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Short communication

Photosynthetic production of glycerol by a recombinant cyanobacterium

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ABSTRACT

Cyanobacteria are prokaryotic organisms capable of oxygenic photosynthesis. Glycerol is an important commodity chemical. Introduction of phosphoglycerol phosphatase 2 from *Saccharomyces cerevisiae* into the model cyanobacterium *Synechocystis* sp. PCC6803 resulted in a mutant strain that produced a considerable amount of glycerol from light, water and CO_2. Mild salt stress (200 mM NaCl) on the cells led to an increase of the extracellular glycerol concentration of more than 20%. Under these conditions the mutant accumulated glycerol to an extracellular concentration of 14.3 mM after 17 days of culturing.

1. Introduction

Cyanobacteria are prokaryotic organisms capable of carrying out oxygenic photosynthesis. They are widely distributed in nature (Whitton, 2012) and have very simple growth requirements (Rippka et al., 1979).

Glycerol is a commodity chemical that is routinely used as a solvent, lubricant, humectant or sweetener, amongst other applications. It is a versatile building block in chemical synthesis (Jérôme et al., 2008) and can be used as a carbon source by many chemo-heterotrophic organisms (Da Silva et al., 2009). Furthermore, numerous processes have recently been developed to convert glycerol into higher value-added compounds (Mattam et al., 2013). Traditionally, fermentative processes would be used for glycerol production from sugars, based on the metabolic capacities of yeasts such as *Saccharomyces cerevisiae* (Wang et al., 2001). In recent years, glycerol has been produced instead as a by-product of biodiesel formation. However, the production of plant-derived biodiesel competes with the use of arable land for food production, and therefore ultimately with food supply.

The development of fully sustainable production processes is highly desirable, preferably those that utilize readily available carbon and energy sources, such as CO_2 and sunlight, and that do not require arable land. Within this context, it would be highly pertinent to engineer glycerol producing cyanobacteria that would convert CO_2 and water into glycerol fueled by (sun)light.

Many yeasts (e.g. *Saccharomyces cerevisiae*) synthesize glycerol in response to adverse environmental conditions, most notably osmotic stress, exploiting its natural properties as an osmoprotectant (Blomberg and Adler, 1992). Additionally, it can also be accumulated extracellularly when dihydroxyacetone phosphate (DHAP) functions as a physiological electron acceptor under anaerobic conditions (Van Dijken and Scheffers, 1986). In either case, glycerol production occurs from the conversion of DHAP in a two-step pathway. Firstly, DHAP is reduced to glycerol-3-phosphate (G3P) in an NADH-dependent reaction, catalyzed by one of the two isozymes of glycerol-3-phosphate dehydrogenase (Gpd 1 and Gpd 2). The subsequent dephosphorylation of G3P, yielding glycerol, can be catalyzed by two alternative phosphoglycerol phosphatase isoforms (*gpp1* and *gpp2*, encoding Gpp1p and Gpp2p, respectively) that are present in *S. cerevisiae*. Expression of *gpp1* is increased under anaerobic conditions (Påhlman et al., 2001), while the levels of phosphoglycerol phosphatase 2 (Gpp2p) are increased in response to osmotic stress (Norbeck et al., 1996). When facing hyperosmotic conditions, the cyanobacterium

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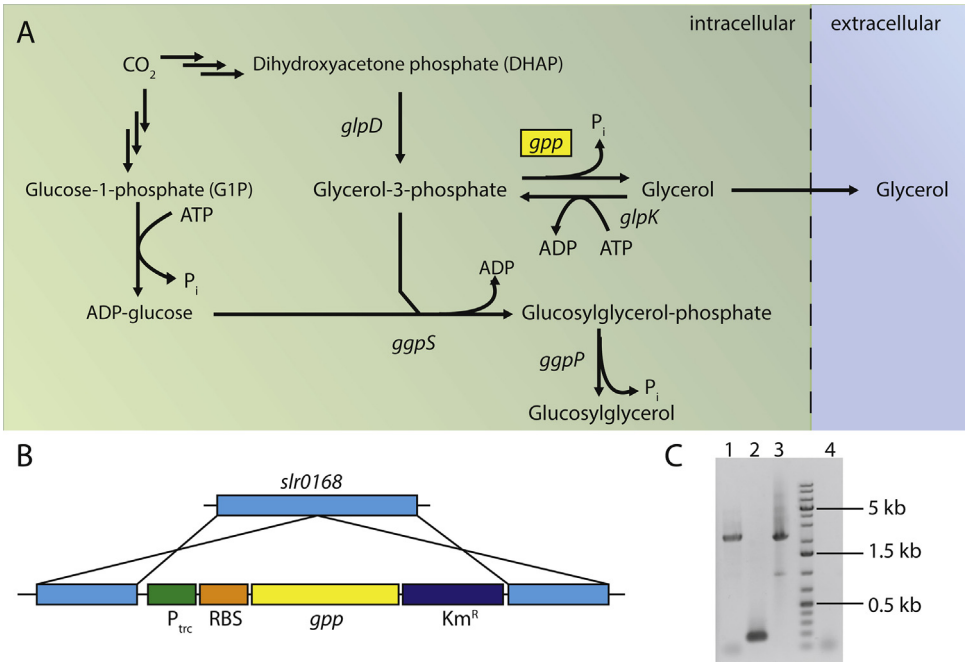

