Identification and Characterization of Diacylglycerol Acyltransferase in Oleaginous Yeast *Rhodosporidium toruloides*

1Zhen Wang, 2Huaiyuan Zhang, 1Lina Zhao and 1,2Yuanda Song

1School of Food Science and Technology, Jiangnan University, Wuxi 214122, Jiangsu, People’s Republic of China
2Colin Ratledge Center for Microbial Lipids, School of Agricultural Engineering and Food Science, Shandong University of Technology, Zibo, 255049, Shandong, People’s Republic of China

**Abstract:** Diacylglycerol acyltransferase (DGAT), which catalyzes TAG formation from DAG and acyl-CoA, has been considered to play a vital role in TAG accumulation in oleaginous microorganisms. The genome of oleaginous yeast *Rhodosporidium toruloides* contains two putative DGAT genes, RtDGATa and RtDGATb, which shared little conserved amino acid coding sequence with each other. Phylogeny tree analysis showed that RtDGATa belonged to DGAT1 family and RtDGATb belonged to DGAT2 family. For functional identification of the DGATs, RtDGATa and RtDGATb were individually expressed in *Saccharomyces cerevisiae* TAG-deficient quadruple mutant (H1246). RtDGATa had obvious preference for monounsaturated fatty acids, however the expression of RtDGATa did not alter the TAG content in *S. cerevisiae* H1246 and it had non-involvement in TAG accumulation according to its mRNA expression level in *R. toruloides*. The expression of RtDGATb could completely resume TAG biosynthesis in *S. cerevisiae* H1246. Substrate preference experiments revealed that RtDGATb preferred unsaturated fatty acids over saturated fatty acids, but not C18:3. Only the expression pattern of RtDGATb was related to the process of fatty acid biosynthesis, suggesting that RtDGATb plays an important role in lipid accumulation in *R. toruloides.*

**Keywords:** Diacylglycerol Acyltransferase, Fatty Acid, Oleaginous, *Rhodosporidium toruloides*, Triglyceride

**Introduction**

In recent years, alternative sources of fuels attracted more and more interests because of petroleum crisis and accumulation of greenhouse gases worldwide. Biodiesel, esterified from vegetable oils or animal fats with lower alcohol by now, acts as an alternative of fossil fuel and biodiesel production, which is commercially available in Europe, the United States and other countries (Li *et al*., 2008; Sitepu *et al*., 2014). The main drawback of this technology is that it conflicts with food resources. Alternative to plant oil and animal fat, oils produced from oleaginous microorganisms can also be used to produce biodiesel, which has several advantages, such as high lipid yield and growth rate over current biodiesel production technologies (Liang and Jiang, 2013). Almost all microbes are intrinsically capable of synthesizing fatty acids as cell membrane components and energy storage materials. However only a few microbes could accumulate intracellular lipids over 20% of their Dry Cell Weight (DCW) and these microorganisms are regarded as oleaginous microorganisms (Ageitos *et al*., 2011; Ratledge and Wynn, 2002).

TAG synthesis, from glycerol-3-phosphate to form TAG, commonly known as the Kennedy pathway, is catalyzed by a series of acyltransferases which contains glycerol-3-phosphate acyltransferase (GPAT; EC 2.3.1.15), lyso-phosphatidic acid acyltransferase (LPAT; EC 2.3.1.51) and diacylglycerol acyltransferase (DGAT; EC 3.2.1.20) (Gong *et al*., 2013). DGAT catalyzes the terminal step in TAG synthesis and has been considered to play a vital role in storage lipid accumulation. There are three major families of DGATs, named membrane-bound type 1, type 2 and a soluble cytosolic type 3 which had rarely been found (Liu *et al*., 2012). DGAT1 and DGAT2 exhibit no sequence homologies to each other. It is
suggested that DGAT2 is the most important enzyme in lipid accumulation and had been utilized to improve the lipid content in several microorganisms in some recent researches. Over expression of DGAT2 in Y. lipolytica led to 3-fold increase in lipid accumulation and its coupling with Acetyl-CoA Carboxylase (ACC) over expression simultaneously improved the lipid content from 11.7 to 61.7% of their biomass in 2-liter baffled stirred-tank bioreactor (Tai and Stephanopoulos, 2013). After genetic transformation of Colletotrichum strain with the CtDGAT2b over expression, total lipid titer in the transformants (73% of DCW) was found to be 1.7-fold more than the wild type (38% of DCW) when grown under standard condition without imposition of any nutrient-stress (Dey et al., 2014). DGAT2 over expression in marine diatom Phaeodactylum tricornutum stimulated more oil bodies and the neutral lipid content increased by 35% and the fatty acid composition showed a significant increase in the proportion of polyunsaturated fatty acids, especially Eicosapentaenoic Acid (EPA) (Niu et al., 2013).

The red yeast R. toruloides has a high capability of growth and lipid biosynthesis on a wide range of carbon sources from glucose, fructose and xylene to glycerol. It can accumulate lipids up to 70% of its biomass under certain conditions and the content of neutral lipids (mainly TAG and DAG) was up to over 90% of total lipids (Jin et al., 2013). However, the molecular mechanism of TAG biosynthesis in R. toruloides had barely been studies. Recently a DGAT2 gene from another red yeast Rhodosporidium diobovatum has been cloned and its function has been confirmed in vivo by expression in the S. cerevisiae TAG-deficient quadruple mutant (Chen et al., 2014). However, not all DGAT genes have been identified in R. diobovatum because of the absence of its genome information. Recently the genome information of R. toruloides has been published (Zhu et al., 2012), so, in this study, we investigated the identification and functional analysis of all putative DGAT genes from R. toruloides and explored the role of DGATs in TAG synthesis in this oleaginous yeast.

