Cross-Feeding between Cyanobacterium *Synechococcus* and *Escherichia Coli* in Artificial Autotrophic-Heterotrophic Co-Culture System Revealed by Integrated Omics Analysis

Jiajia Ma
Tianjin University
Taohong Guo
Tianjin University
Lei Chen
Tianjin University
Xinyu Song
Tianjin University
Weiwen Zhang (wwzhang8@tju.edu.cn)
Tianjin University

Research Article

Keywords: Artificial co-culture system, Interaction mechanism, Metabolomics, Quantitative proteomics, Transcriptomics, Cyanobacteria

Posted Date: December 13th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1147783/v1

Abstract

Background: The light-driven consortia consisted of sucrose-secreting cyanobacteria and heterotrophic species capable of producing valuable chemicals have recently attracted significant attention, and are considered as a promising strategy for green biomanufacturing. In a previous study (Zhang et al, 2020, *Biotechnol Biofuel*, 13:82), we achieved a one-step conversion of CO_2 through sucrose derived from cyanobacteria to fine chemicals by constructing an artificial co-culture system consisting of sucrose-secreting *Synechococcus elongateus cscB*$^+$ and 3-hydroxypropionic acid (3-HP) producing *Escherichia coli* ABKm. Analysis of the co-culture system showed that cyanobacterial cells were growing better than its corresponding axenic culture. To explore the underlaid mechanism and to identify the metabolic modules to further improve the co-culture system, an integrated metabolomics, transcriptomic and proteomic analysis was conducted.

Results: We first explored the effect of reactive oxygen species (ROS) on cyanobacterial cell growth under co-culture system by supplementing additional ascorbic acid to scavenge ROS in CoBG-11 medium. The result showed cyanobacterial growth was obviously improved with additional 1 mM ascorbic acid under pure culture; however, cyanobacterial growth was still slower than that in the co-culture with *E. coli,* suggesting that the better growth of *Synechococcus cscB*$^+$ might be caused by other factors more than just ROS quenching. We then investigated the intracellular metabolite levels in cyanobacteria using LC-MS based metabolomics analysis. The results showed that metabolites involved in central carbon metabolism were increased, suggesting more carbon sources were utilized by cyanobacteria in the co-culture system, which illuminating that enhanced photosynthesis attributes to the higher CO_2 availability produced from co-cultivated heterotrophic partner. To further explore the interaction based on cross-feeding and metabolite exchange, quantitative transcriptomics and proteomics were applied to *Synechococcus cscB*$^+$. Analysis of differentially regulated genes/proteins showed that the higher availability of carbon, nitrogen, phosphate, calcium, Cu^{2+}, Fe^{3+} and co-factors was observed in co-cultivated *Synechococcus cscB*$^+$ during co-cultivation, suggesting the heterotrophic partner in the system might be involved in supplementing CO_2 and improving essential micronutrients necessary to maintain high photosynthetic growth of *Synechococcus cscB*$^+$.

Conclusion: Integrated omics analysis of the interaction mechanism between *S. elongateus* and *E. coli* showed metabolic changes such as enhanced photosynthesis, oxidative phosphorylation, essential micronutrients, and the ROS scavenging occurred at multiple levels of genes, proteins and metabolites, which might be together contributing to the better cell growth of *Synechococcus cscB*$^+$ in co-cultivation. In addition, the results implicated that the co-culture system could be further improved by engineering the modules related to the ROS quenching, carbon metabolism, nitrogen metabolism, Pi transport, metal transport and co-factors biosynthesis. Finally, the light condition, which may influence the cross-feeding metabolites between phototrophic and heterotrophic species, and also affect the oxidative pressure on the *E. coli* strains due to the photosynthesis, could be further optimized to improve cell growth in the co-culture system, eventually leading to high productivity of value-added products.

Introduction

Cyanobacteria with the capability of producing organic matter from CO_2 by using solar energy, have attracted increased attention as environmentally friendly and sustainable “microbial cell factories” for the production of carbohydrate feedstocks to support traditional fermentation processes [12]. Take the sucrose, an easily fermentable feedstock, as example, several cyanobacterial species are capable of synthesizing and secreting sucrose as an osmolyte under appropriate environmental stimuli, such as osmotic pressure [3], and this process can be sustained over a long period of time and at higher levels than that from plant-feedstock such as sugarcane and beet [4, 5]. However, purification of sucrose from cyanobacterial cultivation supernatant is costly and the system is easily contaminated, which creates barriers to any scale-up cultivation [6]. In addition, any application of photosynthetic cell factories in scale-up facilities is always restricted by challenges from harsh environments, suggesting that the adaptability and compatibility of cyanobacterial cell factories should be further improved to facilitate the industrial-scale biomanufacturing [7]. In recent years, increasing evidences suggested that the exchange of essential metabolites between microorganisms could be a crucial process that can significantly affect growth, composition and the structure stability of microbial communities in nature [8, 9]. In aquatic environments, the ecological interaction between photo-autotropic and heterotrophic species is based on cross-feeding and metabolite exchange [10]. In this case, the photo-autotrophs excreted material ranging from targeted photosynthetic intermediates such as glycolate, osmolytes and fatty acids, and extracellular polymeric substance, to the products of cell lysis that can include sugars, proteins, lipids and nucleic acids [11, 12]. In exchange, heterotrophic species are thought to provide essential micronutrients, such as vitamins, amino acids and bioavailable trace metals, necessary to maintain high photosynthetic productivity [9]. In addition, the positive effects on the autotrophs were also observed, which might be attributed to the decrease of oxidative stress by heterotrophs through reactive oxygen species (ROS) scavenging [13, 14]. Inspired by the symbiotic system commonly found in nature, increasing efforts have been made in recent years to design artificial routes of metabolite interchange in order to construct new symbiotic systems with high efficiency and stability [15, 16].

