Enhanced productivity of extracellular free fatty acids by gene disruptions of acyl-ACP synthetase and S-layer protein in *Synechocystis* sp. PCC 6803

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**Abstract**

**Background**: Based on known metabolic response to excess free fatty acid (FFA) products, cyanobacterium *Synechocystis* sp. PCC 6803 preferentially both recycles via FFA recycling process and secretes them into medium. Engineered cyanobacteria with well growth and highly secreted FFA capability are considered best resources for biofuel production and sustainable biotechnology. In this study, to achieve the higher FFA secretion goal, we successfully constructs *Synechocystis* sp. PCC 6803 mutants disrupting genes related to FFA recycling reaction (*aas* gene encoding acyl–acyl carrier protein synthetase), and surface layer protein (encoded by *sll1951*).

**Results**: Three *Synechocystis* sp. PCC 6803 engineered strains, including two single mutants lacking *aas* (KA) and *sll1951* (KS), and one double mutant lacking both *aas* and *sll1951* (KAS), significantly secreted FFAs higher than that of wild type (WT). Certain increase of secreted FFAs was noted when cells were exposed to nitrogen-deficient conditions, BG11-half N and BG11-N conditions, with the exception of strain KS. Under BG11-N condition at day 10, strain KAS strikingly secreted FFAs products up to 40%w/DCW or 238.1 mg/L, with trace amounts of PHB. Unexpectedly, strain KS, with S-layer disruption, appeared to have endured longer in BG11-N growth medium. This strain KS significantly acclimated to the BG11-N environment by accumulating a greater glycogen pool with lower FFA production, whereas strain KA favored higher PHB and intracellular lipid accumulations with moderate FFA secretion.

**Conclusions**: Mutations of both *aas* and *sll1951* genes in *Synechocystis* sp. PCC 6803 significantly improved the productivity of secreted FFAs, especially under nitrogen deprivation.

**Keywords**: Free fatty acid secretion, *Synechocystis* sp. PCC 6803, S-layer protein, Acyl–acyl carrier protein synthetase, Nitrogen deprivation

**Background**

Despite the fact that biofuels presently are more expensive than fossil fuels, their production is growing at an exponential rate across the world. The biotechnological use of cyanobacteria for biofuel production has been classified as third and fourth generations of bioresources generating products, such as biodiesel, alka(e)ne, polyhydroxybutyrates (PHB), fatty alcohols, and energy-containing biomolecules of fatty acids and lipids [1–4]. In the field of biofuel biotechnology, the capacity of cyanobacteria to secrete free fatty acids (FFA) into the growth medium has shown to be useful in omitting the biofuel extraction process. Known strategies to enhance FFA secretion in cyanobacteria and green algae involves...
stressed environment effect, such as osmotic pressure, temperature, pH, and deprived nutrients, or genetically metabolic engineering, or a combination of the two [5–9]. In cyanobacteria, the cellular response mechanisms to FFAs toxicity as a result of accumulations are FFA secretion, FFA recycling, storage [9], and FFA degradation found in yeast and bacteria [10–12]. Genetically modified cyanobacteria with increased FFA secretion have been mainly observed when overexpressing genes related to thioesterase (tesA), catalyzing the conversion of fatty acyl–acyl carrier protein (ACP) to FFA [13], or lipase A (lipA), catalyzing membrane lipid degradation [9] as well as when disrupting aas encoding fatty acyl-ACP synthetase [9, 14, 15]. On the other hand, weakening cell walls of *Synechocystis* 6803 resulted in increased FFA secretion by disturbing genes related to the surface protein S-layer and the peptidoglycan assembly protein, PBP2 [13].

In cyanobacteria, the main substrate for FFA production is acetyl-CoA, a pyruvate intermediate, which is further converted in various pathways, such as the TCA cycle, polyhydroxybutyrate (PHB) synthesis, and fatty acid synthesis via FASII, see Fig. 1. The fatty acyl-ACP intermediate from the FASII system is converted to membrane lipids by phosphotransacylase-type enzymes PIsX (sll1560), PIsC (sll1848), and PIsY [6, 13, 16]. For membrane lipid hydrolysis, the lipase A enzyme, encoded by *lipA* (sll1969), is capable of releasing free fatty acids inside the cells [3, 8, 9, 17]. The FFAs recycling to fatty acyl-ACP occurs via a fatty acyl-ACP synthetase, encoded by aas (sll1609) [3]. Moreover, excess of FFAs may be secreted by rapidly flip–flopping the un-ionized form of FFA through protein channels of membranes, such as efflux transmembrane transporters (sll0180 and sll2131) [18, 19]. For the surface layer (S-layer) on cell walls of cyanobacteria, its disruption results in increased FFA secretion [13]. The functions of S-layer proteins are mainly involved in carbon capture and storage (CCS) and CO₂ diffusion through the cell membranes in relation to bicarbonate (HCO₃⁻) in *Synechocystis* sp. PCC6803 [20]. This S-layer protein has a supportive role for cell wall integrity in *Synechocystis* without any lethal effect in a Δsll1951 strain [21]. The carbon storage form of glycogen, glyco-gen is synthesized from glucose-1-phosphate (G1P) and ADP-glucose intermediates via glucose-1-phosphate acetyltransferase (*gac*) and glucose synthase (*gla1* and *gla2*), respectively, whereas its degradation is catalyzed by glycan phosphorylase (*gxp*) and isomerase (*gxl*) [22]. Under nitrogen deficiency condition, the glycogen pool may eventually be degraded to produce the other carbon storage form polyhydroxybutyrate (PHB) [22, 23]. To cope with environmental stresses with induced cells accumulating energy storage, the cyanobacterial PHB is preferentially produced from acetyl-CoA through multiple enzymes including acetyl-CoA acetyltransferase (*phaA*), acetoacetyl-CoA reductase (*phaB*), and the heterodimeric PHB synthase (*phaEC*) [24–27].

In this study, we successfully created genetically engineered *Synechocystis* sp. PCC 6803 strains with high production of FFAs secreted into the growth medium using knockout (K) mutations of *aas* (A) and/or sll1951 (S), genes encoding fatty acyl-ACP synthetase and S-layer protein, respectively, resulting in strains KA, KS, and KAS. We discovered that a considerable long-term adaptation of the KS strain to nitrogen deprivation (BG₁₁-N) resulted in increased glycogen storage with a comparable PHB pool and decreased FFA production when compared to *Synechocystis* sp. PCC 6803 wild type (WT) cells. Interestingly, the double mutant of the KAS strain released at least 5 times more FFAs than wild type cells while having the lowest PHB accumulation during nitrogen deprivation. The KA strain accumulated more intracellular lipids than the KAS strain, but secreted less FFA. Among all strains investigated, the KA strain showed the highest level of PHB under BG₁₁-N condition.