Fig. 1. (A) Simplified view of conversion of CO_2 into glycerol and glucosylglycerol in *Synechocystis*. (B) Schematic representation of the genetics of the construction of the glycerol-producing mutant. (C) Colony PCR of the glycerol producing strain. 1: PSA002; 2: WT *Synechocystis*; 3: pPSgpp2 (positive control); 4: no template (negative control). The primers used were H1 seg F and H2 seg R.

Synechocystis sp. PCC6803 (*Synechocystis*) accumulates the compatible solute glucosylglycerol in the cytoplasm (Richardson et al., 1983). This osmoprotectant is derived from glycerol-3-phosphate (G3P) and ADP-glucose. Glucosylglycerol phosphate synthase (GgpS) catalyzes the glycosylation of glycerol-3-phosphate to yield glucosylglycerol phosphate. The subsequent dephosphorylation of the latter metabolite to glucosylglycerol is catalyzed by glucosylglycerol phosphate phosphatase (GgpP) (Hagemann and Erdmann, 1994) (Fig. 1A).

Here, we apply a combination of different metabolic engineering approaches to the green biotechnology workhorse *Synechocystis*, aiming at the direct conversion of CO_2 into glycerol. First, we use genetic engineering by expressing a heterologous enzyme catalyzing the dephoshorylation of glycerol-3-phosphate, which leads to an extracellular accumulation of glycerol up to 11.6 mM after 17 days. We then resort to the manipulation of environmental conditions to further increase glycerol production. We impose salt stress with the goal of increasing intracellular glycerol-3-phosphate concentrations. Serendipitously, this was found to already lead to the extracellular accumulation of glycerol in the wildtype strain of *Synechocystis*. We subsequently confirm that these two effects are additive and can be combined, increasing production of glycerol during a similar period by more than 20%. As *Synechocystis* does not require a complex medium for growth, the resulting glycerol is produced in a chemically simple matrix, which will facilitate subsequent downstream processing (Fig. S1) (Samul et al., 2014).

2. Results

2.1. Mutant construction

Wild type *Synechocystis* was transformed with plasmids pPSgpp1 and pPSgpp2, harboring the respective phosphoglycerol phosphatase under control of the strong, constitutive, P_{trc} promoter and a kanamycin resistance cassette. This vector allows for chromosomal integration at the neutral *slr0168* locus (Williams, 1988) (Fig. 1B). Genetic analysis with PCR revealed that transformants harboring *gpp1* grew slowly, and were difficult to segregate (data not shown). In contrast, transformants harboring *gpp2* segregated more readily (Fig. 1C).

2.2. Photosynthetic glycerol production

The resulting mutant, termed *Synechocystis* PSA002 (see Table 1), excreted glycerol to high concentrations into the culture medium, leading to as much as 11.6 mM in a period of 17 days after inoculation (Fig. 2), when the biomass in the culture increases gradually to about 2 gram dry weight per liter. In addition, after prolonged serial cultivation for nearly 20 generations, the glycerol-production phenotype of *Synechocystis* PSA002 remained unaltered, as indicated by the constant ratio of glycerol produced over biomass formed (Fig. 3).

Glycerol was reported to be toxic to *Synechocystis* already at very low concentrations (Ortega-Ramos et al., 2014). Our initial result (see above) could not confirm this. Therefore, we investigated glycerol tolerance of our wild type strain (Fig. 4). It can clearly be seen that we could only observe an inhibitory effect at glycerol concentrations higher than 125 mM. In the study by Ortega-Ramos

Table 1
Strains and primers used in this study.

Strain	Genotype	Comment
Synechocystis sp PCC6803		Obtained from D. Bhaya, Stanford University.
Synechocystis Syn PSA001	Δ*slr0168::Ptrc::gpp1::KmR*	Full segregation could not be achieved.
Synechocystis Syn PSA002	Δ*slr0168::Ptrc::gpp2::KmR*	

Oligonucleotide name	Sequence 5′–3′
H1 seg F	TGTCGCCGCTAAGTTAGA
H2 seg R	CTGTGGGTAGTAAACTGGC