The light-driven artificial consortia consisted of sucrose-secreting cyanobacterium and heterotrophic species have recently attracted significant attention as the alternatives for the utilization of sucrose from cyanobacteria [17]. For example, Ducat et al. constructed a co-culture system consisting of the cyanobacterium *Synechococcus elongatus* PCC 7942 (hereafter as *Synechococcus* 7942) and the heterotrophic bacterium *Halomonas boliviensis*, in which the growth of *H. boliviensis* was supported by sucrose produced by *S. elongatus* 7942 [18]. In another study, Li et al. designed a co-culture system with the sucrose-secreting *S. elongatus* 7942 and three different yeasts to mimic lichen and research the interaction between the autotrophic and heterotrophic strains [13]. More recently, Liu et al. constructed a co-culture system composed of *S. elongatus* 7942 and *E. coli* to produce isoprene and extended the fermentation time of co-cultivation was extended from 100 h to 400 h by adjusting the inoculation ratio between *S. elongatus* 7942 and *E. coli*, in which the production of isoprene was increased sevenfold to

0.4 g/L compared to the axenic culture of *E. coli*[19]. In addition, cyanobacteria other than model species were also utilized, for example, Zhang et al. constructed a microbial consortium consisting of the fast-growing cyanobacterium *Synechococcus elongatus* UTEX 2973 recently identified (hereafter as *Synechococcus* 2973) which are capable of growing under high light and temperature [20], as well as *E. coli* to sequentially produce sucrose and then the platform chemical 3-hydroxypropionic acid (3-HP) from CO_2 [21]. All these studies enlightened us that light-driven co-culture system could be a promising strategy for future CO_2 based biomanufacturing.

To construct light-driven co-culture systems with high efficiency, it is necessary to fully understand the metabolic mechanism underlaid the interaction between autotrophs and heterotrophs. Although several previously studies have showed that the cyanobacterial cell growth could be improved in co-culture system [13], the mechanism is yet to be determined. Moreover, while it is fully expected that the mechanism involves more than just single gene, or even single metabolic module, so far only a few studies utilized global-based omics techniques to explore the interaction mechanism at multi-level of RNA, protein and metabolite [22-24]. Due to the complexity of co-culture structure, the challenge to study on the interaction mechanism is also increased, integrated omics analysis could be a good approach to obtain a "panorama" of cells in the co-culture systems and reveal novel insights into the biological mechanism [25]. For example, Amin et al. analyzed the signaling and interaction between diatom and associated bacteria through integrated metabolite and transcriptomic analysis, in which the tryptophan and indole-3-acetic acid were determined as the key signaling molecules involving in the complex exchange of nutrients [26], demonstrating that the approach of integrated transcriptome, proteome and metabolome should be adopted to explore microbial interactions in the co-culture systems.

In our previous study, Zhang et al.[21] constructed an artificial co-culture system composed of the sucrose-secreting strain *Synechococcus elongatuscscB*$^+$ and the sucrose-utilizing and 3-hydroxypropionic acid (3-HP) -producing strain *E. coli* ABKm. The system was able to produce ~68.29 mg/L 3-HP in 7 days. In spite of the one-step sucrose utilization co-culture system was successfully constructed, productivity and stability of the co-culture systems remain challenging. In this study, an integrated proteomics and transcriptomics approach was employed to analyze the metabolic responses of cyanobacteria to the heterotrophic partner in the artificial co-culture system, which will be valuable to identify the metabolic modules involved in efficiency and stability of the co-culture system and apply them as potential engineering targets to further optimize the system, as well as for guiding cultivation optimization.

Methods

2.1 Strains and culture conditions

The sucrose-secreting strain *SynechococcuscscB*$^+$ (derived from *Synechococcus elongatus* UTEX 2973) and the sucrose-utilizing and 3-HP-producing *E. coli* ABKm strain reported in our previous study were used to construct co-culture system [21]. *SynechococcuscscB*$^+$ was cultivated under 100 μmol photons $m^{-2}s^{-1}$ in

an illuminating shaking incubator (HNYC-202T, Honour, Tianjin, China) at 130 rpm and 37 °C or on BG-11 agar plates in an incubator (SPX- 250B-G, Boxun, Shanghai, China) [27]. *E. coli* ABKm strain were grown on LB medium or agar plates with appropriate antibiotics added to maintain plasmids at 37 °C in a shaking incubator (HNY-100B, Honour, Tianjin, China) at 200 rpm or in an incubator, respectively. Co-culture medium (hereafter as CoBG-11) was used to construct co-culture system according to the previous study [21], in which 150 mM NaCl, 4 mM NH_4Cl and 3 g/L 2-[[1,3-dihydroxy-2-(hydroxymethyl) propan-2-yl] amino] ethanesulfonic acid (TES) were supplemented into the BG-11 medium. The pH value was adjusted with NaOH to 8.3.