**Results**

*Synechocystis* sp. PCC 6803 engineered strains and their growth under stress conditions

First, the sll1951 gene of WT and KA strains (Table 1) was disrupted through the integral insertion of a 0.9 kb fragment of a kanamycin cassette gene (*kmr*) to generate a knockout of sll1951 (KS) and a knockout of aas/sll1951 (KAS) strains (Fig. 2A). To confirm the segregation and location of the insertions (Fig. 2B, C), PCRs using gDNA of each strain as template and selected specific primers were performed (Table 2). Both strains KS and KAS contained the *kmr* fragment with a size of about 0.9 Kb, compared to those of WT and KA with *kmr* fragment, Fig. 2B-a, C-a. In addition, PCR products with SII1951_F and SII1951_R primers confirmed the correct size of 3.0 Kb in strain KS, whereas it was 2.1 Kb in WT (Fig. 2B-b). The SII1951_UF and Km_SR primers confirmed the expected size of about 1.1 Kb in strain KAS comparing with no band in WT (Fig. 2C-b). When we amplified the fragment by primers SII1951_UF and SII1951_R, the PCR products gave a 3.2 Kb band in both KS and KAS strains, while it showed a 2.3 Kb band in the WT (Fig. 2B-c, C-c).

Cell growths of the KA and KAS strains were lower than that of the wild type (WT) cells under BG₁₁ growth conditions, although the KS strain exhibited a similar tendency as WT (Fig. 3A). It was intriguing to see that the oxygen evolution rates of all engineered strains were significantly higher than those of WT cells (Fig. 3B). Furthermore, the KS strain accumulated equivalent levels of chlorophyll *a* and carotenoids as the WT strain (Fig. 3C,
D). The KA and KAS strains showed lower quantities of chlorophyll $a$ and carotenoids, in agreement with their respective growth. On the other hand, all strains could grow similar to WT in BG11 with half concentration of NaNO$_3$ (BG$_{11}$-half N), with the exception of the KAS strain, which showed a slightly lower growth after 9 days (Fig. 4A). Under this growth condition, the KA and KS strains contained more chlorophyll $a$ and carotenoids after 9 days (Fig. 4B, C). Images of cell culture in BG$_{11}$-half N clearly demonstrated that strain KAS showed a lighter green color than the other strains (Fig. 4D), reflected in a lower chlorophyll $a$ content (Fig. 4B). In line with growth and chlorophyll $a$ content, KS and KA cell cultures showed a more deep green color under half
N growth condition. When BG11 lacking NaNO₃ condition (or BG11-N) was applied to all strains (Fig. 5). Strain KS showed the highest growth level (Fig. 5A). The chlorophyll a levels were comparable between the strains, with the exception of KAS which contained a lower amount (Fig. 5B). However, the carotenoid levels were relatively stable under BG11-N condition (Fig. 5C). It is clear from the images of cell cultures grown in BG11-N that all engineered strains remained green for at least 3 days before becoming yellow compared to WT cells, particularly strain KS (Fig. 5D). The KAS strain had a deep yellowish cell culture from days 5 to 7, whereas strain KS strain showed a deep yellowish cell culture from days 5 to 15.

Contents of intracellular lipids, extracellular FFAs, PHB and glycogen under normal and stressed conditions

All engineered strains secreted more FFAs into the BG11 growth medium, Table 3. Strains KA and KAS notably contained higher total amounts of intracellular lipids and extracellular FFAs by about 35.8 and 39.0%w/DCW, respectively, than WT cells (23.6%w/DCW), in particular at day 5. In addition, we observed that all engineered strains had certain total yields (mg/L) that were higher than WT at days 5 and 10, particularly in strain KAS produced about 178.5 and 336.9 mg/L, respectively (Table 3). After exposing the cells to reduced levels of nitrogen (BG11-half N), all engineered strains produced higher levels of intracellular lipids than observed in WT cells, in particular strain KA at day 5 and KAS at day 10 with 39.0 and 44.8%w/DCW, respectively (Fig. 6A). The certain increase of FFA secretion of all engineered strains was also noted under this condition with the highest level in strain KAS at day 10, about 28.2%w/DCW (Fig. 6B), representing 376.2 mg/L or 53.3 mg/10¹¹ cells (Table 4). On the other hand, when the strains were grown in BG11-N medium, we discovered that the KS strain accumulated extracellular FFAs at the same level, either %w/DCW or mg/L, as the WT, whereas the KA and KAS strains showed increased level (Fig. 6B and Table 4). The results indicate that strain KAS preferentially secreted FFAs into medium up to 40.4%w/DCW or 238.1 mg/L after a long period (10 days) of nitrogen deprived condition (BG11-N) rather than accumulated intracellular lipids (30.4%w/DCW) when compared to strain KA, 45.5 and 18.0%w/DCW% of intracellular lipids and extracellular FFAs contents, respectively (Fig. 6A–C).

We also determined polyhydroxybutyrate or PHB contents of all strains under BG11, BG11-half N, and BG11-N growth conditions at day 10 (Fig. 7A). Unexpectedly, a substantial increase in PHB content occurred in all strains under BG11-N conditions, with the exception of strain KAS, which showed a low level equivalent to that under BG11 condition. Not all strains were affected by the BG11-half N condition, only strain KAS showed a 2.3 fold-increase in PHB accumulation when compared to WT cells. On the other hand, the glycogen content of all engineered strains were higher than in WT cells under BG11 condition, especially in strain KA with 21.4%w/DCW. When the BG11-N condition was applied, the increased levels of glycogen were observed in strains KS and KAS, compared to under BG11 medium. It is interesting that strain KS showed similar glycogen content under both BG11-half N and BG11-N growth conditions.