Materials and Methods

Microorganism Strains and Culture Medium

The R. toruloidesstrain CGMCC 2.1389 was bought from China General Microbiological Culture Collection Center (CGMCC) and R. diobovatum CICC 31994 was from China Center of Industrial Culture Collection (CICC). S. cerevisiae mutantstrain H1246 (dga1Δ lro1Δ are1Δ are2Δ) kindly provided by prof. Stymne from Swedish University of Agricultural Sciences was used for functional complementation of putative DGAT genes in R. toruloides and R. diobovatum. E.coli Top10 was kept in our lab and used for plasmid construction.

For lipid accumulation of R. toruloides CGMCC 2.1389 in a fermentor, the nitrogen limited medium contained (g/L): Glucose 60, (NH4)2SO4 0.1, yeast extract 0.75, KH2PO4 0.4 and MgSO4·7H2O 1.5. After the medium was sterilized at 121°C for 30 min, it was supplemented with 1% (v/v) trace element solution contained (g/L): CaCl2·2H2O 4.0, FeSO4·7H2O 0.55, citric acid·H2O 0.52, ZnSO4·7H2O 0.10, MnSO4·H2O 0.076 and 18 M H2SO4 100 uL (Wu et al., 2011). All chemical reagents was from Sinopharm Group CO., LTD (Beijing, China) and belonged to analytic grade.

For functional expression in S. cerevisiae H1246, transformants harboring each plasmid were grown at 30°C in synthetic minimal medium containing 2% raffinose, 0.67% yeast nitrogen base (with ammonium sulfate) and appropriate amino acid contents according to the protocol (Invitrogen).

Construction of Plasmids and S. cerevisiae Mutant Strains

Standard molecular genetic techniques were employed throughout this study. Total RNA of R. toruloides CGMCC 2.1389 and R. diobovatum CICC 31994 was isolated using TRizol Reagent (TaKaRa, Dalian, China) according to the manufacturer’s instruction and reverse transcribed into cDNA. DGAT gene fragments were obtained by PCR using their cDNA as template with the corresponding primers (Table 1) and then these DNA fragments were cloned into expression vector pYES2/NT C using restriction endonuclease BamHI and EcoRI, the plasmids used in this study were showed in Table 1.

The resulting plasmids pYES2/NT C, pYES2-DGA1, pYES2-RdDGAT2, pYES2-RdDGATa and pYES2-RdDGATb were transformed into S. cerevisiae H1246 (TAG-deficient quadruple mutant) using the PEG/lithium acetate method (Elble, 1992). S. cerevisiae H1246 harboring empty vector pYES2/NT C was the negative control and S. cerevisiae H1246 harboring pYES2-DGA1 was the positive control strains. Transformants were selected by plating on complex synthetic minimal medium agar lacking uracil and grown at 30°C for 2-3 days. The expression of DGAT cDNA and DGA1 was induced under transcriptional control of the GAL1 promoter. For protein expression, transformants were inoculated in synthetic minimal medium until OD600 reached to 0.4 and then induced by supplementing 2% (w/v) galactose and cultivated for a further 90 h at 28°C. The growth curve of mutant strains was monitored by measuring their OD600 and explored the role of DGATs in TAG synthesis in this oleaginous yeast.
Table I. Plasmids and microorganism strains used in this study

| Plasmids | Use, relevant characteristic(s) and source |
|----------|-------------------------------------------|
| pYES2/NT C | Ampicillin resistance, transformation vector for S. cerevisiae, stored in our lab |
| pYES2-DGA1 | Ampicillin resistance, S. cerevisiae DGA1 gene cloned to BamHI/EcoRI-cut pYES2/NT C for positive control |
| pYES2-RdDGAT2 | Ampicillin resistance, R. diobovatum RdDGAT2 gene cloned to BamHI/EcoRI-cut pYES2/NT C for another positive control |
| pYES2-RtDGATa | Ampicillin resistance, R. toruloides putative RtDGATa gene cloned to BamHI/EcoRI-cut pYES2/NT C |
| pYES2-RtDGATb | Ampicillin resistance, R. toruloides putative RtDGATb gene cloned to BamHI/EcoRI-cut pYES2/NT C |

| Strains | Use, relevant characteristic(s) and source |
|----------|-------------------------------------------|
| E. coli Top10 | E. coli host for DNA manipulations |
| R. toruloides CGMCC 2.1389 | From China General Microbiological Culture Collection Center |
| R. diobovatum CICC 31994 | From China Center of Industrial Culture Collection |
| H1246 | Saccharomyces cerevisiae H1246 (MATα::his3 are2-α::Leu2 dga1-α::KanMX4 Iro1-α::TRP1 ADE2) |
| HY1 | S. cerevisiae H1246 harboring empty plasmid pYES2/NT C |
| HY2 | S. cerevisiae H1246 harboring plasmid pYES2-DGA1 |
| HY3 | S. cerevisiae H1246 harboring plasmid pYES2-RdDGAT2 |
| HY4 | S. cerevisiae H1246 harboring plasmid pYES2-RtDGATa |
| HY5 | S. cerevisiae H1246 harboring plasmid pYES2-RtDGATb |

**Lipid Extraction and Fatty Acid Analysis**

Total lipids were extracted from 100mg of lyophilized biomass with a solvent mixture of chloroform: Methanol (2:1, v/v) according to the modified method (Bligh and Dyer, 1959). TAGs were separated from total lipids by thin layer chromatography (TLC) using a solvent system of hexane: Diethyl ether: Acetic acid (70:30:1, v/v/v). Individual lipid spots were visualized by exposing the silica gel plates (Qingdao, China) to iodine vapor and TAGs were scraped off the plates, eluted with 1 mL chloroform for three times and then submitted to methyl esterification. Total Fatty Acid (TFA) isolation and fatty acid composition were analyzed as their methyl esters as described by (Zhang et al., 2013a) using C15:0 as internal standard [17]. Each sample was performed in triplicate.

