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Enhancement of lipid production in *Synechocystis* sp. PCC 6803 overexpressing glycerol kinase under oxidative stress with glycerol supplementation

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ABSTRACT

In this study, the effect of glycerol kinase overexpression in *Synechocystis* sp. PCC 6803 on lipid content was investigated. The glycerol kinase overexpressing *Synechocystis* cells (OE) had a higher lipid content than the wild type. The OE treated with phenol up to 1 mM showed a slight increase in the cell biomass whereas the total lipid production increased considerably (0.39 ± 0.012 g/L) as compared to that of the wild type (0.26 ± 0.01 g/L). The supplementation of 12 g/L glycerol to BG11 medium increased the lipid content of phenol treated OE from 22 to 35% with the increase of lipid production from 0.39 ± 0.012 to 0.69 ± 0.035 g/L. The RT-PCR analysis revealed that the expression of *glpK* was upregulated from 1.3 to 2.4 and from 1.89 to 3.64-fold after phenol treatment and glycerol supplementation respectively.

1. Introduction

The significance of alternative fuel has attracted worldwide attention due to its high demand. The excessive consumption of fossil fuels and increasing global warming leads to the depletion of fossil fuel and increased emissions of greenhouse gas which eventually affects the global climate (Sivaramakrishnan and Incharoensakdi, 2018a). Hence, the future fuel demand draws the attention of researchers towards the renewable feedstock for fuels. The biofuels from cyanobacteria and algae are the important renewable sources which are being used by developed countries (Sivaramakrishnan and Incharoensakdi, 2018b). Cyanobacteria are the attractive prokaryotic bacteria for biofuel production and can be grown in wastewater (Parmar et al., 2011). Cyanobacteria are being considered as the excellent source for the biodiesel production with benefits including the CO_2 mitigation and high oil yield (Karpagam et al., 2015). Moreover, cyanobacterial lipids can be increased by modifying lipid biosynthetic pathway. The *Synechocystis* sp. is the important cyanobacterium, which is the model organism for the photosynthetic prokaryotes. The source and genetic information are already available for the genetic manipulation of *Synechocystis* sp. (Hendry et al., 2016). Various enzymes of lipid biosynthetic pathway have been studied, but no reports were found on glycerol kinase overexpression in *Synechocystis* sp. for the lipid enhancement. Glycerol kinase is an important enzyme catalyzing the conversion of glycerol to glycerol-3-phosphate (first reaction of Kennedy pathway). Overexpression of glycerol kinase improves the lipid production in *Saccharomyces cerevisiae* (Ko et al., 2013).

Nutrient deficiency stress is the common condition to increase the lipid content in cells (Lia et al., 2018). On the other hand, oxidative stress which produces reactive oxygen species (ROS) can also enhance the lipid content as reported in the *Scenedesmus* sp. treated with H_2O_2 (Sivaramakrishnan and Incharoensakdi, 2017a). Several chemicals are involved in the oxidative stress, whereas no literature is available concerning the phenol induced oxidative stress effect on lipid content. Phenol is the strong oxidant which readily forms phenoxy radicals (ROS). The ROS generated by phenol causes lipid peroxidation and damages the cell membranes (Paliwal et al., 2015). In living organisms there are various antioxidant enzymes such as superoxide dismutase, catalase, glutathione-*S*-transferase and ascorbate peroxidase to defend against the oxidative stress (Silvaa et al., 2018). Apart from the stress induction, supplementation of valuable nutrients also improves the biomass and lipid content. Heterotrophic cultivation of *Chlorella* sp. using crude glycerol efficiently produces the biodiesel (Katiyar et al., 2017).

This work focuses on the engineering of *Synechocystis* sp. to produce high lipid content by glycerol kinase gene overexpression. So far, there has been no report on the glycerol kinase overexpression in *Synechocystis* sp. The overexpression of glycerol kinase in marine diatom *Fistulifera solaris* JPCC DA0580 improves the lipid productivity by 12% (Muto et al., 2015). The glycerol kinase involved in diglyceride

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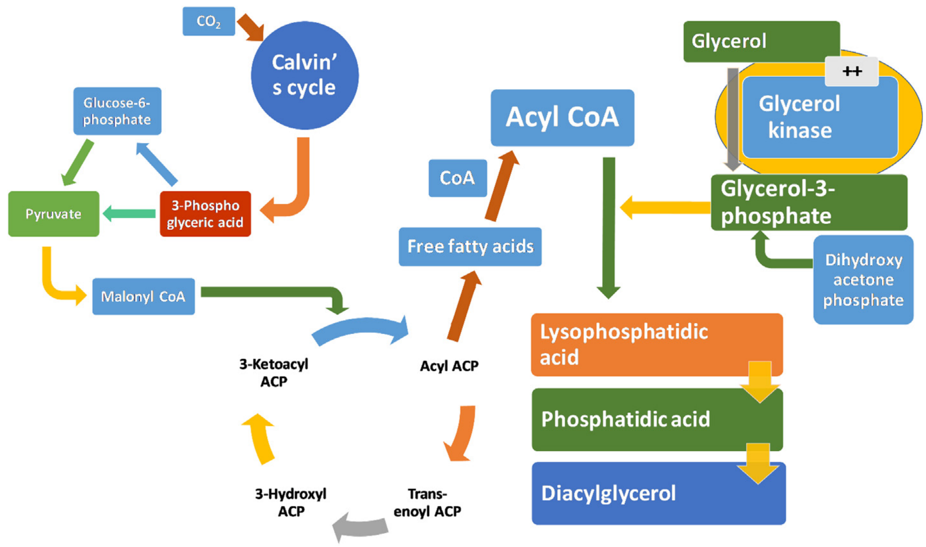