Table 1 Strains and plasmids used in this study

| Name | Relevant genotype | Reference |
|------|-------------------|-----------|
| Cyanobacterial strains | | |
| Synechocystis sp. PCC 6803 | Wild type | Pasteur culture collection |
| Control WT (WTc) | cmr and kmr integrated at region of native psbA2 gene in Synechocystis genome | [9] |
| KA | cmr integrated at region of native aas gene in Synechocystis genome | [9] |
| KAOL | cmr integrated at region of native aas gene in Synechocystis genome lipA, kmr integrated at region of native psbA2 gene in Synechocystis genome | [9] |
| KS | kmr integrated at region of native sll1951 gene in Synechocystis genome glpD, Rubisco; rbcL, rbcX, rbcS, kmr integrated at region of native Rubisco gene in Synechocystis genome | This study |
| KAS | kmr integrated at region of native sll1951 gene in Synechocystis genome cmr integrated at region of native aas gene in Synechocystis genome | This study |
| Plasmids | | |
| pJSKm | P₁₇₋sll1951-cmr; plasmid containing kmr between the flanking region of sll1951 gene | This study |
Moreover, transcript levels of genes related to fatty acid synthesis, its degradation, PHB synthesis and glycogen degradation were monitored in cells at day 10 of growth (Fig. 8). Under BG11 condition (Fig. 8A), the accA transcript levels, related to the initial step of fatty acid synthesis, were slightly increased in strains KA and KAS. The plsX transcript level, which is related to membrane lipid synthesis, was greatly elevated in strain KAS. The lipA transcript levels, related to membrane lipid hydrolysis, were increased in strains KS and KA but decreased
in strain KAS. For PHB synthesis, the phaA transcript levels were slightly upregulated in all engineered strains corresponded to higher PHB contents when compared to WT cells. The glgX transcript amounts, related to glycogen degradation, showed a significant upregulation in strain KS, whereas decreased levels were observed in strains KA and KAS, compared to WT cells under BG11 growth condition. In addition, similar aas transcript levels, related to FFA recycling reaction, of WT and KS cells were observed. On the other hand, the higher ratio values of transcript/16 s band intensity of accA, aas, phaA and glgX in WT cells were noted under BG11-N condition when compared to those under BG11 condition, in Fig. 8B. The transcript levels of accA, aas, plsX, lipA, phaA and glgX in strain KS were higher than those in WT cells. For strain KA, only the lipA transcript level was increased, whereas similar or decreased levels were observed for the other genes.

**Discussion**

To increase free fatty acid (FFA) secretion, genetically engineered cyanobacteria are considered as a promising option. However, FFAs secretion as a consequence of excessive production of FFAs may generate toxicity and damage the cells by randomly diffuse across the membranes, in particular short chain FFAs, generating reactive oxygen species (ROS) and a highly oxidative stressful environment for the cells [28–30]. Some recent reports addressed the crucial consequences associated with higher FFA secretion after modifying the cyanobacterium *Synechocystis* sp. PCC6803 by gene disruption, such as *aas, sll1951* encoding surface layer (S-layer) protein, and *slr1710* encoding peptidoglycan assembly protein, or by overexpression of heterologous *tesA* encoding thioesterase, or combination strategies of *aas* inactivation either with *tesA* or *lipA* overexpression [9, 13–15]. In this study, we created a *Synechocystis* sp. PCC6803 engineered strain with double gene disruptions of *aas* and *sll1951*, encoding S-layer, resulting in significantly increased secreted FFA content under nitrogen deprived conditions.

The hemolysin-like protein (HLP) Sll1951, surface layer protein (S-layer), is the outermost cell component in archaea and bacteria (Fig. 1). Especially, in Gram-negative bacteria including cyanobacteria, the S-layers are closely associated with the lipopolysaccharide on the outer membrane, while some S-layers in archaea are mushroom-like subunits (reviewed in [31]). Recently, several functions of S-layer in cyanobacteria have been addressed including a barrier against the adsorption of some toxic compounds and antibiotics, such as CdCl₂, CuSO₄, antibiotics (kanamycin, ampicillin), a component related to mobility in some motile species, a template of natural mineral formation process on surface in some species living in high mineral habitats [32–34]. The *sll1951* deletion mutation in *Synechocystis* 6803 had similar growth rate and

### Table 2: Primers used in this study

| Name          | Sequence (5′ to 3′) | Purpose of primer | Expected size | Cycles/Tm | Reference |
|---------------|--------------------|------------------|---------------|-----------|-----------|
| Km_FKpnI      | TAGAGAGTGCTTCTAGAAACATCGAGCA | PCR for km'      | 939           | 30/60.0 °C | This study |
| Km_RKpnI      | TAGAGAGTCTGCTCAAGAATCTGATG   | PCR for km'      | This study    | This study |
| Km_SR         | TAGAGATGACCTGCTACAGGCT       | PCR for km'      | This study    | This study |
| Sll1951_F     | TAGAGAGTGGAAGATGCAAATATACCT | PCR for sll1951  | 1980          | 35/56.3 °C | This study |
| Sll1951_R     | TAGAGAGTGGAAGATGCAAATATACCT | PCR for sll1951  | This study    | This study |
| Sll1951_UF    | TAGAGAGTGGAAGATGCAAATATACCT | PCR for sll1951  | This study    | This study |
| RTGlglX_F360  | GAGCTTACTGCAAGGACGGA        | RT-PCR for glgX  | 360           | 35/56.0 °C | This study |
| RTGlglX_R360  | GCGGACTTCTGCAAGGACGGA       | RT-PCR for glgX  | 30/56.0 °C   | (BG11-N)  |
| RTphaA_F420   | TCAGCGCGGATAGAATTGGAGCAAGATAT | RT-PCR for phaA  | 420           | 35/53.5 °C | (BG11-N)  |
| RTphaA_R420   | CAAACAAGTTCAAATCTGCGAGGGTTT | RT-PCR for phaA  | 30/53.5 °C   | (BG11-N)  |
| RTlipA_F379   | TTGGCGAGCAAGTGAAAACAAATCT | RT-PCR for lipA  | 379           | 34/55.0 °C | (BG11-N)  |
| RTlipA_R379   | CATGGACCGACAGGCAAAATTCACT | RT-PCR for lipA  | 28/55.0 °C   | (BG11-N)  |
| RTaccA_F428   | ATGATCGACGAGGGAGGT          | RT-PCR for accA  | 428           | 35/58.0 °C | (BG11-N)  |
| RTaccA_R428   | TGGAGTAGCAAGGACGGA          | RT-PCR for accA  | 32/58.0 °C   | (BG11-N)  |
| RT16sRNA_F521 | AGTCTGACGAGTAACGTAATGAG    | RT-PCR for 16 s  | 521           | 24/56.0 °C | (BG11-N)  |
| RT16sRNA_R521 | GTCAAGCTTCGTTGTAAGTTAT      | RT-PCR for 16 s  | 22/56.0 °C   | (BG11-N)  |
| RTaas_F307    | GTTGTATATTGCGGATCAAG        | RT-PCR for aas   | 307           | 38/54.5 °C | (BG11-N)  |
| RTaas_R307    | TTCCTCGGGGGGAAAGGGGAGG      | RT-PCR for aas   | 33/54.5 °C   | (BG11-N)  |
| RTPlslX_F     | AAGGGTGTGGGAATGGAAGGA       | RT-PCR for PlsX  | 488           | 35/52.7 °C | (BG11-N)  |
| RTPlslX_R     | AAGTAGGTGCTCCTCTCCG         | RT-PCR for PlsX  | 32/52.7 °C   | (BG11-N)  |
carotenoid content to WT cells under photoautotrophic growth condition [21]. This is in agreement with our result under BG11 growth condition, strain KS or Synechocystis lacking sll1951, grew-like WT cells with similar accumulation of both chlorophyll a and carotenoids, except higher photosynthetic efficiency (Fig. 3). A S-layer disruption in Synechocystis did not generate any severe effects on cell growth and photosynthesis. More strikingly, we observed increased growth of strain KS grown in BG11 without NaNO3 (BG11-N) medium with green colored cell cultures (Fig. 5). As known for cyanobacterial chlorosis process, cells turn blue–green to yellow color during nitrogen deprivation, because phycobilisomes, as well as chlorophyll a, are degraded leading to decreased photosynthetic activities [35–37]. Therefore, our observations indicate that the lack of S-layer may enhance the exchange or transport activities of some essential compounds which consequently helps the cells to survive under nitrogen deprived conditions. Although it was previously shown that the Δsll1951 mutant of Synechocystis sp. PCC 6803 may secret high quantities of protein into the medium [22], further experimental data and research are still needed to determine how nitrogen deprivation and S-layer disruption are related. For the other aspect, the strain KS, Synechocystis lacking the S-layer protein, may thrive better in lower nitrogen environments, since the production of the S-layer protein certainly consumes substantial amounts of nitrogen. In addition, although we found a lower cell growth under BG11 condition of both strains KA, Synechocystis lacking aas gene, and KAS when compared with WT cells, they all showed similar growth under BG11-N condition.