**R. toruloides CGMCC 2.1389 Fermentation and DGAT Expression Pattern**

For analyzing lipid accumulation and expression pattern of putative DGAT genes in oleaginous yeast R. toruloides, bioreactor scale fermentation was carried out in a 2.8L ferment or contained 2L nitrogen limited medium (0.5 vvm aeration, pH 5.6, 28°C, 250 rpm agitation). Culture samples were collected at different time for biomass and lipid analysis and total RNAs were also extracted from yeast cells according to the manufacturer’s instruction. Genomic DNA was removed from total RNAs with gDNA Eraser (TaKaRa) at 37°C for 15 min. After total RNAs were reverse transcribed into cDNA using the PrimeScript® RT reagent Kit (TaKaRa), 2 uLcDNAs was used for quantitative Real Time-PCR amplification using the SYBR Green I master mix Kit (TaKaRa). Reactions were run in a Mini Opticon Thermal Cycler (Bio-Rad). DGAT cDNA fragments were amplified with primer pairs listed in S1 Table and 18S cDNA was used as an internal standard. Gene expression analyses were performed with the previous method (Livak and Schmittgen, 2001). Each sample was performed in triplicate. Nile Red fluorescence assays of yeast cells were carried out according to the method described previously (Dey et al., 2014).

**Results**

**Identification and Protein Sequence Analysis of R. toruloides DGATs**

According to annotated genome of oleaginous yeast R. toruloides, two deduced DGAT sequences, RtDGATa (protein ID: 726) and RtDGATb (protein ID: 1957), were found from the published genome database (http://genome.jgi.doe.gov/Rhoto1/Rhoto1.home.html). RtDGATa and RtDGATb are 698 and 349 amino acid residuals in length respectively, sharing different domains and subfamilies. The analytical results using the conserved domain database of National Center for Biotechnology Information (NCBI) showed that RtDGATb possessed a lysophospholipidacyl transferase (LPLAT) super family domain. The putative DGAT amino acid sequences from R. toruloides and other DGATs from animals, plants, fungi and microorganisms, cover three DGAT families (DGAT1, DGAT2 and cytosolic DGAT3), were used for construction of phylogenetic tree. All 36DGAT protein sequences were clearly classified to DGAT1, DGAT2 and DGAT3 families with extremely strong bootstrap support
(Fig. 1). As showed in Fig. 1, RtDGATa was clustered within DGAT1 family and had substantial similarity with Acyl-CoA: Cholesterol acyltransferase from S. cervisiae (ScARE). However RtDGATb was distinctly separated into DGAT2 family and had the largest similarity with RdDGAT2 from R. diobovatum, which had been proved to belong to DGAT2 family (Chen et al., 2014). Multiple sequence alignment of DGAT2 from seven organisms was performed (Fig. 2). RtDGATb contained six highly conserved motifs that were identified as signature motifs within the DGAT2 family by (Cao, 2011), namely Motif 1 (PH Block), Motif 2 (PR Block), Motif 3 (GGE Block), Motif 4 (RGFA Block), Motif 5 (VPFG Block) and Motif 6 (G Block).

Fig. 1. Phylogenetic analysis of homolog sequences of RtDGAT from plant, fungi and microorganisms. Multiple sequences were aligned by Clusta IX 2.1 multiple alignment software. The phylogenetic tree was generated using MEGA 6.06 software. Bootstrap values are expressed in the percentages of 1000 replicates on the nodes. Arabidopsis thaliana (At), Brassica napus (Bn) var. napus; Caenorhabditis elegans (Ce), Chlamydomonas reinhardtii (Cr), Candida tropicalis (Ct), Glycine max (Gm), Homo sapiens (Hs), Jatropha curcas (Jc), Mus musculus (Mm), Nicotiana tabacum (Nt), Oryza sativa Japonica (Os), Phaeodactylum tricornutum (Pt), Ricinus communis (Rc), Rhodosporidium diobovatum (Rd), Rhodosporidium toruloides (Rt) Saccharomyces cerevisiae (Sc), Sesamum indicum (Si), Trypanosomagrayi (Tg), Tropaeolum majus (Tm), Umbelopsis ramanniana (Ur), Vernicia fordii (Vi), Vernonia galamensis (Vg) Sc ARE, S. cerevisiaeacy l-CoA: Sterol acyltransferase.
R. toruloides DGATs Recovered TAG Synthesis in S. cervisiae Mutant

Four genes, DGA1 (DGAT2 homolog), LRO1 (encoding phospholipid: Diacylglycerol acyltransferase), ARE1 and ARE2 (both involved in steryl ester synthesis) were found in the yeast S. cervisiae which contributing to TAG biosynthesis (Sandager et al., 2002). A TAG-deficient quadruple mutant of these four genes in S. cervisiae (S. cervisiae H1246) is an impeccable platform for identification of DGAT activity from various organisms (Liu et al., 2012). To affirm whether RtdGAta and RtdGAtb indeed encoded proteins with DGAT activity, RtdGAta and RtdGAtb were expressed individually in S. cervisiae H1246 to obtain mutant strains HY4 and HY5. In addition, empty vector and pYES2-DGA1 harboring DGA1 from S. cervisiae were transformed into S. cervisiae H1246 as negative (HY1) and positive controls (HY2) respectively (Table 1).