For construction of co-culture system, the exponential phase *SynechococcuscscB*$^+$ (OD_{750}≈1.0) was collected and inoculated into 25 mL of CoBG-11 and grown at 30 °C for 48 h to an OD_{750} of 0.5. *E. coli* was cultivated in CoBG-11 with 1 g/L sucrose for 48 h, and then the cells were collected and resuspended in deionized water and inoculated into the 25 mL *SynechococcuscscB*$^+$ culture grown on CoBG-11. To separate two species in the co-culture system, the dialysis bag (diameter is 36 mm, molecular weight cut-off is 14 kDa, respectively, biosharp, Hefei, China) was used. The *E. coli* ABKm was incubated in the dialysis bag, while the *SynechococcuscscB*$^+$ was incubated outside in the flask. The pre-treatment of dialysis bags was according to a previous study with some modifications [28]. Briefly, the dialysis bag was cut into small pieces of appropriate length (approximately 10 cm), which were boiled for 10 minutes with a large volume of 1 mmol/L EDTA (pH 8.0). And then the dialysis bags were boiled with distilled water for 10 minutes for twice. The prepared dialysis bag was autoclave sterilized before using.

The cell density was measured at OD_{750} using a UV-1750 spectrophotometer (Shimadzu, Kyoto, Japan). The co-cultivated *SynechococcuscscB*$^+$ was counted by a hemocytometer under a microscope (BX43, Olympus, Shinjuku, Tokyo, Japan) after series dilution.

2.2 Determination of H_2O_2 concentration

H_2O_2 Quantitative Assay Kit (Water-Compatible) (Sangon Biotech, Shanghai, China) was used to analyze the content of H_2O_2 in the medium supernatant. Under acidic conditions, H_2O_2 oxidizes Fe^{2+} ions into Fe^{3+} ions, which then combine with dye molecules to form Fe^{3+}-dye complex. The formed complex has the maximum absorption wavelength at 560 nm (or 595 nm) and the absorption value is proportional to the concentration of H_2O_2, which was detected by spectrophotometer (Thermo Fisher Scientific Oy, Vantaa, Finland).

2.3 LC-MS based metabolomics analysis

Liquid chromatography-mass spectrometry (LC-MS) based targeted metabolomics was performed according to the protocol described previously [29]. Cells (5 OD_{730} unit) were harvested at 48 h via centrifugation at 7380 rpm for 5 min at 4 °C (Eppendorf 5430R), quenched, and extracted rapidly with 900 µL of 80:20 methanol/water (v/v; -80 °C pretreated) and then frozen in liquid nitrogen. Intracellular

metabolites were extracted via the freeze/thaw cycle for three times. The aforementioned extraction process was repeated with another 500 μL 80:20 methanol/water (v/ v). The supernatant was combined and filtered through a 0.22 μm syringe filter. The solvents were removed using a vacuum concentrator system (ZLS-1, Hunan, China), and 100 μL of ddH2O was added and mixed well. LC-MS analysis was conducted using an Agilent 1260 series binary HPLC system equipped with a Synergi Hydro-RP (C18) 150 mm × 2.0 mm ID, 4 μm 80 Å particle column (Phenomenex, Torrance, CA, U.S.A.), and an Agilent 6410 triple quadrupole mass analyzer equipped with an electrospray ionization (ESI) source. Data were acquired using the Agilent Mass Hunter work-station LC/QQQ acquisition software (version B.04.01), and chromatographic peaks were subsequently integrated via the Agilent Qualitative Analysis software (version B.04.00). All data of metabolomic profiling was first normalized by the internal control and the cell numbers of the samples. Each condition analysis consisted of four biological replicates and three technical replicates.

2.4 Transcriptomic analysis of cyanobacterial responses to *E. coli* in co-culture system

Considering the characteristics of transcriptomics technology and the accuracy of transcriptomics data, dialysis bags were used to separate cyanobacteria and *E. coli* to construct the co-culture system. For transcriptomic analysis, 5 OD_{750} of co-cultured and ascorbic acid treated axenic *Synechococcus*$cscB^+$ were collected respectively for extracting RNA samples; meanwhile, the same amount of *Synechococcus*$cscB^+$ cultivated under axenic was used as control. The transcriptomics analysis was conducted by GENEWIZ (Suzhou, China). There three biological replicates for each sample, and two statistic parameters which are fold change>1.5 and *Q*-value (fdr or padj)≤0.05 were used to determine differentially regulated genes.

2.5 Quantitative proteomics analysis of cyanobacterial responses to *E. coli* in co-culture system

The same weight of four-day co-cultured strains were sampled for proteome analysis. The samples were enzymatically digested by trypsin, following marked by isobaric tags for relative and absolute quantification (iTRAQ) technique, the samples were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The axenic *Synechococcus* 2973 cells with same incubation time were used as control. The technical service and data of quantitative proteomics were provided by BGI (Shenzhen, China). Three biological replicates for each sample were used. In the case of unmatched biological replicates, two statistic parameters, fold change>1.2 (the average ratio of the nine comparison groups) and *P*-value<0.05 (t-test of nine comparison groups) were used to screen differentially regulated proteins.