We demonstrate a significant increase of intracellular lipids and FFA secretion in all engineered strains (Table 3). It was worth to note that strain KAS showed the highest capacity to produce total contents of
intracellular lipids and secreted FFAs, about 39.0%w/DCW or 178.5 mg/L at day 5, when compared, e.g., with strain KA [9]. Although strain KS secreted a lower level of FFAs, about 11.1%w/DCW or 15.0 mg/L compared to the other engineered strains, a higher FFA secretion was noted when compared to WT cells at days 5 and 10, in agreement with a previous report of a Δsll1951 strain of Synechocystis sp. PCC 6803 with higher FFA secretion [13]. Under BG11 growth condition, the aas and sll1951 gene disruptions slightly induced PHB accumulations.
when compared to that in WT cells but with a more significant increase in glycogen pool size, particularly in strain KA, about 21.4%w/DCW, 5.7 fold increase compared to WT cells (Fig. 7). This suggests that the deletion of aas, involved in FFA recycling process, can enhance the glycogen accumulation as carbon storage in *Synechocystis*. This is supported by the lower glgX transcript level, related to glycogen degradation, in strain KA compared to that in WT cells (Fig. 8A). For strain KS, the disruption of slt1951 seemed to stimulate glycogen and membrane...
lipid degradation, as evidently demonstrated by high glgX and lipA transcript levels compared to WT cells.

Nutrient (nitrogen) deficiency was addressed in this study to gain more understanding of carbon storage and fatty acid and lipids syntheses by applying BG11-half N and BG11-N growth conditions for 15 days (Figs. 4 and 5). For the BG11-half N condition, increased total contents of intracellular lipids and secreted FFAs were noted in all engineered strains compared to WT cells, particularly in strain KA at day 5 (60.0%/DCW) and KAS (73.0%/DCW) at days 5 and 10, respectively (Fig. 6A, B). Reduced nitrogen level (BG11-half N) did not significantly affect PHB content, except a lower level in strain KS and a higher content in strain KAS (Fig. 7A). The dramatic increase of glycogen accumulation was apparently induced by the lower nitrate condition employed, especially in strain KA (Fig. 7B). These results may suggest that higher glycogen accumulation contributes to higher growth and intracellular pigment contents under limited nitrogen supply, BG11-half N condition (Fig. 4), in agreement with earlier studies on glycogen metabolism under similar level to that of WT for 5 days, with enhanced carbon storages of glycogen, about 7.8 fold increase compared to WT, and decreased PHB levels, about 0.9 fold (Figs. 7 and 9). Only accA transcript level, involved in the initial step of fatty acid synthesis, was upregulated in KS and KAS strains (Figs. 8B and 9). Since nitrogen is a vital element substantially contributed in biomolecules and cofactors, its deficiency considerably affects cellular mechanisms which force cell coping to this stress for prolonging life by mainly synthesizing energy-containing molecules and increasing carbon or nitrogen source storage, such as glycogen, PHB, and lipid [37, 40–43]. It was noted that the KA strain could cope nitrogen deprivation stress by relatively balancing its carbon storages, lipid and fatty acid syntheses, and FFA secretion (Fig. 9). However, the critical issue for FFA-producing cyanobacteria would result in a rich carbon supply for several other microorganisms. Aseptic production strategy on large scale are, therefore, essential for preventing contamination, and continuous fermentation would offer an appropriate solution.

Conclusions

Increased levels of FFA secretion were achieved in engineered strains of Synechocystis sp. PCC 6803 (KA, KS, and KAS) by affecting the aas gene encoding acyl-ACP synthase in FFA recycling and sll1951 gene encoding

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### Table 3

Yields of intracellular lipids and extracellular FFAs of all strains under normal BG11 condition