Meanwhile, A RtdDGAt2 from other red yeast R. diobovatum which had been discussed in previous study (Chen et al., 2014) was also considered as another positive control (HY3). The results of Western blot analysis showed that all proteins were expressed in S. cervisiae H1246 and their expression levels were undifferentiated (Fig. 1). After cultivation for 96 h in synthetic minimal medium, yeast cells were harvested for lipid isolation and analysis. Total lipid was separated by TLC and the results showed that TAG was at undetectable level in S. cervisiae H1246 with empty vector and with pYES2-RtdGAta, whereas expression of RtdGAtb recovered TAG biosynthesis as did the expression of DGA1 and RtdDGAT2 (Fig. 3A). However the TAG content in S. cervisiae strain HY5 was up to 3.6% of cell dry weight and was lower than mutant strain HY2 (5.8%) and HY3 (7.7%). The trend of TFA content in respective strains was similar with TAG content, TFA biosynthesis in HY5 (8.9%) was also less than HY2 and HY3 (Fig. 3A).

Acyl-CoA Preference Analysis of R. toruloides DGATs

To determine the possible substrate preference of RtdGAta and RtdGAtb for those fatty acids which are naturally present in R. toruloides, S. cervisiae mutant strains were inoculated in synthetic minimal medium with different fatty acids supplement (C18:2 or C18:3) and added 2% (w/v) galactose as the inducer for protein expression. The results of growth curve and lipid biosynthesis were shown in Fig. 3 and 4. α-linolenic acid (C18:3) supplement did not alter the growth trend of all S. cervisiae mutant strains compared to cultivation mode devoid of supplement, however the growth situation with linoleic acid (C18:2) supplement had significant difference between DGAT active strains and DGAT inactive strains. In the early period of cultivation, addition of linoleic acid suppressed the yeast cell proliferation in DGAT inactive strains, but it also surprisingly promoted the biomass upsurge at the later period of the culture cycle and final OD_{600} reached about 24.0 in DGAT inactive strains (Fig. 4).
Fig. 3. TFA and TAG synthesis of \textit{S. cervisiae} mutant cultivated in synthetic minimal medium with fatty acid supplement to final concentration of 1.0 mM. The sample was harvested after 96 h culture, total lipid was separated by TLC and fatty acid methyl ester was determined by GC-MS. A, no supplement; B, C18:2 supplement; B, C18:3 supplement.

Fig. 4. Growth curve of \textit{S. cervisiae} mutant cultivated in synthetic minimal medium with fatty acid supplement to final concentration of 1.0 mM (●, no supplement; ■, added C18:2; ▲, added C18:3). Data are mean±S.D. from triple biological replicates.

At the end of cultivation with fatty acid supplement, intracellular lipid was isolated from freeze-dried yeast cells and TAG was separated from total lipids by TLC. C18:2 supplementation apparently increased the TFA content in all mutant strains, although, addition of C18:3 had little influence on TFA and TAG contents (Fig. 3B and 3C). However fatty acid supplement obviously changed the fatty acid composition of TFA (Fig. 2) and TAG (Table 2) in \textit{S. cervisiae} mutant strains. Compared with fatty acid profile of TAG and TFA in HY2 and HY3, RtDGATb preferred monounsaturated fatty acid (C16:1 and C18:1) to saturated fatty acid (C16:0 and C18:0). For these addition experiments of fatty acids, C18:2 could be accumulated over 20% of total fatty acids in all strains, expression of RdDGAT2 led to the highest content of C18:2 of TAG and the lowest content was found in strain HY2 with DGA1 expression. There was very low C18:3 content in TAG (less than 2%), supplementation of C18:3 had little effect on fatty acid composition of TAG (Table 2). For fatty acid profile of TFA, mutant strains with an active DGAT also had tremendously low content of C18:3, however, C18:3 was accumulated more than 10% of TFA (Fig. 2) in DGAT inactive mutants. RtDGATb preferred monounsaturated fatty acid to saturated fatty acid and had no preference for C18:3. The expression of RtDGATa also alter the fatty acid profile in \textit{S. cervisiae} mutant strains (Fig. 2). RtDGATa had significant preference for C16:1 and C18:1 and had no preference for C16:0, C18:0, C18:2 and C18:3.
Fig. 5. The lipid accumulation and DGATs gene expression pattern in *R. toruloides*. (A) TFA (▲) and TAG (◆) content of *R. toruloides* cultivated in a fermentor. (B) DGATs gene expression in *R. toruloides*. (C) Lipid bodies where neutral lipid accumulate were visualized in yeast cells with the fluorescent dye Nile Red. BF, Bright-field image; FR, image of Nile Red fluorescence. Data are mean ± S.D. from triple biological replicates.