2.6 Quantitative real-time PCR analysis

For RNA extraction, the 2 OD_{750} cells of *Synechococcus cscB*$^+$ under axenic culture and co-culture were collected and centrifuged at 7,830 rpm and 4 ℃ for 5 mins. The total RNA samples were extracted using Direct-zol™ RNA Miniprep kit (ZYMO RESEARCH, CA, USA) according to the instruction, and then reverse transcribed as cDNA template using HiScript® II Q RT SuperMix for qPCR (+gDNA wiper) reagent

(Vazyme, China). The quantitative real-time PCR (qPCR) reactions were performed according to the methods described previously [30]. Briefly, the 10 μL reaction system was composed of 5 μL of 2 × ChamQ Universal SYBR qPCR Master Mix (Vazyme, China), 3 μL of RNase-free H_2O, 0.5 μL (5 μM) of each upstream primer and downstream primer and 1 μL of appropriately diluted cDNA template. The relative changes in gene expression from qPCR experiment could be analyzed using the $2^{-\Delta\Delta CT}$ method [31].

Results And Discussion

3.1 Effect of ROS quenching on cyanobacterial cell growth in co-culture system

With the potential to react with biomolecules including nucleic acids, proteins and lipids, ROS can cause oxidative stress to cells, leading to cellular damage [32]. Previous studies pointed to the possibility that heterotrophic bacteria can remove ROS in medium to improve cell growth of cyanobacteria in co-culture system, eventually enhancing the stability of co-culture system [13, 14]. To verify this hypothesis, ascorbic acid was added into CoBG-11 medium to decrease ROS generation through directly scavenge $O_2^{\bullet-}$, $^{\bullet}OH$ and reduce H_2O_2 to water [33]. Different concentrations of ascorbic acid, 0.1 mM, 1 mM, 2 mM were added into pure cultural *SynechococcuscscB*$^+$ respectively in CoBG-11 [34, 35], the analysis showed that *SynechococcuscscB*$^+$ grew better with additional 1 mM ascorbic acid in CoBG-11 compared with 0.1 mM ascorbic acid supplementation, while cell growth was inhibited after three days when supplemented 2 mM ascorbic acid **(Fig. 1A)**. Consistently, the analysis showed that H_2O_2 content was significantly decreased with ascorbic acid added (**Fig. 1B)**; however, it is unclear why the cells growth was arrested when 2 mM supplementary ascorbic acid led to almost no detectable H_2O_2 at 4 days. More importantly, although cyanobacterial growth was obviously improved with additional 1 mM ascorbic acid under pure culture, cyanobacterial growth was still slower than that in co-culture with *E. coli,* suggesting that the better growth of *SynechococcuscscB*$^+$ might be caused by other factors more than just ROS quenching.

3.2 Analysis of key metabolites in *S. elongateus cscB*$^+$ during co-cultivation by target LC-MS metabolomics

As the stability and productivity in the co-culture system was dependent on the cyanobacterial sucrose production, intracellular levels of key metabolites within *Synechococcus cscB*$^+$ cells were investigated. LC-MS based metabolomics approach, which has been employed previously to comparatively analyze cellular metabolism in the engineered cyanobacterial strains [29, 36], was applied to compare co-cultivated and pure cultural *Synechococcus cscB*$^+$. As shown in **Fig. 2**, twenty-one metabolites of cyanobacterial metabolism involve in glycolysis, amino acid, and the citric acid (TCA) cycle were chemically classified. Comparative analysis showed that the intracellular contents of FBP, F6P, E4P, R5P and acetyl-CoA were increased, suggesting more carbon sources were utilized in co-cultivated *Synechococcus cscB*$^+$. Five amino acids, lysine (Lys), serine (Ser), valine (Val), alanine (Ala) and phenylalanine (Phe) were found with significant up-regulation during co-cultivation condition. Three metabolites involved in TCA cycle including citric, malate and succinate, were also showed significant increases in co-cultivation condition.

One possible explanation for the increased metabolites in glycolysis, amino acid, and TCA cycle is that increasingly available CO_2, possibly from the respiration of heterotrophic cells, contributes to cyanobacterial better cell growth during co-cultivation, consistent with a previous finding that enhanced CO_2 fixation and oil production in co-culturing green algal *Chlorella* and yeast *Saccharomyces cerevisiae* system [37]. The results were also consistent with a more recent study which found salt stress redirect the fixed CO_2 toward sucrose production rather than biomass and glycogen accumulation in engineered *Synechococcus* 2973 [38]. In addition, the results of LC-MS also implicated that the enhanced CO_2 fixation could be used as an engineering target for further improving sucrose production in the co-cultivated cyanobacteria by modulating sucrose production pathway.

3.3 Analysis of cyanobacterial metabolic responses to *E. coli* in co-cultivated *S. elongateus* by transcriptomics

For the interaction based on cross-feeding and metabolite exchange, early studies have shown that heterotrophic bacteria could also be involved in providing essential micronutrients, such vitamins, amino acids and bioavailable trace metals, necessary to maintain high photosynthetic productivity [39]. To explore the mechanism underlaid the increased cell growth of co-cultivated cyanobacteria, and to determine the factors necessary for the stability and fermentation performance, transcriptomics between pure cultural cyanobacteria (C) and co-cultural cyanobacteria with *E. coli* (D) was applied to analyze the interaction mechanism of cyanobacteria responses to the heterotrophic partner in co-culture system. With a cutoff of 1.5-fold change and a *p* value of statistical significance less than 0.05, we found 120 genes up-regulated and 104 genes down-regulated as a result of co-cultivation, respectively. The reliability and accuracy of the transcriptomics data was independently verified by real-time quantitative PCR (qRT-PCR) (**Suppl Table S1**), the correlation coefficient R^2 was 0.9086 (**Fig. S1**), indicating the transcriptomics data collected in this study is of very high accuracy. The analysis showed that a large fraction of up-regulated transcripts was affiliated with photosynthesis and oxidative phosphorylation (25%), signal transduction and membrane transport (13%), translation (10%), genetic information processing (6%), metabolism of cofactors and vitamins (3%) role categories (**Fig. 3A**, **Suppl Table S2**), suggesting the key central metabolism of cyanobacterial cells was affected by the presence of *E. coli* in the co-culture system.