| Strains | Intrapcellular lipids (A) | Extracellular FFAs (B) | Total (A + B) | Intrapcellular lipids (A) | Extracellular FFAs (B) | Total (A + B) | Note |
|---------|--------------------------|------------------------|--------------|--------------------------|------------------------|--------------|------|
| Start of cultivation |
| WT      | 12.4 ± 1.13               | 5.30 ± 0.22            | 17.7 ± 1.35   | 7.69 ± 0.23               | 0.68 ± 0.05            | 8.37 ± 0.28   | This study |
| KS      | 10.9 ± 0.50               | 4.46 ± 0.12            | 15.4 ± 0.62   | 5.45 ± 0.25               | 0.89 ± 0.02            | 6.34 ± 0.27   | This study |
| KA      | 17.6 ± 0.24               | 4.52 ± 1.17            | 22.1 ± 1.41   | 10.6 ± 0.14               | 1.35 ± 0.35            | 11.9 ± 0.49   | [9]   |
| KAS     | 18.1 ± 1.27               | 7.42 ± 0.66            | 25.5 ± 1.93   | 10.8 ± 1.36               | 2.22 ± 0.20            | 13.0 ± 1.56   | This study |
| Day 5 of cultivation |
| WT      | 16.7 ± 1.37               | 6.8 ± 1.35             | 23.6 ± 2.72   | 126.9 ± 7.26              | 7.81 ± 1.64            | 134.7 ± 8.90  | This study |
| KS      | 17.2 ± 1.34               | 11.1 ± 1.25            | 28.3 ± 2.49   | 154.2 ± 14.6              | 15.0 ± 1.62            | 169.2 ± 16.2  | This study |
| KA      | 20.6 ± 0.65               | 15.2 ± 1.12            | 35.8 ± 1.77   | 137.4 ± 0.57              | 21.1 ± 0.84            | 158.5 ± 14.1  | [9]   |
| KAS     | 23.0 ± 0.48               | 16.0 ± 1.02            | 39.0 ± 1.50   | 155.8 ± 0.87              | 22.7 ± 2.16            | 178.5 ± 3.03  | This study |
| Day 10 of cultivation |
| WT      | 15.5 ± 0.52               | 5.6 ± 0.46             | 21.1 ± 0.97   | 143.2 ± 5.30              | 31.0 ± 1.91            | 174.2 ± 7.21  | This study |
| KS      | 19.2 ± 1.42               | 9.1 ± 0.49             | 28.3 ± 1.91   | 177.1 ± 15.0              | 50.1 ± 2.37            | 227.2 ± 17.4  | This study |
| KA      | 16.4 ± 0.70               | 13.9 ± 0.64            | 30.3 ± 1.43   | 255.2 ± 20.0              | 108.4 ± 11.4           | 363.6 ± 31.4  | [9]   |
| KAS     | 16.3 ± 0.48               | 12.1 ± 1.02            | 28.4 ± 1.50   | 245.2 ± 15.8              | 91.7 ± 10.7            | 336.9 ± 26.5  | This study |

Data represent mean ± S.D., n = 3. For superscript, means with the different letter are significantly different with the significance level at P < 0.05.
surface layer of outer membranes resulting in significant increases of both intracellular lipids and secreted FFAs. Strain KAS with non-functional \textit{aas} and \textit{sll1951}, showed considerably a higher FFA-secreting under both BG11 and nitrogen deprived growth conditions (BG11-half N and BG11-N) with less PHB accumulation.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig6}
\caption{Contents of total intracellular lipids (A) and extracellular FFAs (B), and total contents of total intracellular lipids and extracellular FFAs (C) of WT, KS, KA, and KAS \textit{Synechocystis} sp. PCC 6803 strains growing in BG11-half N and BG11-N at 0, 5 and 10 days, respectively. The error bars represent standard deviations of means (mean ± S.D., \(n=3\)). Means with the same letter are not significantly different with the significance level at \(P<0.05\).}
\end{figure}
Interestingly, disrupting the S-layer did not affect cell growth, it even improved under nitrogen deficiency conditions. FFA-producing and excreting cyanobacterial cells are promising cell factories for biotechnology applications including biofuel production.

### Materials and methods

#### Strains and culture conditions

The host propagation, *Escherichia coli* DH5α strain, was grown either on agar plate or in liquid medium of Luria Bertani (LB) containing 35 µg/mL of kanamycin (Km) and 35 µg/mL of chloramphenicol (Cm) at 37 °C. *Cyanobacterium Synechocystis* sp. PCC 6803 cells were grown in BG11 medium on rotary shaker at 28 °C and continuous light illumination of 50 µmol photons m⁻² s⁻¹. Two engineered strains of *Synechocystis* KA (Δaas) and KAOL (KA with overexpressing lipA) were obtained as described previously [8, 9]. In this study, the Δsll1951 mutant (KS) and Δsll1951_Δaas mutant (KAS) were constructed (Table 1). All strains were cultured in BG11 medium containing 35 µg/mL of kanamycin and 35 µg/mL of chloramphenicol.

#### Constructions of recombinant plasmids

To construct the recombinant pSKm plasmid, plet1.2 blunt end vector was used to insert a kanamycin resistance cassette gene (km) fragment between *sll*1951 sequences. The *sll*1951 fragment with its designed size of about 1980 bp was amplified by PCR using a pair of primers; Sll1951_F and Sll1951_R (Table 2). After that, the *sll*1951 fragment was introduced into a pJet1.2 vector by blunt end ligation generating a pJetS vector. The antibiotic kanamycin resistance cassette gene (km) fragment was amplified by PCR using pEERM_Km vector from the previous study as the template [44], and used Km_FKpnI and Km_RKpnI as the primers (Table 2). Both of km fragment and pletS vector were digested with the same restriction KpnI enzyme and subsequently ligated by T4 DNA ligase. The recombinant plasmid pJSKm was confirmed by agarose gel electrophoresis. To get the recombinant plasmid pJSKm, the constructed pJetS vector was transformed into *Escherichia coli* DH5α competent cells, and plasmid DNA was isolated from E. coli DH5α transformants and analyzed by agarose gel electrophoresis.

#### Table 4

Yields of extracellular FFAs of all strains under normal BG11, BG11-half N, and BG11-N condition

| Strains | Extracellular FFA titer (mg/L) | Extracellular FFA (mg/10¹¹ cells) |
|---------|-------------------------------|-----------------------------------|
|         | Start | Day 5 | Day 10 | Start | Day 5 | Day 10 |
| **BG11-half N condition** | | | | | | |
| WT      | 0.53 ± 0.12<sup>a</sup> | 18.21 ± 6.10<sup>b</sup> | 29.52 ± 5.73<sup>h</sup> | 1.46 ± 0.19<sup>a</sup> | 6.11 ± 1.99<sup>p</sup> | 5.30 ± 1.09<sup>h</sup> |
| KS      | 1.20 ± 0.09<sup>c</sup> | 28.81 ± 6.55<sup>i</sup> | 101.43 ± 8.05<sup>n</sup> | 2.17 ± 0.09<sup>h</sup> | 9.93 ± 2.58<sup>e</sup> | 17.8 ± 1.80<sup>h</sup> |
| KA      | 1.25 ± 0.02<sup>sc</sup> | 92.38 ± 19.25<sup>n</sup> | 182.54 ± 10.80<sup>l</sup> | 2.43 ± 0.59<sup>h</sup> | 34.3 ± 6.80<sup>h</sup> | 32.0 ± 1.05<sup>l</sup> |
| KAS     | 1.73 ± 0.07<sup>n</sup> | 62.38 ± 2.18<sup>l</sup> | 376.19 ± 63.57<sup>l</sup> | 4.04 ± 0.28<sup>c,d</sup> | 20.0 ± 0.64<sup>n</sup> | 53.3 ± 0.46<sup>l</sup> |
| **BG11-N condition** | | | | | | |
| WT      | 0.77 ± 0.08<sup>d</sup> | 6.81 ± 1.66<sup>g</sup> | 30.24 ± 7.33<sup>k</sup> | 1.72 ± 0.18<sup>h</sup> | 13.6 ± 3.01<sup>l</sup> | 15.1 ± 3.39<sup>g</sup> |
| KS      | 1.04 ± 0.15<sup>h</sup> | 8.24 ± 1.20<sup>i</sup> | 32.14 ± 7.50<sup>h</sup> | 2.54 ± 0.38<sup>e</sup> | 7.26 ± 1.29<sup>d</sup> | 7.11 ± 2.06<sup>g</sup> |
| KA      | 1.19 ± 0.02<sup>sc,b</sup> | 34.57 ± 4.88<sup>k</sup> | 65.87 ± 3.64<sup>h</sup> | 2.67 ± 0.08<sup>g</sup> | 28.9 ± 4.78<sup>h</sup> | 32.9 ± 1.72<sup>g</sup> |
| KAS     | 1.73 ± 0.02<sup>n</sup> | 22.86 ± 1.78<sup>i</sup> | 238.10 ± 36.67<sup>l</sup> | 3.94 ± 0.13<sup>c</sup> | 23.9 ± 1.82<sup>i</sup> | 149 ± 22.6<sup>k</sup> |

