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Alleviation of reactive oxygen species enhances PUFA accumulation in *Schizochytrium* sp. through regulating genes involved in lipid metabolism

Sai Zhang[a,1], Yaodong He[a,1], Biswarup Sen[a,1], Xiaohong Chen[b], Yunxuan Xie[a], Jay D. Keasling[c], Guangyi Wang[a,b,d,*]

[a] Center for Marine Environmental Ecology, School of Environmental Science and Engineering, Tianjin University, Tianjin 300072, China
[b] State Key Laboratory of Systems Engines, Tianjin University, Tianjin 300072, China
[c] Berkeley Center for Synthetic Biology, University of California, Berkeley, CA 94720-3224, USA
[d] Key Laboratory of Systems Bioengineering (Ministry of Education), Tianjin University, Tianjin 300072, China

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ABSTRACT

The unicellular heterotrophic thraustochytrids are attractive candidates for commercial polyunsaturated fatty acids (PUFA) production. However, the reactive oxygen species (ROS) generated in their aerobic fermentation process often limits their PUFA titer. Yet, the specific mechanisms of ROS involvement in the crosstalk between oxidative stress and intracellular lipid synthesis remain poorly described. Metabolic engineering to improve the PUFA yield in thraustochytrids without compromising growth is an important aspect of economic feasibility. To fill this gap, we overexpressed the antioxidative gene superoxide dismutase (SOD1) by integrating it into the genome of thraustochytrid *Schizochytrium* sp. PKU#Mn4 using a novel genetic transformation system. This study reports the ROS alleviation, enhanced PUFA production and transcriptome changes resulting from the SOD1 overexpression. SOD1 activity in the recombinant improved by 5.2–71.6% along with 7.8–38.5% decline in ROS during the fermentation process. Interestingly, the total antioxidant capacity in the recombinant remained higher than wild-type and above zero in the entire process. Although lipid profile was similar to that of wild-type, the concentrations of major fatty acids in the recombinant were significantly ($p \leq 0.05$) higher. The PUFA titer increased up to 1232 ± 41 mg/L, which was 32.9% higher ($p \leq 0.001$) than the wild type. Transcriptome analysis revealed strong downregulation of genes potentially involved in β-oxidation of fatty acids in peroxisome and upregulation of genes catalyzing lipid biosynthesis. Our results enrich the knowledge on stress-induced PUFA biosynthesis and the putative role of ROS in the regulation of lipid metabolism in oleaginous thraustochytrids. This study provides a new and alternate strategy for cost-effective industrial fermentation of PUFA.

1. Introduction

As a significant component of cellular membranes, polyunsaturated fatty acids (PUFA) are known to play vital structural and functional roles in humans including regulation of immunity (Armenta and Valentine, 2013) and coronary heart disease (Lopez-Huertas, 2010). A typical ω–3 PUFA – docosahexaenoic acid (DHA, C22:6) – has been recommended to prevent coronary heart disease, hypertension, diabetes, renal disease, rheumatoid arthritis and other diseases (Simopoulos, 1997). With an increasingly better understanding of the health benefits of PUFA, their demand is rising enormously. Although fish oils have long been the main source of PUFA (Pike and Jackson, 2010), their supply is largely limited by fish stocks (Lahsen and Iddya, 2014). The risks of potential contamination, unpleasant odors, and multifarious purification procedures further impact the fish oils market (de Oliveira Finco et al., 2017). Therefore, microbial sources of PUFA are currently being developed as an alternative source (Shi et al., 2017). Of these, thraustochytrids, a unicellular heterotrophic fungus-like marine protist (Leyland et al., 2017), are becoming an increasingly promising source of PUFA on account of their fast growth rate and high content of DHA (Aasen et al., 2016).

Much work focused on the thraustochytrid culture optimization demonstrated significant improvements in their biomass and DHA yields (Chen et al., 2016; Jiang et al., 2017; Ludevese-Pascual et al., 2016; Lung et al., 2016; Patil and Gogate, 2015; Zhao et al., 2017; Zhu et al., 2008). The biomass can reach up to 171.5 g/L under the optimal cultivation condition (Bailey et al., 2003), and with modified medium the total fatty acids (TFA) could account for 83.84% of biomass (Li

* Corresponding author at: Center for Marine Environmental Ecology, School of Environmental Science and Engineering, Tianjin University, Tianjin 300072, China.
E-mail address: gywang@tju.edu.cn (G. Wang).
[1] These authors contributed equally to this work.

