. (B) The estimation of relative expression levels was based on $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T = (C_T$ gene of interest—C_T internal control) overexpressing *ScACC1* gene of YA103ps—(C_T gene of interest—C_T internal control) control YA103g. C_T represents the cycle number at which a sample reaches a predetermined threshold signal value for the specific target gene. All the experiments were performed in triplicate.

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domain. The binding sites for carboxybiotin and acetyl-CoA are also present in the CT domain at residues 1578–1628 and 1873–1892, respectively.

Comparative analysis of the primary structure of ACCs has demonstrated a highly conserved organization of the multidomains underpinning their relevant catalytic functions. With regard to sequence homology, the cloned gene from *L. starkeyi* resembles the fungal *ACC* involved in malonyl-CoA production. In addition, the absence of an N-terminal extension required for a canonical mitochondrial targeting signal indicated that the LsACC1 protein is categorized as a multi-domain ACC belonging to the small family of biotin-dependent carboxylase.

Accumulation of G3P by overexpression of GPD1 in S. cerevisiae did not improve lipid accumulation

As we know, G3P is a key intermediate in biosynthesis of TAG, and YA103 strain where GPP activity is completely abolished (deletion of *GPP1* and *GPP2*) has been already shown to accumulate G3P.[23] So we used the YA103 strain bearing the empty vector pX2-GAL (YA103g strain) as a control instead of the untransformed strains in order to enable direct comparison with the same strains bearing the plasmid pX2-GPD1 for *GPD1* overexpression (YA103p strain). QPCR analysis of the transgenic yeasts confirmed five-fold expression levels of *GPD1* to control levels (Fig. 3(A)). An obvious increase (1.5-fold) of G3P was obtained in strain YA103p compared with YA103g (Fig. 4). Our results were similar to Nguyen et al.[34] To determine the influence of accumulation of G3P on cell growth and lipid accumulation in YA103, the DCW and lipid productivity of YA103g and YA103p were compared in SD-L medium. However, no differences concerning the lipid content could be detected between both strains (Table 2), most probably due to the lack of a concomitant excess of fatty acids, the second component needed for TAG synthesis.

This hypothesis was experimentally proven when YA103g and YA103p were cultured on SD-L medium by feeding external oleic acid (Table 2). The YA103p

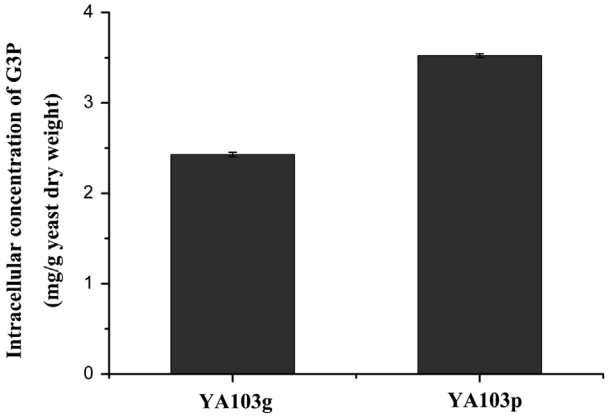

Fig. 4. Intracellular accumulation of G3P in transgenic yeasts.
Notes: Cells were cultivated in SD-L in shake flasks and harvested for extraction of G3P in the mid-log phase of growth. Data shown are the average values from three independent experiments including the standard deviations.

overexpressing *GPD1* gene led to a lipid content of 13.0%, a 37% increase from a lipid content of 9.5% in the background strain YA103g. Notably, lipid productivity also increased from 292.5 to 340 mg/L. So, in the *S. cerevisiae*, the fatty acids were the main element for TAG synthesis rather than G3P. We can overexpress the *ACC* gene which was the rate-limiting enzyme in fatty acid biosynthesis and increase total lipid in YA103.