Data represent mean ± S.D., n = 3. For superscript, means with the different letter are significantly different with the significance level at P < 0.05.

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**Fig. 7** Contents of polyhydroxybutyrate (PHB) (A) and glycogen (B) of *Synechocystis* sp. PCC 6803 WT, KS, KA, KAS, and KAOL strains cultured under BG11-half N and BG11-N at day 10. The error bars represent standard deviations of means (mean ± S.D., n = 3). Means with the same letter are not significantly different with the significance level at P < 0.05.
ligase, and generated the recombinant pJSKM plasmid (Table 1).

**Transformation of Synechocystis cells**

Two host cells including *Synechocystis* sp. PCC 6803 wild type (WT) and KA strains were grown in BG11 medium until an optical density of 0.3–0.5. The cells were harvested by centrifugation at 5000 rpm (2516 × g) for 10 min. The cell pellets were washed by fresh BG11 medium and harvested by centrifugation at 5000 rpm (2516 × g) for 10 min. The 1 µg of recombinant plasmids were separately added into condensed WT and KA cells and incubated at 28 °C for 6 h and inverted the tubes every 2 h. Then, the condensed cells were spread on a 0.45 µm sterile nitrocellulose membrane placed over BG11 agar plate overnight and then transferred that membrane to place over BG11 agar containing 35 µg/mL chloramphenicol or both of 35 µg/mL kanamycin and 35 µg/mL chloramphenicol depending on their host cells. Obtained colonies were collected and examined for gene location and segregation by PCR analysis using specific pairs of primers (Table 2).

**Cell cultivation and nitrogen deficiency treatments**

Cell culture with mid-log phase of growth was harvested by centrifugation at 6000 rpm (3622 × g) for 10 min and transferred into various nitrogen deficiency conditions including BG11 medium containing 17.6 mM NaNO₃.
BG11 medium containing 50% NaNO₃ concentration (8.8 mM NaNO₃) or BG11-half N, and BG11 medium without NaNO₃ (BG11-N). The OD₇₃₀ at beginning of cultivation was about 0.1 and continuously cultured for 15 days.

Determinations of cell growth and pigment contents

*Synechocystis* cell growth was monitored by a spectrophotometer during cultivation. The pigment contents including chlorophyll *a* (chl *a*) and carotenoid were extracted and determined as described previously [45, 46]. One milliliter of cell culture was harvested and centrifuged at 6,000 rpm (3622 × g) for 10 min. *N,N*-dimethylformamide (DMF) was added into a fraction of cell pellets to extract the pigments. After a quick centrifugation, the pigments in the supernatant were determined by measuring the absorbances (Abs) at 461, 625 and 664 nm using a spectrophotometer, and calculated according to [45, 46]. The results are normalized to cell numbers corresponding to 1.0 × 10⁸ of the cells.

Measurement of oxygen evolution rate

Five mL of cell culture were centrifuged at 6000 rpm (3622 × g) for 10 min. Cell pellets were resuspended by adding 2 mL of fresh BG11 medium and incubated in the darkness for 30 min. After that, the cell suspension was measured for oxygen evolution by Clark-type oxygen electrode (Hansatech instruments, UK) at room temperature.
Lipid extraction

Ten mL of cell culture was harvested by centrifugation at 6000 rpm (3622 × g) for 10 min. Lipids, which are represented as intracellular lipids and extracellular free fatty acids, respectively, were extracted from the cell pellets and supernatant fraction. The lipids were extracted according to the Bligh and Dyer method [47] with slight modification. A glass tube containing cell pellets was filled with 1 mL of a 2:1 chloroform (CHCl₃): methanol (CH₃OH) solution, and the supernatant fraction was added with a 5 mL solvent solution. The reaction mixture tube was then incubated in a water bath at 37 °C for 2 h. Then, one mL of 0.88% (v/v) potassium chloride (KCl) was added and vortexed for few seconds. After centrifugation of the reaction mixture tube at 3000 rpm (906 × g) for 5 min, the lower organic phase containing lipids was collected. Then, the chloroform was evaporated at 70 °C.

Determinations of total lipid and free fatty acid contents

The total lipid and extracellular free fatty acid contents were determined by potassium dichromate oxidation reaction method [48]. The 0.5 mL of K₂Cr₂O₇ (0.18 M) and sulfuric acid were added into the glass tube of extracted lipids. The reaction mixture was heated at 105°C for 30 min. After the mixture was cooled down to room temperature, distilled water (0.5 mL) was added before measuring the absorbance at 600 nm (Abs₆₀₀) using spectrophotometer. The canola oil was used as a commercial standard, prepared as same as sample. The unit of lipid content was represented by the percentage of lipids to dry cell weight (%/wDCW). Dry cell weight (DCW) measurement was performed by dehydrating harvested cell pellets in the 60-70 °C oven until obtaining a constant dry weight.