The analysis showed significant increases in transcripts levels of phosphate transport system permease genes M744_04015 (*pstS, sphX*), and *M744_04030* (*pstA*), and iron transporter (for example, bicarbonate transporter M744_01215, calcium/sodium antiporter M744_01815, P-type Cu^{2+} transporter M744_04705), suggesting higher availability of these important micronutrients for *Synechococcus cscB*$^+$ during co-cultivation condition [40]. Meanwhile, the gene M744_08450 (c*mpD* encoding a bicarbonate transporter) was significantly down-regulated, which illuminated that CO_2 availability might be increased due to the fact that *E. coli* ABKm might provide additional CO_2 for co-cultivated *Synechococcus cscB*$^+$. In addition, *M744_08660* (*modA*) encoding a molybdate transport system substrate-binding protein was also found down-regulated. Molybdenum is an essential component of the cofactors of many metalloenzymes including nitrate reductase and Mo-nitrogenase [41], the down-regulation of *modA* might suggest that more

nitrogen available for *Synechococcus cscB*$^+$ during co-cultivation as well. Varkey et al. described the up-regulation in several translational, ribosomal biogenesis and transcriptional proteins under oxidative stress in marine *Synechococcus* isolates [42]. However, many transcripts involved in ribosomal proteins (M744_13675, M744_13670, M744_05205, M744_00735, M744_05210, M744_12320, M744_05195, M744_05180, M744_05185), tRNA synthetases (M744_03935, M744_5340) and RNA binding protein (*M744_12800*) were down-regulated in our study, which likely due to the reduced ROS content in co-culture system [13]. The down-regulated transcripts in co-cultivated *Synechococcus cscB*$^+$ compared with under axenic condition were also demonstrated (**Suppl Table S3**).

Consistent with abovementioned results, significant decreases in transcripts levels of oxidative stress related genes were found in co-cultivated *Synechococcus cscB*$^+$, including two genes (M744_11065 and M744_01810) encoding high light-inducible proteins (Hli), M744_03995 encoding antioxidant protein and M744_07160 encoding heme oxygenase, indicating possible reduced oxidative stress in co-cultivated *Synechococcus cscB*$^+$. High light-induced proteins are critical for the energy dissipation mechanism to resist oxidative stress in cyanobacteria [43]. He et al. found the gene expression of 4 *hlis* genes were induced under low temperature, strong light stress and nutrient deficiency condition in *Synechocystis* sp. PCC 6803 (hereafter as *Synechocystis* 6803), and the *hli* knockout mutant strain could not be survival under strong light, suggesting that the high light-induced protein may play a photoprotective role [44]. The ferredoxin-dependent heme oxygenase catalyzes the degradation of heme to produce biliverdin IXα with the release of ferrous iron [45]. Cheng et al. found the down-regulation of heme oxygenase gene might reduce the release of detrimental free iron that causes oxidative stress [46]. Consistent with these results, our finding that the down-regulation of these four genes that the better growth of *Synechococcus cscB*$^+$ in co-culture system was partially attributed to the quenching of ROS by heterotrophic partner *E. coli* ABKm. In a previous study, Vance et al. found that the phospholipid/cholesterol/gamma-HCH transport system permease protein (MlaE) was down-regulated after exposure to a high bisphenol A concentration, which might inhibit phospholipid transport, and subsequently altered the spontaneous diffusion of the membrane to eventually caused membrane damage [47]. Interestingly, the relative expression of M744_01095 (*mlaE*) was also increased in co-cultivated *Synechococcus cscB*$^+$, which also suggested that ROS induced membrane damage was relieved by the presence of the heterotrophic partner.

In cyanobacteria, the secretory (Sec) pathway is critical for proteins transportation across the plasma membrane and thylakoid membrane [48]. The core of translocase in Sec pathway is a protein channel assembled by heterotrimeric membrane protein complex SecYEG and ATPase SecA oligomers, SecA is used as a molecular motor [49]. It was estimated that 82% of translocated proteins in *Synechocystis* 6803 contain a Sec signal peptide [50]. The expression of M744_13645 (*secE*) was up-regulated in co-cultivated *Synechococcus cscB*$^+$. In addition, the relative expression of M744_09155 (*yidC*) was increased in co-cultivated *Synechococcus cscB*$^+$ as YidC protein mediates integration of membrane integral proteins in bacteria and thylakoid membrane [51]. The increased expression levels of *secE* and *yidC* were consisted

with the phenotype of better cyanobacterial growth as translocase is responsible for the insertion of the photosystem integral membrane proteins into the thylakoid membrane in cyanobacteria [52].