Table 2. Fatty acid profile of TAG in *S. cervisiae* H1246 mutant strains harboring each plasmid cultivated with different fatty acid supplement

| Strains          | C16:0       | C16:1       | C18:0       | C18:1       | C18:2       | C18:3       |
|------------------|-------------|-------------|-------------|-------------|-------------|-------------|
| No supplement    |             |             |             |             |             |             |
| HY2              | 22.40±0.89  | 36.25±1.23  | 10.34±1.03  | 31.00±1.56  | -           | -           |
| HY3              | 19.44±0.96  | 38.88±1.02  | 7.02±0.23   | 36.65±1.35  | -           | -           |
| HY5              | 15.4±0.86   | 36.88±0.95  | 9.02±0.15   | 38.65±2.04  | -           | -           |
| Added C18:2      |             |             |             |             |             |             |
| HY2              | 14.13±0.35  | 27.32±0.65  | 9.66±0.24   | 24.10±1.86  | 24.73±1.21  | -           |
| HY3              | 11.01±0.13  | 28.62±0.43  | 7.03±0.09   | 26.12±1.07  | 27.25±0.95  | -           |
| HY5              | 9.73±0.46   | 29.10±1.32  | 7.25±0.39   | 29.11±0.99  | 25.64±0.43  | -           |
| Added C18:3      |             |             |             |             |             |             |
| HY2              | 21.01±0.23  | 38.10±2.58  | 11.02±0.49  | 29.20±0.68  | 0.88±0.52   | -           |
| HY3              | 16.14±0.86  | 40.88±3.21  | 7.32±0.36   | 34.25±0.48  | 1.48±0.79   | -           |
| HY5              | 14.64±1.03  | 39.88±1.09  | 8.82±0.08   | 35.45±1.57  | 1.29±0.45   | -           |

Data are mean±S.D. from triple biological replicates.-, not detected

Table 3. Fatty acid composition of TAG in *R. toruloides* CGMCC 2.1389 cultivated in fermentor with nitrogen limited medium

| Culture time (h) | C16:0 | C18:0 | C18:1 | C18:2 | C18:3 | other |
|------------------|-------|-------|-------|-------|-------|-------|
| 11               | 30.92±1.23 | 14.80±0.37 | 35.50±1.02 | 15.88±0.12 | 1.61±0.01 | 1.29±0.02 |
| 24               | 29.89±2.14 | 12.41±0.57 | 42.94±1.15 | 8.81±0.31 | 2.58±0.02 | 1.29±0.01 |
| 36               | 30.80±1.45 | 13.83±0.29 | 42.54±2.01 | 9.91±0.04 | 1.68±0.02 | 1.25±0.04 |
| 48               | 28.74±1.25 | 11.91±0.10 | 49.48±1.89 | 5.71±0.09 | 2.89±0.00 | 1.27±0.13 |
| 60               | 28.40±1.86 | 11.78±0.25 | 49.96±1.81 | 5.54±0.11 | 2.99±0.01 | 1.32±0.02 |
| 72               | 28.88±1.49 | 11.83±0.43 | 49.82±1.67 | 5.58±0.12 | 2.64±0.02 | 1.24±0.10 |
| 84               | 30.06±2.04 | 12.35±0.37 | 48.17±1.59 | 5.96±0.08 | 2.18±0.02 | 1.29±0.07 |
| 96               | 28.05±2.18 | 12.64±0.26 | 47.60±1.09 | 8.52±0.06 | 2.08±0.03 | 1.11±0.09 |
| 108              | 27.53±1.84 | 12.46±0.20 | 50.19±1.35 | 6.40±0.12 | 2.27±0.00 | 1.15±0.11 |
| 120              | 27.35±1.76 | 11.63±0.19 | 49.65±0.98 | 7.85±0.13 | 2.43±0.03 | 1.09±0.06 |
The Relationship between DGATs Gene Expression Pattern and Lipid Accumulation in R. toruloides

To explore the role of DGATs in the process of lipid accumulation in R. toruloides, this yeast was cultivated in a fermentor with nitrogen limited medium and the lipid biosynthesis and DGATs gene expression pattern was determined. Following the consumption of glucose in the medium, biomass increased continuously until glucose was exhausted and final biomass was up to 23 g L\(^{-1}\) (Fig. 3). TFA and TAG increased sharply at cultivation time from 11 h to 48 h and at the later period of cell growth TFA and TAG contents increased very slowly. The final TFA content was near 70% of DCW and TFA production was up to 16 g L\(^{-1}\) (Fig. 5A). Lipid bodies in yeast cells were visualized with the fluorescent Nile Red and results were shown in Fig. 5C. There were only 1-2 small lipid bodies inside each spindle yeast cell at the earlier period of fermentation and lipid bodies grew bigger in size by continuous TAG biosynthesis later on. At the end of fermentation, a large lipid body was almost the size of whole yeast cell with TAG content up to 53% of DCW. The fatty acid composition of TAG and TFA was analyzed by GC and the results were showed in Table 3 and 2. Only C18:1 content in TAG and TFA was gradually increased and up to about 50% of total fatty acid. In addition, fluorogenic quantitative PCR was used to determine the mRNA expression level of DGTA genes in R. toruloides. The transcript level of RdDGATa was almost unchanged during the process of fermentation but the trend of RdDGATb mRNA was related to the rate of lipid accumulation (Fig. 5B). The expression level of RdDGATb increased from inoculation to 36 h, peaked at 36 h and then decreased to a low level in the following cultivations.