Determination of PHB contents by HPLC

Five mL of cell culture were harvested by centrifugation at 6000 rpm (3622 × g), 10 min. One hundred µL of adipic acid (20 mg/mL) and 800 µL of concentrated H₂SO₄ was added into the tube of cell pellets and further boiled at 100 °C for 1 h for converting of PHB to crotonic acid. After that, 50 µL of the reaction mixture was diluted with 1.20 mL of ultrapure water. Then, one mL of solution was filtered through PP Syringe filter 0.45 microns, 13 mm. and collected in a glass vial for HPLC analysis (Shimadzu HPLC LGE System, Japan). A carbon-18 column with inert sustain 3 µm (GL-Sciences, Japan) was used and performed with a flow rate of 1.0 mL/min. The running buffer was 30% (v/v) acetonitrile in 10 mM KH₂PO₄ at pH 2.3. The amount of crotonic acid was detected at 210 nm of UV detector. The commercial standard of crotonic acid was prepared as same as samples. The PHB content is represented as a percentage of PHB per dried cell weight (%w/DCW).

Determination of glycogen content

One mL of cell culture was harvested by centrifugation at 6000 rpm (3622 × g), 10 min. Cell pellets were collected, and mixed with 600 µL of 30% (v/v) KOH. The mixture was then heated at 90 °C for 1 h. The supernatant was separated by centrifugation at 12,000 rpm (14,489 × g) for 10 min, then it was transferred into a 1.5 mL microcentrifuge tube. After adding 900 mL of the absolute ethanol into the solution tube, it was incubated at −20 °C for overnight to precipitate glycogen. The glycogen sediment fraction was harvested by centrifugation at 12,000 rpm (14,489 × g) 4 °C for 10 min, and completely dried at 60 °C for overnight. After that, the sediment was dissolved with one mL of 10% (v/v) H₂SO₄. To determine glycogen content, the dissolved sample (0.5 mL) was taken to mix with 1 mL of anthrone solution (2 g/L anthrone dissolved in concentrated H₂SO₄). The reaction mixture was vigorously vortexed, and subsequently heated at 90 °C for 10 min. The sample solution was then measured by spectrophotometer at the absorbance of 625 nm. A commercial glycogen standard (Sigma-Aldrich) was prepared and used for calibrations. The unit of glycogen content presented represents by the percentage of glycogen per the dried cell weight (%/wDCW).

Reverse transcription polymerase chain reaction

Fifteen mL of cell culture was harvested by centrifugation at 6000 rpm (3622 × g), 10 min, and the total RNA was extracted using 1 mL of TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA). After that, the isolated RNAs were treated with RNasel-free DNAseI (Fermentas, Life Sciences, Canada) to remove any DNA contaminants and then converted RNAs to cDNA using ReverTra Ace® qPCR RT Master Mix (Toyobo, Osaka, Japan). Then, the cDNA was used as a template for PCR analysis of genes involved in lipid biosynthesis and neighboring pathways including glgX, phaA, accA, aas, plsX, and lipA. The 16 s rRNA was used as reference. All RT-PCR primers used in this study are listed in Table 2. For PCR condition, it was first started by 98 °C for 3 min, followed by proper cycles of each gene at 98 °C for 15 s, the primer melting temperature (Tm) for 35 s, 68 °C for 15 s to extend the DNA strand, and 68 °C for 5 min at the last step. The cycle numbers and Tm of each primer pair are shown in Table 2. PCR products were verified by electrophoresis on 1.2% (w/v) agarose gels and the intensity of bands.
was detected using a Syngene Gel Documentation (SYNGENE, Frederick, MD).

Abbreviations
AAS: Acyl–acyl carrier protein synthetase; ACP: Acyl carrier protein; Car: Carotenoids; Chl a: Chlorophyll a; CO2: Carbon dioxide; DCW: Dry cell weight; DMF: N,N-dimethylformamide; FFA: Free fatty acid; h: Hour; lipA: Lipase A; m: Meter; µg: Microgram; mL: Milliliter; min: Minute; nm: Nanometer; OD: Optical density; PCR: Polymerase chain reaction; pLX: Putative acyltransferase; PHB: Polyhydroxybutyrate; rpm: Revolutions per minute; s: Seconds; S-layer: Surface layer protein; WT: Wild type.

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Author contributions
KE responsible for study conception, experimenter, data collection and analysis, manuscript preparation. PL study conception and manuscript revision. SJ study conception, supervision, and design, critical revision and manuscript writing, and final approval of the manuscript. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