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et al., 2015) with PUFA content as high as 76.5% (Marchan et al., 2017). Interestingly, from these studies, it was evident that cell proliferation and fatty acids (FA) yield are enhanced under an oxygen-rich environment (Jakobsen et al., 2008; Ren et al., 2010); however, PUFA gets easily oxidized due to their high degree of unsaturation (Else and Kraffe, 2015; Guichardant et al., 2011). Thus, lipid peroxidation not only reduces the content of PUFA but also activates the accumulation of high levels of reactive oxygen species (ROS) (Johansson et al., 2016; Ruenwai et al., 2011). Besides lipids, these ROS cause severe oxidative damage to proteins and DNA resulting in the loss of protein function and even cell death (Shi et al., 2017). However, they could be scavenged by different antioxidative defense components under a steady physiological state (Finkel and Holbrook, 2000). Both non-enzymatic and enzymatic mechanisms have been implicated in the detoxication process of ROS (Apel and Hirt, 2004; Gupta et al., 2013; Sun et al., 2016). Based on these mechanisms, several strategies have been adopted to enhance removal of ROS (Apel and Hirt, 2004).

Apart from the external addition of antioxidants (Ren et al., 2017), overexpression of antioxidative enzymes also seems to be an alternative way of alleviating ROS. Of these enzymes, superoxide dismutase (SOD) acts as the first line of defense against ROS (Yanase et al., 2009) and converts superoxide radical (O_2^-) into hydrogen peroxide (H_2O_2). Various transgenic plants that expressed increased amounts of SOD have been developed in past to better tolerate the oxidative stress (Tang et al., 2006; Zambounis et al., 2002). Overexpression of *sod1* gene and *sod2* gene in *Streptomyces peucetius* enhanced secondary metabolites as a result of the increased transcriptional level of regulatory genes and biomass increment (Bashistha et al., 2011). Although metabolic engineering seemed an effective and promising way to alleviate ROS, no attempts have yet been made, to our knowledge, which demonstrates the effect of oxidative stress in thraustochytrids at the molecular level. Despite the knowledge of a versatile transformation system for thraustochytrids (Hong et al., 2013; Sakaguchi et al., 2012; Sun et al., 2015), only a few studies were focused on the improvement of FA productivity through gene manipulation (Table S1). Up to now, there are no comprehensive studies focused on ROS quenching to enhance PUFA productivity in thraustochytrids.

To elucidate the effect of oxidative stress alleviation on PUFA production, we first designed a genetic transformation system for a previously isolated high PUFA yielding thraustochytrid strain – *Schizochytrium* sp. PKU#Mn4 (Liu et al., 2014). In an attempt to alleviate oxidative stress, we integrated the SOD1 gene overexpression cassette driven by a newly isolated promoter into the PKU#Mn4 genome at the 18S rDNA loci. The recombinant strain exhibited increased PUFA production resulting from ROS alleviation and increased total antioxidant capacity (T-AOC). Further transcriptomic analysis revealed strong regulation of key genes implicated in the FA metabolism. This study highlights an alternative direction for enhanced PUFA productivity in thraustochytrids and provides the framework for a cost-effective industrial fermentation process.

2. Materials and methods

2.1. Strains and culture conditions

Schizochytrium sp. PKU#Mn4 (GenBank accession number JX847360), previously isolated from mangrove leaves from Pearl River Delta region of China (Liu et al., 2014), was used in this study. The strain was maintained on 2% modified Vishniac's (MV) agar plates (Damare and Raghukumar, 2006) prepared with artificial seawater (ASW) (Nagano et al., 2009) at room temperature and subcultured every 4 weeks. The seed culture was prepared by cultivating a single colony from the agar plate in M4 medium (20 g/L glucose, 0.25 g/L KH_2PO_4, 1.5 g/L peptone, 1 g/L yeast extract, dissolved in ASW, pH = 7) (Jain et al., 2005) at 28 °C on an orbital shaker at 170 rpm for 36 h. The seed culture was subsequently transferred to 100 mL shake flask containing 40 mL fresh M4 medium at a final concentration of 5%, which was then cultivated on an orbital shaker for 4 days under the same conditions until harvest.