Discussion

The lipid content in red yeast R. toruloides was up to 70% of its biomass under certain conditions and total lipids mainly composed with neutral lipids (mainly TAG and DAG) (Jin et al., 2013). DGAT, which catalyzes TAG formation from DAG and fatty acyl-CoA, is the terminal step for lipid accumulation. This oleaginous yeast cell contains two putative DGAT proteins, RdDGATa and RdDGATb, which shared little conserved amino acid with each other. Phylogeny tree analysis showed that RdDGATa belonged to DGAT1 family and RdDGATb belonged to DGAT2 family. To confirm whether these two proteins showed the DGAT activity RdDGATa and RdDGATb from this yeast were expressed in S. cerevisiae TAG-deficient quadruple mutant (H1246). The results showed that RdDGATa did not have DGAT activity and did not enhance the sterol ester biosynthesis capacity of the complemented S. cerevisiae HY4, although RdDGATa belonged to DGAT1 family and shared substantial similarity with ScARE. Furthermore the level of sterol ester in R. toruloides wild type was extremely low (Jin et al., 2013) and these results were consistent with mRNA expression level of RdDGATa in R. toruloides.

However, RdDGATb of DGAT2 family could completely resume TAG biosynthesis in S. cerevisiae H1246 and the lipid content in S. cerevisiae HY5 was lower than mutant strains HY2 and HY3. This may due to the higher DGAT activity of RdDGATb than RdDGATa because the subtle differences of RdDGAT2 and RdDGATb amino acid residues, although the sequences of RdDGAT2 and RdDGATb are highly similar. Knockout of DGATs, especially DGAT2, in microbial cells obviously resulted in diminution of TAG synthesis. In Y. lipolytica ATCC 20362 strain, the total lipids of DCW in DGAT2 mutant decreased to 36% of the wild-type strain (Zhang et al., 2012). The atf2-disrupted Rhodococcus opacus mutant exhibited a decrease in TAG accumulation (from 40-60% to 25-30%, w/w) and approximately ten-fold increase in glycerogen formation in comparison with the wild-type strain (Hernández et al., 2013). DGAT mutant experiments suggested that the reaction catalyzed by DGAT is an important regulatory factor in lipid biosynthesis. In recent years, it has been considered that the genetic engineering of DGAT of lipid biosynthesis pathway could be a promising approach to increase the storage lipid content in plants, microalgae and fungi including yeasts for economic production of lipid feedstock (Table 4). Over expression of DGAT2 in Y. lipolytica led to total lipid production increase from 8.7% of DCW to 33.8% (Tai and Stephanopoulos, 2013). In oleaginous bacteria R. opacus PD 630mutant strain with atf2-overexpression (WS/DGAT, wax ester synthase/acyl-CoA: diacylglycerol acyltransferase), TAG accumulation was promoted about 10% (Hernández et al., 2013). Total lipid titer by the transformed Colletotrichum (lipid content,73% of DCW) was found to be 1.7-fold more than the wild type (lipid content, 38% of DCW) due to functional activity of DGAT2 from oleaginous yeast Candida tropicalis SY005 when grown understand condition without imposition of any nutrient-stress (Dey et al., 2014). As showed in Table 4, many DGAT2 family members have been used for improving lipid accumulation. In this study, RdDGATa did not alter the TAG content in S. cerevisiae mutant and it could not be the metabolic target for improving the TAG biosynthesis in R. toruloides. However RdDGATb could restore the TAG synthesis in S. cerevisiae TAG-deficient quadruple mutant. Furthermore the mRNA expression level of RdDGATb was associated with lipid accumulation in R. toruloides and RdDGATb was up regulated before the onset of TAG accumulation and might functionally contribute to the accumulation of large amount of TAGs, suggesting that RdDGATb may participate in lipid biosynthesis and plays a key impact for TAG accumulation in oleaginous yeast.
Table 4. Genetic engineering of DGAT for increasing lipid accumulation in various organisms

| Host                          | DGAT source | DGAT family | Lipid change                  | Reference                         |
|-------------------------------|-------------|-------------|--------------------------------|-----------------------------------|
| *Y. lipolytica*               | *Y. lipolytica* | DGAT2       | From 8.7% of DCW to 33.8%     | Tai and Stephanopoulos (2013)      |
| *P. tricornutum*              | *P. tricornutum* | DGAT2       | Neutral lipid increased by 35% | Niu et al. (2013)                 |
| *Rhodotorula glutinis*        | *Vernicia fordii* | DGAT2       | 6.24-fold increase            | Chen et al. (2015)                |
| *Chlamydomonas reinhardtii*   | *Chlamydomonas* | DGAT2       | 2.5-fold increase             | Iwa et al. (2014)                 |
| *Rhodococcus opacus*          | *Rhodococcus opacus* | WSDGAT     | Approximately 10% increase of TAG | Hernandez et al. (2013)          |
| *S. cerevisiae*               | *Arabidopsis thaliana* | DGAT2       | 3 to 9-fold increase of TAGs   | Bouvier-Nave et al. (2000)        |
| *C. tropicalis*               | *Candida tropicalis*SY0 | DGAT2       | From 38% of DCW to 73%        | Dey et al. (2014)                 |
| *Chlamydomonas reinhardtii*   | *Brassica napus* | DGAT2       | From 12.33% to 18.76%         | Ahmad et al. (2015)               |
| *Petunia*                     | *Vernonia galamensis* | DGAT1       | From 1.85% of DCW to 2.5% in leaves | Li et al. (2010)                |
| *Arabidopsis thaliana*        | *Jatropha curcas* | DGAT1       | 30-41% increase               | Mista et al. (2013)              |
| *Arabidopsis thaliana*        | *Thraustochytrium aureum* | DGAT2       | Nearly 2-fold increase of oleic acid | Zhang et al. (2013b)            |
| *Soybean*                     | *U. ramanniana* | DGAT2       | From 25 to 26.5%              | Lardizabal et al. (2008)         |
| *Tobacco*                     | *Arabidopsis thaliana* | DGAT2       | Lipid in leaves increased by 20-fold | Andrianov et al. (2010)        |
| *Maize*                       | *Maize*      | DGAT1       | Seed oil increased by 41%     | Zheng et al. (2008)              |

Similar results were also found in other oleaginous diatom *P. tricornutum* (Gong et al., 2013). Further researches are required to investigate whether over expression of DGAT genes alters lipid accumulation in *R. toruloides*.