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References
1. Quintana N, Van der Kooy F, Van de Rhee MD, Voshol GP, Verpoorte R. Renewable energy from cyanobacteria: energy production optimization by metabolic pathway engineering. Appl Microbiol Biotechnol. 2011;91:471–90.
2. Gao Q, Wang W, Zhao H, Lu X. Effects of fatty acid activation on photosynthetic production of fatty acid-based biofuels in Synechocystis sp. PCC 6803. Biotechnol Biofuels. 2012;5:17.
3. Kaczmarzyk D, Fulda M. Fatty acid activation in cyanobacteria mediated by acyl–acyl carrier protein synthetase enables fatty acid recycling. Plant Physiol. 2010;152:1598–610.
4. Wang W, Liu X, Lu X. Engineering cyanobacteria to improve photosynthetic production of alkætkines. Biotechnol Biofuels. 2013;6:69.
5. Yang F, Xiang W, Li T, Long L. Transcriptome analysis for phosphorus starvation-induced lipid accumulation in Scenedesmus sp. Sci Rep. 2018;8:16420.
6. Towijit U, Songruk N, Lindblad P, Incharoensakdi A, Jantaro S. Co-overexpression of native phospholipid-biosynthetic genes plsX and pIIF enhances lipid production in Synechocystis sp. PCC 6803. Sci Rep. 2018;8:13510.
7. Minyuk G, Sidorenko V, Solovchenkii A. Effect of nitrogen source on the growth, lipid, and valuable carotenoid production in the green microalga Chromochloris zofingiensis. J Appl Phycol. 2020;32:923–35.
8. Eungrasamee K, Maio R, Incharoensakdi A, Lindblad P, Jantaro S. Improved lipid production via fatty acid biosynthesis and free fatty acid recycling in engineered Synechocystis sp. PCC 6803. Biotechnol Biofuels. 2019;12:8.
9. Eungrasamee K, Incharoensakdi A, Lindblad P, Jantaro S. Overexpression of lipA or glpD_0692 in the Synechocystis sp. PCC 6803 mutant lacking the aas gene enhances free fatty acid secretion and intracellular lipid accumulation. Int J Mol Sci. 2021;12(21):11468.
10. Iram SH, Cronan JE. The β-oxidation systems of Escherichia coli and Salmonella enterica are not functionally equivalent. J Bacteriol. 2006;188:599–608.
11. Leber C, Polson B, Fernandez-Moya R, Da Silva NA. Overproduction and secretion of free fatty acids through disrupted neutral lipid recycle in Saccharomyces cerevisiae. Metab Eng. 2015;28:54–62.
12. Ferreira R, Teixeira PG, Siwers V, Nielsen J.Redirection of lipid flux toward phospholipids in yeast increases fatty acid turnover and secretion. Proc Natl Acad Sci USA. 2018;115:1262–7.
13. Liu X, Sheng J, Curtis R III. Fatty acid production in genetically modified cyanobacteria. Proc Natl Acad Sci USA. 2011;108(17):6899–6904.
14. Takatani N, Use K, Kato A, Ikeda K, Kojima K, Aichi M, Maeda SI, Omata T. Essential role of acyl-ACP synthetase in acclimation of the cyanobacteria Synechococcus elongatus strain PCC 7942 to high-light conditions. Plant Cell Physiol. 2015;56:1608–15.
15. Ruffing AM, Jones HD. Physiological effects of free fatty acid production in genetically engineered Synechococcus elongatus PCC 7942. Biotechnol Bioeng. 2012;109:2190–9.
16. Weer D, Muller C, Gaspers C, Frentzen M. Characterisation of acyltransfersases from Synechocystis sp. PCC 6803. Biochem Biophys Res Commun. 2005;334(4):1127–34.
17. Paoletti L, Lu YJ, Schujman GE, de Mendoza D, Rock CO. Coupling of fatty acid and phospholipid synthesis in Bacillus subtilis. J Bacteriol. 2007;189(16):5816–24.
18. Gao Q, Tan X, Lu X. Characterization of a key gene in membrane lipid cycle in Synechocystis sp. PCC 6803. Chin J Biotechnol. 2012;28(12):1473–81.
19. Hamilton JA, Kamp F. How are free fatty acids transported in membranes? Is it by proteins or by free diffusion through the lipids? Diabetes. 1999;48(12):2255–69.
20. Bellefleur MP, Wanda SY, Curtis R. Characterizing active transportation mechanisms for free fatty acids and antibiotics in Synechocystis sp. PCC 6803. BMC Biotechnol. 2019;19(1):1–17.
21. Jansson C, Northen T. Calcifying cyanobacteria—the potential of biomimeralization for carbon capture and storage. Curr Opin Biotechnol. 2010;21(3):365–71.
22. Trautner C, Vermaas WF. The sll1951 gene enhances free fatty acid secretion and intracellular lipid accumulation in genetically engineered Synechocystis sp. PCC 6803. Biotechnol Biofuels. 2013;6:45.
23. Koch M, Doello S, Gutekunst K, Forchhammer K. PhB is produced from glycogen turn-over during nitrogen starvation in Synechocystis sp. PCC 6803. Int J Mol Sci. 2019;20:1942.
24. Dutt V, Srivastava S. Novel quantitative insights into carbon sources for synthesis of poly-3-hydroxybutyrate in Synechocystis PCC 6803. Photosynth Res. 2018;136:303–14.
25. Wu GF, Wu QY, Shen ZY. Accumulation of poly-β-hydroxybutyrate in cyanobacterium Synechocystis sp. PCC6803. Biocatalysis. 2001;7(62):85–90.
26. Khetkorn W, Incharoensakdi A, Lindblad P, Jantaro S. Enhancement of poly-3-hydroxybutyrate production in Synechocystis sp. PCC 6803 by overexpression of its native biosynthetic genes. Biocatalysis. 2016;214:761–8.
27. Hauf W, Schlebusch M, Hüge J, Kopka J, Hagemann M, Forchhammer K. Metabolic changes in Synechocystis PCC 6803 upon nitrogen-starvation: excess NADPH sustains polyhydroxybutyrate accumulation. Metabolites. 2013;3:101–18.

28. Koch M, Beierdenz KW, Forchhammer K. On the role and production of polyhydroxybutyrate (PHB) in the cyanobacterium Synechocystis sp. PCC 6803. Life. 2020;10(4):47.

29. Desbois AP, Smith VJ. Antibacterial free fatty acids: activities, mechanisms of action and biotechnological potential. Appl Microbiol Biotechnol. 2010;85:162942.

30. Ruffing AM. Improved free fatty acid production in cyanobacteria with Synechococcus sp. PCC 7002 as host. Front Bioeng Biotechnol. 2014;2:17.

31. Kato A, Use K, Takatani N, Ikeda K, Matsuura M, Kojima K, Achi M, Maeda S, Omata T. Modulation of the balance of fatty acid production and secretion is crucial for enhancement of growth and productivity of the engineered mutant of the cyanobacterium Synechococcus elongatus. Biotechnol Biofuels. 2016;9:91.

32. Schuster B, Sleytr UB. Biomimetic interfaces based on S-layer proteins, lipid membranes and functional biomolecules. J R Soc Interface. 2014;11:20140232.

33. Schulz-Lam S, Harauz G, Beveridge TJ. Participation of a cyanobacterial S layer in fine-grain mineral formation. J Bacteriol. 1992;174(24):7971–81.

34. Sakiyama T, Ueno H, Homma H, Numata O, Kuwabara T. Purification and characterization of a hemolysin-like Protein, Sll 1951, a nontoxic member of the RTX protein family from the cyanobacterium Synechocystis sp. strain PCC 6803. J Bacteriol. 2006;188:3535–42.

35. Sakiyama T, Arai H, Suzuki T, Shirawa Y. Functions of a hemolysin-like protein in the cyanobacterium Synechocystis sp. PCC 6803. Arch Microbiol. 2011;193:565–71.

36. Wang RT, Stevens CLR, Myers J. Action spectra for photoreactions I and II of photosynthesis in the blue-green alga Anacystis nidulans. Photochem Photobiol. 1977;25(1):103–8.

37. Göril M, Sauer J, Baier T, Forchhammer K. Nitrogen-starvation-induced chlorosis in Synechococcus PCC 7942: adaptation to long-term survival. Microbiology. 1998;144(9):2449–58.

38. Schwarz R, Forchhammer K. Acclimation of unicellular cyanobacteria to macronutrient deficiency: emergence of a complex network of cellular responses. Microbiology. 2005;151(8):2503–14.

39. Joseph A, Aikawa S, Sasaki K, Matsuda F, Hasunuma T, Kondo A. Increased biomass production and glycogen accumulation in apcE gene deleted Synechocystis sp. PCC 6803. AMB Express. 2014;4:17.

40. Luan G, Zhang S, Wang M, Lu X. Progress and perspective on cyanobacterial green photosynthesis: molecular and physiological insights. J Photochem Photobiol B Biol. 2014;145:426–39.

41. Görl M, Sauer J, Baier T, Forchhammer K. Nitrogen induction of sugar catabolic gene expression in Synechocystis sp. PCC 6803. AMB Express. 2014;4:17.

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