3.4 Analysis of cyanobacterial metabolic responses to *E. coli* in co-cultivated *S. elongateus* by quantitative proteomics

The low correlation between mRNA and protein expression has been found and well discussed in previous studies, which might be caused by the widespread post-transcriptional regulation mechanism[53, 54]. For example, Nie et al. found that correlation of mRNA expression and protein abundance was affected at a fairly significant level by multiple factors related to translational efficiency [55]. In order to fully identify the interaction mechanism in the co-culture system, the quantitative iTRAQ proteomics was used to analyze cell responses of *Synechococcus cscB*$^{+}$ adapt to *E. coli* in co-culture system. Three *Synechococcus cscB*$^{+}$ samples from the co-culture (E1, E2, E3) and three from the axenic culture (C1, C2, C3) were collected after cultivation of 96 h, respectively, and the differential profiles of proteins in *Synechococcus cscB*$^{+}$ were identified by setting comparison groups of E1 vs. C1, E1 vs. C2, E1 vs. C3, E2 vs. C1, E2 vs. C2, E2 vs. C3, E3 vs. C1, E3 vs. C2, E3 vs. C3. The proteomic analysis identified a total of 914,635 spectra, among which 21,603 unique spectra met the 1% FDR filter and were matched to 2,206 unique proteins in the genome. In addition, a good coverage was obtained for a wide MW range for protein (**Fig. 4A**). Most of the identified proteins were with good peptide coverage, ~89% of the proteins were with more than 10% of the sequence coverage and ~87% were with more than 20% of the sequence coverage (**Fig. 4B**). Among the functional categories, the “general function prediction only” was the top detected functional category, representing 13.43% of all the identified protein (**Fig. 4C**). This result is consisted with the previous finding that approximately 45% of proteins in the cyanobacterial genome are hypothetical proteins [56]. Other frequently detected functional categories included “translation, ribosome structure and biogenesis” (9.42%), “amino acid transport and metabolism” (8.67%), “posttranslational modification, protein turnover, chaperones” (8.51%), “signal transduction mechanism” (7.17%), “carbohydrate transport and metabolism” (6.51%).

All 251 differentially expressed proteins were divided into 21 secondary branches of pathways based on the KEGG database classification, in which 181 differentially regulated proteins are related to cell metabolism, including energy metabolism (10.36%), metabolism of cofactors and vitamins (10.36%), carbohydrate metabolism (10.36%), and amino acid metabolism (4.38%) (**Fig. 5A**). The number of up- and down-regulated proteins in each annotated pathway was shown in **Fig. 5B**. The KEGG enrichment analysis suggested that seven pathways were significantly enriched (P-value<0.05) in the up-regulated differential proteins, including “two-component system”, “nitrogen metabolism”, “biofilm formation-*E. coli*”, “lipoic acid metabolism”, “sulfur relay system”, “ABC transporters”, “glyoxylate and dicarboxylate metabolism” (**Fig. 5C**), while only “ABC transporters” was significantly enriched (P-value<0.05) in the down-regulated differential proteins (**Fig. 5D**).

3.5 Cyanobacteria responses to co-culture systemdeciphered by proteomics

As demonstrated in the transcriptomics analysis that the large fraction (25%) of transcripts involved in photosynthesis and oxidative phosphorylation were significantly increased during co-cultivation, the differentially expressed proteins involved in the energy metabolism pathway was also identified (**Suppl Table S4**). In co-cultivated *Synechococcus cscB*$^{+}$, the increase of protein abundances for energy metabolism enzymes, such as ferredoxin (PetF, M744_01325), phycobiliproteins terminal rod linker (CpcD, M744_11425), photosystem II reaction center H (PsbH, M744_01910), photosystem II D1 protein (PsbA, M744_00850), NAD(P)H-quinone oxidoreductase subunit 4 (NadhD, M744_05920), and NAD(P)H-quinone oxidoreductase subunit 5 (NadhF, M744_01470) were observed, suggesting that more NADPH and ATP generated from photosynthesis [57], which is likely due to the elevated C and/or N availability compared with the axenic control, as discussed above. Meanwhile, increased protein abundance of the light-independent prochlorophyll reductase subunit B (ChlB) (M744_07280), which catalyzes the conversion of prochlorophyll to chlorophyll a [58], was also found, suggesting that cyanobacterial photosynthesis might be improved during the co-cultivation. These results are well consistent with our findings based on the transcriptomics analysis.

Nitrogen metabolism, either from nitrate or ammonium, governs the turnovers of the macromolecules that regulate metabolic pathways, eventually affecting energy production and carbon skeleton [59]. Through the quantitative proteomics analysis, three nitrate/nitrite transport system ATP-binding proteins of M744_10450 (NrtB), M744_10455 (NrtC), and M744_10460 (NrtD) and two ferredoxin-nitrite reductases (M744_10440 and M744_07195) were found up-regulated in the co-cultivated *Synechococcus cscB*$^{+}$, suggesting that the nitrite uptake in co-cultivated *Synechococcus cscB*$^{+}$ was enhanced. The ammonium is incorporated into carbon skeletons through glutamine synthetase (M744_02210), which was also found up-regulated in co-cultivated *Synechococcus cscB*$^{+}$. Significant up-regulation in the nitrogen uptake and assimilation were evident with higher photosynthesis and better cyanobacterial growth during the co-cultivation condition.