Fig. 1. The schematic representation of hypothetical glycerol kinase pathway (Abbreviation: ACP-Acyl carrier protein; CO_2-carbon dioxide).

synthesis pathway is presented in Fig. 1 (modified from Wang et al., 2016). In this study, the copy of glycerol kinase (*glpK*) gene was integrated into *Synechocystis* sp. to produce glycerol kinase overexpressing strain. The influence of glycerol kinase overexpression on lipid content in *Synechocystis* sp. was investigated. Additionally, the effect of phenol induced oxidative stress together with glycerol supplementation on the lipid content of *Synechocystis* sp. overexpressing glycerol kinase was determined. Oxidative stress is necessary to improve the fatty acid content which can induce more lipid storage by diglyceride synthesis, whereas glycerol serves as substrate to produce glycerol-3-phosphate which is the first precursor for the synthesis of diglyceride. Hence, it is likely that the improved fatty acids content by oxidative stress coupled with glycerol supplementation in glycerol kinase overexpressing strain can improve its overall lipid content.

2. Materials and methods

2.1. Strains and culture conditions

Synechocystis sp. (*Synechocystis* PCC 6803) was obtained from the Pasteur Institute, France and grown in BG11 medium (Rippka et al., 1979; Sivaramakrishnan and Incharoensakdi, 2017b) 30 °C on a shaker at 160 rpm with continuous radiance at 50 µmol photons/m^2/s using fluorescent white light. For the screening of engineered strains, 30 µg/ml of chloramphenicol was used on agar BG11 plates. The screened engineered strain was also grown with the same concentration of chloramphenicol in BG11 medium. All the cyanobacterial cultures with the initial OD_{730} of 0.1 under ambient CO_2 condition were used. For the glycerol supplementation experiments, different concentration of glycerol was added to the BG11 medium. The *Escherichia coli* DH 5α used to construct the plasmids was grown in LB medium at 37 °C.

2.2. Plasmid construction

The overexpression of the slr1672 *glpK* gene, which is involved in the initial step of diglyceride synthesis in *Synechocystis* sp., was constructed using pEERM plasmid. The gene sequence was obtained from the Cyanobase – Kazusa Genome Resources and selected with the functional specifications. The pEERM plasmid contained a chloramphenicol resistance cassette and the *psbA2* flanking region, which serve as a strong promoter for the *Synechocystis* sp. The *glpK* gene was amplified by PCR from corresponding genomic DNA (*Synechocystis* sp.) with specific primers (*glpK*_F: AGCGACTAGTATGACAGCAAAACATA ATC with *SpeI* restriction site and *glpK*_R: CCGCTGCAGTCACTGGTCA ACG with *PstI* restriction site). The amplified *glpK* PCR products were digested and ligated into the pEERM cloning vector at the *SpeI* and *PstI* restriction sites of plasmid. The flanking region of *psbA2* region in pEERM with *glpK* fragment (pEERM_ *glpK*) was used to replace the native *psbA2* in the *Synechocystis* sp. The ligated plasmids were then transferred to DH5α cells by the heat shock method. The plasmid containing DH5α was further cultivated in LB medium containing chloramphenicol. The plasmids were extracted from DH5α cells (overnight culture) by using a plasmid extraction kit (Geneaid Biotech).

2.3. Transformation of Synechocystis sp. with pEERM_glpK plasmid

The transformed *Synechocystis* sp. containing *glpK* was obtained by natural transformation. The *Synechocystis* sp. culture (OD_{730} of 0.4 to 0.5) was centrifuged (2790 g) and the harvested cells were resuspended in 500 µl of fresh BG11 medium without antibiotic in a microcentrifuge tube. The 10 µg/10 µl plasmid was added to the prepared cell suspension and mixed gently. The mixture was kept under the fluorescent white light for 6 h with gentle mixing every 2 h for the natural transformation. After 6 h incubation the cell suspension was spread on a BG11 medium plate containing chloramphenicol (30 µg/ml). The engineered cells were obtained from plates within 2 to 3 weeks of incubation. The obtained engineered colonies were grown for seven generations to ensure the cells survival.

2.4. Biomass, carbohydrate, protein and lipid determination

The biomass content was determined by measuring dry cell weight (DCW) (Sivaramakrishnan and Incharoensakdi, 2017b). Carbohydrate content analysis was done by phenol-sulfuric acid method (Sivaramakrishnan and Incharoensakdi, 2017b). Protein content was determined by Bradford method (Bradford, 1976) and the lipid was extracted by using chloroform/methanol (1:2) (Sheng et al., 2011). The oil extraction yield was calculated using the Eq. (1)

$$\text{Oil yield (\%)} = \frac{\text{Weight of lipid extract (g)}}{\text{Weight of algal biomass (g)}} \times 100 \quad (1)$$

2.5. Phenolic treatment

The phenolic treatment experiments were conducted in BG11 medium supplemented with different concentrations of phenol (0, 0.5,