2.2. Construction of EGFP and superoxide dismutase (SOD) expression cassettes

We identified a polyubiquitin promoter region with the length of 1484 bp in the whole genome dataset of *Schizochytrium* sp. PKU#Mn4. The *egfp* gene was expressed under this promoter region. Polyubiquitin promoter was amplified from PKU#Mn4 genomic DNA using primers pup-F2 and pup-gfp-R, *egfp* gene was amplified from PX458 (plasmid #48138, Addgene, USA) with primers pup-gfp-F and gfp-cyc-R, and the CYC1 terminator was amplified from pGAPZαA (Invitrogen, USA) with gfp-cyc-F and cyc-sc-R (Table S2). The three fragments (polyubiquitin promoter, *egfp* gene, and CYC1 terminator) were then ligated seamlessly by Overlap Extension PCR with the outer primer pair pup-FU-BamHI/cyc-FU-SalI and designated PGC. Vector pMG201M (Dubeau et al., 2009), containing the neomycin-resistant gene (*neo*^{r}), was digested with BamHI to remove the *codA* gene and designated pNeoR. The PGC segment was subcloned into pNeoR at the BamHI and SalI site and designated pNeoR-PGC, which was used for transformation and EGFP gene expression (Fig. S1a). To confirm the fragments obtained by fusion PCR, they were ligated to pEASY-Blunt vector (TransGen Biotech Inc., Beijing) and transformed into *Escherichia coli* DH5α (Tiangen Inc, Beijing). The plasmid was extracted using TIANprep Mini Plasmid Kit (TIANGEN Inc., Beijing) and sequenced with primers M13F and M13R at AuGCT Inc., Beijing.

The SOD overexpression cassette was integrated into the genome by 18S rDNA homologous recombination. The *sod1* gene was amplified from PKU#Mn4 genome with pup-sod-F and sod-cyc-R and was ligated seamlessly with polyubiquitin promoter and CYC1 terminator by Overlap Extension PCR. The 18S rDNA fragment of PKU#Mn4 was amplified using primers 18S001 and 18S13 (Honda et al., 1999) and cloned into pGM-T vector (Tiangen, China). The plasmid was designated pGMT-18S. The insert DNA was confirmed by sequencing (AuGCT Inc., Beijing) pGMT-18S with primers M13F and M13R. The neomycin resistant gene (*neo*^{r}) was amplified from pNeoR with primers NeoR-F-XbaI and NeoR-R-PstI. The *neo*^{r} gene and *sod1* expression cassette were cloned into pGMT-18S and designated pGMT-18S-NeoR-PSC (Fig. 2**b**). The resulting PCR fragments were confirmed by sequencing as described above. The vector pGMT-18S-NeoR-PSC was linearized with ApaI REase digestion before transformation. The PCR protocols used in this study are provided in Table S3.

2.3. Preparation of competent cells, transformation, and screening

The seed culture, prepared as above, was transferred to fresh M4 medium and grown to the exponential phase (OD_{660} = 7–8). One mL culture was centrifuged (4000 × *g*, 4 °C, 10 min) and the resulting cell pellet was washed twice with the ice-cold sterile water, then treated with 25 mM DTT in 10 mM PBS for 10 min and washed with 1 M ice-cold sterile sorbitol to remove the remaining DTT. The cells were then placed into 2 mL enzyme medium and incubated at 28 °C for 5 h to further weaken the cell wall. The enzyme medium (Cheng et al., 2012) with modification, containing 0.7 M KCl, 20 g/L pectinase, and 20 g/L snailase, was prepared with 10 mM PBS and sterilized by filtration. Cells were then collected from enzyme medium by centrifugation and washed twice with 1 M ice-cold sterile sorbitol to remove the remaining medium. A 250 μL aliquot of ice-cold sterile sorbitol was added to the resuspended competent cells. Approximate 5×10^6 competent cells were mixed with around 2 μg circular or linearized plasmids in a 0.1 cm cuvette for electroporation. Electroporator (Bio-Rad, Gene Pulser Xcell, USA) were set to exponential decay, 1000 V, 50 μF and 500 Ω.

One mL fresh M4 medium was added immediately after electroporation and the resulting cells were incubated at 28 °C for 20 h before