**Conclusion**

Two putative DGAT genes from oleaginous yeast *R. toruloides*, *RtDGATa* and *RtDGATb*, were individually expressed in *S. cerevisiae* TAG-deficient quadruple mutant (H1246) and the results showed that *RtDGATa* had no DGAT activity, but *RtDGATb* could completely resume TAG biosynthesis in *S. cerevisiae* H1246. Substrate preference experiments revealed that *RtDGATb* preferred unsaturated fatty acids over saturated fatty acids but had no preference for C18:3. The expression pattern of *RtDGATb* was associated with the process of fatty acid biosynthesis and it suggested that *RtDGATb* may play as a rate-limiting step in lipid accumulation in *R. toruloides*.

**Acknowledgement**

This work was supported by the National Natural Science Foundation of China (31271812), the National High Technology Research and Development Program of China (863 Program 2012AA02 2105C).

**Author Contributions**

**Yuanda Song**: Conceived and designed the experiments and wrote the paper.

**Zhen Wang and Huaiyuan Zhang**: Performed the experiments, analyzed the data, contributed reagents/materials/analysis tools and wrote the paper.

**Lina Zhao**: Analyzed the data, contributed reagents/materials/analysis tools and wrote the paper.

**Ethics**

This article is original and contains unpublished material. The corresponding author confirms that all the other authors have read and approved the manuscript and no ethical issues involved.

**References**

Ageitos, J.M., J.A. Vallejo, P. Veiga-Crespo and T.G. Villa, 2011. Oily yeasts as oleaginous cell factories. Applied Microbiol. Biotechnol., 90: 1219-27. DOI: 10.1007/s00253-011-3200-z

Ahmad, I., A.K. Sharma, H. Daniell and S. Kumar, 2015. Altered lipid composition and enhanced lipid production in green microalga by introduction of brassica diacylglycerol acyltransferase 2. Plant Biotechnol. J., 13: 540-550. DOI: 10.1111/pbi.12278

Andrianov, V., N. Borisjuk, N. Pogrebnyak, A. Brinker and J. Dixon et al., 2010. Tobacco as a production platform for biofuel: Over expression of *Arabidopsis DGAT* and LEC2 genes increases accumulation and shifts the composition of lipids in green biomass. Plant Biotechnol. J., 8: 277-87. DOI: 10.1111/j.1467-7652.2009.00458.x

Bligh, E.G. and W.J. Dyer, 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol., 37: 911-917. DOI: 10.1139/o59-099

Bouvier-Nave, P., P. Benveniste, P. Oelkers, S.L. Sturley and H. Schaller, 2000. Expression in yeast and tobacco of plant cDNAs encoding acyl CoA:diacylglycerol acyltransferase. Eur. J. Biochem., 267: 85-96. DOI: 10.1046/j.1432-1327.2000.00961.x

Cao, H., 2011. Structure-Function Analysis of Diacylglycerol Acyltransferase Sequences from 70 Organisms. BMC Res. Notes, 4: 249-249. DOI: 10.1186/1756-0500-4-249

Chen, Y., Q. Cui, Y. Xu, S. Yang and M. Gao, 2015. Effects of tung oilseed *FAD2* and *DGAT2* genes on unsaturated fatty acid accumulation in *Rhodotorula glutinis* and *Arabidopsis thaliana*. Molecular Genet. Genom., 290: 1605-1613. DOI: 10.1007/s00438-015-1011-0
Chen, Z., Liu, P., Liu, Y., Tang, H., Chen, Y., Zhang, L. (2014). Identification and characterization of a type-2 diacylglycerol acyltransferase (DGAT2) from *Rhodopseudomonas diobovatum*. Antonie Van Leeuwenhoek, 106: 1127-1137. DOI: 10.1007/s10482-014-0282-5

Dey, P., N. Mall, A. Chattopadhyay, M. Chakraborty and M.K. Maiti, 2014. Enhancement of lipid productivity in oleaginous *Colletotrichum* fungus through genetic transformation using the yeast CtDGAT2b gene under model-optimized growth condition. PLoS One, 9: e111253-e111253. DOI: 10.1371/journal.pone.0111253

Elble, R., 1992. A simple and efficient procedure for transformation of yeasts. Bio Techniques, 13: 18-20. PMID: 1503765

Gong, Y., J. Zhang, X. Guo, X. Wan and Z. Liang et al., 2013. Identification and characterization of PtDGAT2B, an acyltransferase of the DGAT2 acyl-Coenzyme A: Diacylglycerol acyltransferase family in the diatom *Phaeodactylum tricornutum*. FEBS Lett., 587: 481-487. DOI: 10.1016/j.febslet.2013.01.015

Hernández, M.A., A. Arabalaza, E. Rodriguez, H. Gramajo and H.M. Alvarez, 2013. The atf2 gene is involved in triacylglycerol biosynthesis and accumulation in the oleaginous *Rhodococcus opacus* PD630. Applied Microbiol. Biotechnol., 97: 2119-2130. DOI: 10.1007/s00253-012-4360-1