Phosphorus is a vital nutrient for cyanobacterial growth, which impacts the synthesis of cyanobacterial extracellular polymeric substances and also appears to induce significant changes in the synthesis of polysaccharides, as well as membrane lipids [60]. In the co-cultivated *Synechococcus cscB*$^{+}$, the proteins involved in phosphate transport system, including M744_04030 (PstA), M744_04035 (PstB), M744_04025 (PstC), M744_04020 (PstS) and M744_04015 (SphX), were found up-regulated by 2.08-, 2.12-, 3.80-, 2.67- and 4.75-fold, respectively. The up-regulation of all four phosphate transporters in the co-cultivated *Synechococcus* cscB^{+} might be due to the increased consumption of Pi in the form of NAPDH or ATP, which contributes to further cell growth. Consistently, the increased transcripts level of *M744_04015* (*sph*X) and *M744_04030* (*pst*A), were also found at transcription level. Aside from dissolved inorganic phosphorus, dissolved organic phosphorus is used by cyanobacteria via alkaline phosphatase [60]. Two alkaline phosphatases (M744_09635 and M744_11635) in the co-cultivated *Synechococcus cscB*$^{+}$ were found up-regulated by 2.89- and 1.40- fold, respectively, suggesting that the cyanobacteria were able to acquire more phosphorus for cell growth during the co-cultivation condition [40].

Two up-regulated proteins M744_05990 and M744_04340 annotated respectively as xylose-5-phosphate/fructose 6-phosphate phosphotransketolase (Xfp) and pyruvate-ferredoxin/flavodoxin oxidoreductase (Por), were identified in the co-cultivated *Synechococcus cscB*$^{+}$. Xfp plays a key role in glycolysis, catalyzing the conversion of X5P or F6P to acetyl phosphate [61], while Por is responsible for the oxidation process of pyruvate to generate acetyl-CoA [62]. The up-regulation of Xfp and Por indicated CO_2 fixation might be enhanced in the co-cultivated *Synechococcus cscB*$^{+}$, well-consistent with the increased acetyl-CoA content in the metabolomic analysis discussed above. In addition, three bicarbonate transporters, including M744_08440 (CmpB), M744_08445 (CmpC), and M744_08450 (CmpD) were also found down-regulated in the quantitative proteomics data, also consistent with the transcriptomic analysis. The *cmp* operon (*cmpA*, *cmpB*, *cmpC*, *cmpD*) in *Synechococcus* 7942 has been confirmed to encode a component of the ABC-type HCO^{3-} transporter BCT1, and its transcription was activated at low CO_2 concentrations [63, 64]. The down-regulation of these three bicarbonate transporters indicated that the concentration of CO_2 in the co-culture system might be higher than that under pure culture conditions, due to the fact that *E. coli* ABKm might secret CO_2 to the system, which was also found in transcriptomics.

The exchange of essential micronutrients, such as vitamins, amino acids and bioavailable trace metal, from heterotrophic bacteria to cyanobacteria during co-cultivation was observed above at the transcript level. Pathway enrichment analysis of the proteomic data indicated that *Synechococcus cscB*$^{+}$ had maximized the uptake and utilization of Fe^{3+} and thiamine to improve cell growth during the co-cultivation condition. Significant decrease in protein abundances of Fe^{3+} transporter, including AfuA (M744_05470) and AfuB (M744_09555) [65], indicated increased availability of this important micronutrient for *S. elongateus cscB*$^{+}$ during the co-cultivation condition. The up-regulation of thiamine metabolism proteins was also found in the co-culture system. The cysteine desulfurase (M744_03415) catalyzes the conversion of L-cysteine to L-alanine and sulfur, the released sulfur was then transferred into scaffold protein to assemble Fe-S clusters [66], while the Fe-S clusters participate in electron transfer, iron-sulfur storage, regulation of gene expression, photosynthesis, and enzyme activity in all kingdoms of life [67]. The up-regulation of M744_03415 was observed, consistent with the cyanobacterial growth and functional performance during co-cultivation. Thiamine pyrophosphate (TPP) acts as a cofactor for several enzymes in key cellular metabolic pathways such as glycolysis, the pentose phosphate pathway and the citric acid cycle (TCA) [68]. Proteomic analysis showed that the phosphomethylpyrimidine synthase (ThiC) (M744_11180), an essential enzyme for TPP biosynthesis, was up-regulated in the co-cultivated *Synechococcus cscB*$^{+}$, suggesting that enhanced carbon metabolic activity in *Synechococcus cscB*$^{+}$. In addition, up-regulation was also observed for 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate synthase (MenD) (M744_09410), which is involved in the biosynthesis of menaquinone and phylloquinone. Menaquinones play important roles in electron transport and oxidative phosphorylation, while phyloquinone is the secondary electron carrier in the photosystem I [69], their up-regulation was in general consisted with the improved growth of *Synechococcus cscB*$^{+}$.

Moreover, the down-regulation of five proteins related to oxidative stress, including Hli protein (M744_01810 and M744_11065), *fur* family transcription regulator (Fur) (M744_05500 and M744_12665), and monothiol glutaredoxin (M744_10930) were found in the co-cultivated *Synechococcus cscB*$^{+}$ by our quantitative proteomics analysis. The two Hli proteins were also identified in transcriptomic analysis. The transcription regulator Fur, as an iron uptake regulator, is responsible for controlling the gene expression of siderophore biosynthesis and iron transport [70]. In previous studies, monothiol glutaredoxin was proved to protect against oxidative stress by regulating iron homeostasis [71]. The crosstalk between controlling iron homeostasis and defending against ROS was previously confirmed in *E. coli*, demonstrating that the lack of iron regulation may lead to oxidative stress [72]. Thus, the down-regulation of these five proteins indicated the positive effect on cell growth during the co-cultivation was attributed to the decrease of oxidative stress in *Synechococcus cscB*$^{+}$ by a heterotrophic cells capable of ROS scavenging, which was also consistent with the our previous finding that the gene expression of three types of catalases, including hydroperoxidase I (*kat*G), hydroperoxidase II (*kat*F), and hydroperoxidase III (*kat*E) were significant induced in *E.coli* during the co-cultivation condition [21].