LsACC1 expression analysis and overexpression of the gene in S. cerevisiae

The yeast *L. starkeyi* AS 2.1560 was cultured in a nitrogen-deficient medium with a C/N molar ratio of 160. The time courses of DCW and the lipid content of the bath culture are shown in Fig. 5(A). DCW and lipid contents for the 12-h, 48-h, and 96-h samples were 0.55 g/L and 17.3%, 7.21 g/L and 33.8%, and 12.97 g/L and 45.3%, respectively. Our findings indicate that the 12-h sample was suitable to represent the early stage of lipid production, and the 48-h sample was

Table 2. Lipid content, lipid productivity, and DCW of transgenic *S. cerevisiae.*

Strain	Plasmid(s)	Lipid content (%)	Lipid productivity (mg/L)	DCW (g/L)
YA103g	pX2-GAL	5.9 ± 0.5	235.0 ± 17.6	4.02 ± 0.10
YA103p	pX2-GPD1	5.9 ± 0.1	243.3 ± 4.4	4.12 ± 0.02
YA103l	pLSA1.0	7.3 ± 0.1	287.5 ± 2.5	3.93 ± 0.08
YA103g + FA	pX2-GAL	9.5 ± 0.1	292.5 ± 13.6	3.09 ± 0.14
YA103p + FA	pX2-GPD1	13.0 ± 0.4	340.0 ± 9.6	2.61 ± 0.04
YA103pl	pX2-GPD1 + pLSA1.0	9.6 ± 0.8	308.3 ± 17.6	3.22 ± 0.15
YA103ps	pX2-GPD1 + pSCA1.0	10.2 ± 0.9	315.0 ± 23.6	3.08 ± 0.03

Notes: Lipid content is expressed as lipid productivity (mg/L)/DCW (mg/L) × 100. Results are the mean ± standard deviations of triplicate. FA: oleic acid.

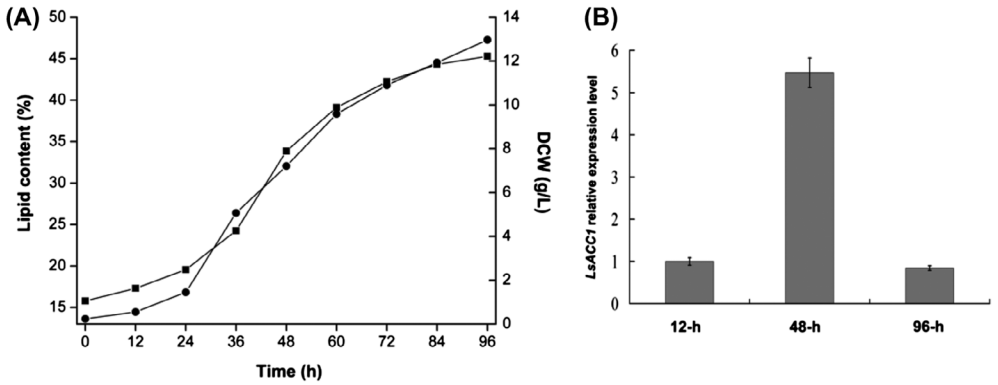

Fig. 5. The lipid content and DCW during cell growth of *L. starkeyi* AS 2.1560 in the nitrogen-deficient medium and qPCR analysis of *LsACC1* expression in 12-h, 48-h, and 96-h samples.