Iwai, M., K. Ikeda, M. Shimojima and H. Ohta, 2014. Enhancement of extraplastidic oil synthesis in *Chlamydomonas reinhardtii* using a type-2 diacylglycerol acyltransferase with a phosphorus starvation-inducible promoter. Plant Biotechnol. J., 12: 808-819. DOI: 10.1111/pbi.12210

Jin, G., Y. Zhang, H. Shen, X. Yang and H. Xie et al., 2013. Fatty acid ethyl esters production in aqueous phase by the oleaginous yeast *Rhododendron toruloides*. Bioreosur Technol., 150: 266-270. DOI: 10.1016/j.biotechlett.2013.10.023

Lardizabal, K., R. Effertz, C. Levering, J. Mai and M.C. Pedroso et al., 2008. Expression of *Umbelopsis ramanniana* DGAT2A in seed increases oil in soybean. Plant Physiol., 148: 89-96. DOI: 10.1104/pp.108.123042

Li, Q., W. Du and D. Liu, 2008. Perspectives of microbial oils for biodiesel production. Applied Microbiol. Biotechnol., 80: 749-756. DOI: 10.1007/s00253-008-1625-9

Li, R., K. Yu, T. Hatanaka and D.F. Hildebrand, 2010. *Vernonia* DGATs increase accumulation of epoxy fatty acids in oil. Plant Biotechnol. J., 8: 184-195. DOI: 10.1111/j.1467-7652.2009.00476.x

Liang, M.H. and J.G. Jiang, 2013. Advancing oleaginous microorganisms to produce lipid via metabolic engineering technology. Prog. Lipid Res., 52: 395-408. DOI: 10.1016/j.plipres.2013.05.002

Liu, Q., R.M. Siloto, R. Lehrer, S.J. Stone and R.J. Weselake, 2012. Acyl-CoA:diacylglycerol acyltransferase: Molecular biology, biochemistry and biotechnology. Prog. Lipid Res., 51: 350-377. DOI: 10.1016/j.plipres.2012.06.001

Livak, K.J. and T.D. Schmittgen, 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods, 25: 402-408. DOI: 10.1006/meth.2001.1262

Misra, A., K. Khan, A. Niranjan, P. Nath and V.A. Sane, 2013. Over-expression of *JcDGAT1* from *Jatropha curcas* increases seed oil levels and alters oil quality in transgenic *Arabidopsis thaliana*. Phytochemistry, 96: 37-45. DOI: 10.1016/j.phytochem.2013.09.020

Niu, Y.F., M.H. Zhang, D.W. Li, W.D. Yang and J.S. Liu et al., 2013. Improvement of neutral lipid and polyunsaturated fatty acid biosynthesis by overexpressing a type 2 diacylglycerol acyltransferase in marine diatom *Phaeodactylum tricornutum*. Mar Drugs., 11: 4558-4569. DOI: 10.3390/md11114558

Ratledge, C. and J.P. Wynn, 2002. The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. Adv. Applied Microbiol., 51: 1-51. DOI: 10.1016/S0065-2164(02)51000-5

Sandager, L., M.H. Gustavsson, U. Stahl, A. Dahlqvist and E. Wiberg, 2002. Storage lipid synthesis is non-essential in yeast. J. Biol. Chem., 277: 6478-6482. DOI: 10.1074/jbc.M109109200

Sitepu, I.R., L.A. Garay, R. Sestric, D. Levin and D.E. Block et al., 2014. Oleaginous yeasts for biodiesel: Current and future trends in biology and production. Biotechnol. Adv., 32: 1336-1360. DOI: 10.1016/j.biotechadv.2014.08.003

Tai, M. and G. Stephanopoulos, 2013. Engineering the push and pull of lipid biosynthesis in oleaginous yeast *Yarrowia lipolytica* for biofuel production. Metab. Eng., 15: 1-9. DOI: 10.1016/jymben.2012.08.007

Wu, S., X. Zhao, H. Shen, Q. Wang and Z.K. Zhao, 2011. Microbial lipid production by *Rhodopseudomonas toruloides* under sulfate-limited conditions. Bioreosur Technol., 102: 1803-1807. DOI: 10.1016/j.biotechnol.2010.09.033

Zhang, C., U. Iskandarov, E.T. Klotz, R.L. Stevens and R.E. Cahoon et al., 2013a. A thraustochytrid diacylglycerol acyltransferase 2 with broad substrate specificity strongly increases oleic acid content in engineered *Arabidopsis thaliana* seeds. J. Exp. Bot., 64: 3189-3200. DOI: 10.1093/jxb/ert156

Zhang, H., H.G. Damude and N.S. Yadav, 2012. Three diacylglycerol acyltransferases contribute to oil biosynthesis and normal growth in *Yarrowia lipolytica*. Yeast, 29: 25-38. DOI: 10.1002/yea.1914
Zhang, H., L. Zhang, H. Chen, Y.Q. Chen and C. Ratledge et al., 2013b. Regulatory properties of malic enzyme in the oleaginous yeast, Yarrowia lipolytica and its non-involvement in lipid accumulation. Biotechnol. Lett., 35: 2091-2098. DOI: 10.1007/s10529-013-1302-7

Zheng, P., W.B. Allen, K. Roesler, M.E. Williams and S. Zhang et al., 2008. A phenylalanine in DGAT is a key determinant of oil content and composition in maize. Nat Genet., 40: 367-372. DOI: 10.1038/ng.85

Zhu, Z., S. Zhang, H. Liu, H. Shen and X. Lin et al., 2012. A multi-omic map of the lipid-producing yeast Rhodosporidium toruloides. Nat Commun., 3: 1112-1112. DOI: 10.1038/ncomms2112