Notes: (A) Symbols: ■ denotes the lipid content (%); ● denotes the accumulation of DCW. (B) The estimation of relative expression levels was based on $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T = (C_T$ gene of interest—C_T internal control) overexpressing *LsACC1* gene of 48-h and 96-h—(C_T gene of interest—C_T internal control) control 12-h. C_T represents the cycle number at which a sample reaches a predetermined threshold signal value for the specific target gene. All the experiments were performed in triplicate.

selected to represent the middle stage of lipid production. Finally, the 96-h sample was used for the late stage. The expression profiles of *LsACC1* during the lipid fermentation process were analyzed by the qPCR method. The expression level of 48-h sample was fivefold higher than the 12-h sample. However, the mRNA level dropped in the 96-h sample (Fig. 5(B)). QPCR analysis of *LsACC1* showed that the expression levels were upregulated during 48-h sample, which was consistent with the rapid accumulation of lipid. Liu et al. [35] reported that the expression of *LsACC1* was upregulated during the lipid accumulation progress according to proteome analysis of *L. starkeyi*. For the experiment results, we supposed that the expression levels of the *LsACC1* increased along with the lipid accumulation, because the gene is first committed step in fatty acid biosynthesis. However, in the last stage of lipid accumulation, the abundance of LsACC1 was enough for fatty acid biosynthesis, so the expression levels of mRNA decreased quickly.

The conversion of acetyl-CoA to malonyl-CoA by the ACC was the rate-limiting step of fatty acid biosynthesis in many organisms including yeast.[36] To test the function of LsACC1, we overexpressed *LsACC1* in *S. cerevisiae* to increase the pool of malonyl-CoA for biosynthesis fatty acyl-CoA. Plasmid-based overexpression of *LsACC1* led to a lipid content of 7.3%, a 24% increase from a lipid content of 5.9% in the YA103 g strain (Table 2). Notably, total lipid productivity also increased from 235 to 287.5 mg/L. This outcome proved the enzyme activity of the gene.

Heterologous expression of ACC with GPD1 gene in the S. cerevisiae *YA103*

To compare the ability of *LsACC1* and *ScACC1* gene to regulate fatty acids and lipid biosynthesis in *S. cerevisiae*, the two mutants YA103pl (transformed with pX2-GPD1 and pLSA1.0) and YA103ps (transformed with pX2-GPD1 and pSCA1.0) were transformed, respectively. The expression level of *ScACC1* in YA103ps was fourfold higher than the control strain YA103g (Fig. 3(B)). Meanwhile, the expression level of *GPD1* in both transformants is both more than fivefold than YA103g (Fig. 3(A)), and the lipid contents were significantly higher than that of YA103p, which were a result of the overexpression of the *LsACC1* and *ScACC1* under the control of the *GAL1* promoter (Table 2). However, the lipid content of two ACC-overexpressing YA103pl and YA103ps showed no significant differences (Table 2), most likely due to differences in codon usage between *L. starkeyi* and *S. cerevisiae*. Meanwhile, the feed of external oleic acid can accumulate more lipid than overexpression of *LsACC1* in the yeast YA103p.

In general, overexpression of *LsACC1* in YA103p showed an overall enhancement in lipid production, where the highest accumulation resulted in a 63% increase in total lipid content (from 5.9 to 9.6%), so the gene can yield malonyl-CoA for acyl-CoA synthesis and subsequently increase the TAG synthesis. This result is similar to previous reports found in fungi, plants, and *E. coli*. In fungi, the heterologous expression of an oleaginous fungus, *Mucor rouxii ACC* in a

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non-oleaginous yeast, *Hansenula polymorpha* showed a 40% increase in total fatty acid content.[18] In addition, targeting of the homomeric *Arabidopsis* ACC to plastids of *Brassica napus* resulted in an increase in the enzyme activity by approximately 20-fold with only a 5% increase in the total oil content of the seeds.[17] It has been also demonstrated that the overexpression of the ACC in *E. coli* led to a threefold increase, and co-expression of ACC and malic enzyme resulted a 5.6-fold increase in intracellular lipids.[37]

Taken together, we overexpressed the *LsACC1* gene in YA103p led to a lipid content of 9.6%, but it was still low in contrast to the oleaginous yeast. Therefore, the low levels of lipid content found in the *S. cerevisiae* transformant may be explained by constraints of other metabolic processes involved in lipid accumulation in addition to acetyl-CoA carboxylation.

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Disclosure statement

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