Conclusion

In our previous study, we constructed a one-step conversion system from CO_2, and sucrose synthesized and secreted by cyanobacteria to a fine chemical 3-HP by including a sucrose-secreting cyanobacterial *Synechococcus cscB*$^{+}$ and a 3-HP producing *E. coli* ABKm in a co-culture system maintaining for one week, and achieved a 3-HP production of 68.29 mg/L [21]. However, the stability and 3-HP productivity in the co-culture system are still low, probably due to the low cyanobacterial biomass and sucrose productivity in the co-culture system, and even the low metabolite exchange between the two partner cells in the system [73]. To further improve the functional performance of the co-culture system, an integrated omics analysis was conducted in this study to determine the interaction mechanism between cyanobacterium *Synechococcus* and *E. coli*.

The stability in the autotrophy-heterotrophy co-culture system dependents on the cyanobacterial growth and sucrose production. In this study, the decreased level of several oxidative stress related proteins was found in transcripts level (*M744_11065*, *M744_01810*, *M744_03995* and *M744_07160*) and proteomics level (M744_01810, M744_11065, M744_05500, M744_12665 and M744_10930), suggesting the possible reduced oxidative stress in co-cultivated *Synechococcus cscB*$^{+}$. In addition, the increased expression of transcript related to phospholipid/cholesterol/gamma-HCH transport system (*M744_01095*), and decreased expression of many transcripts involved in ribosomal proteins (*M744_13675*, *M744_13670*, *M744_05205*, *M744_00735*, *M744_05210*, *M744_12320*, *M744_05195*, *M744_05180*, *M744_05185*), tRNA synthetases (*M744_03935*, *M744_5340*) and RNA binding protein (*M744_12800*) were found in co-cultivated *Synechococcus cscB*$^{+}$, also suggesting that ROS induced membrane damage was relieved by the presence of the heterotrophic partner. All these finding illuminated us that the antioxidative system of *E. coli* could be further enhanced through overexpressing the major ROS-scavenging enzymes, for

example superoxide dismutase, catalase, glutathione peroxidases and thioredoxin [74], to improve cyanobacterial cell growth and productivity during co-cultivation. Previous studies have shown that heterotrophic species could provide essential micronutrients, such as vitamins, amino acids and bioavailable trace metals, necessary to maintain high photosynthetic productivity in various co-culture systems [9]. In this study, the higher availability of carbon, nitrogen, phosphate, calcium, Cu^{2+}, Fe^{3+} and co-factors in co-cultivated *Synechococcus cscB*$^{+}$ during co-cultivation were identified by the integrated metabolomics, transcriptomics and proteomics analysis, which therefore with great promise as the potential targets to improve the fermentation performance of the co-culture system consisted with photoautotrophic and heterotrophic species.

Light condition is critical for the cell growth of cyanobacteria through photosynthesis [75], especially under co-cultivation, as the cell concentration increases, light-shading caused by the heterotrophic species might reduce cyanobacterial exposure to light and thus the photosynthetic activity [76]. Meanwhile, the enhanced photosynthesis and oxidative phosphorylation identified by the integrated omics also illuminated light condition might be further optimized to improve the stability and efficiency of artificial co-culture system. More importantly, cyanobacteria are often inhibited by ROS produced from the imbalance between light absorption and light utilization during the process of photosynthesis [77]. Although in co-culture system, the inhibition of oxidative pressure on cyanobacteria can be reduced by the heterotrophic species [13, 21], the growth of heterotrophic species can be inhibited by ROS when exposed to high densities of cyanobacteria in the light [17, 78]. Considering the light availability may influence the cross-feeding metabolites between phototrophic and heterotrophic species including oxygen and carbon dioxide through photosynthesis and respiration, and also affect the oxidative pressure on the *E. coli* strains due to the photosynthesis [25], the optimal light conditions could also be critical to the high cell growth in the co-culture system.

In conclusion, the results showed that many metabolic changes, including enhanced photosynthesis, oxidative phosphorylation, and essential micronutrients, were discovered at multiple levels, not only the ROS scavenging, might be responsible for the better cell growth of *Synechococcus cscB*$^{+}$ during co-cultivation (**Fig. 5**). We thus proposed that the metabolic modules related to the ROS quenching, carbon metabolism, nitrogen metabolism, Pi transport, metal transport and co-factors biosynthesis could be potential engineering targets to further improve stability and fermentation performance in this co-culture system.

Declarations

Acknowledgements

None.

Authors' contributions

XYS and WZ designed the research; JJM and THG performed the major experiments and wrote the draft manuscript; and JJM, THG, LC, XYS and WZ analyzed the data, drafted and revised the manuscript. All authors read and approved the final manuscript. JJM and THG contribute equally.

Funding

This research was supported by Grants from the National Key Research and Development Program of China (Grants No. 2019YFA0904600, 2018YFA0903000, 2018YFA0903600 and 2020YFA0906800), the National Natural Science Foundation of China (nos. 31901016, 31770035, 31770100, 31972931, 91751102 and 21621004), and Tianjin Synthetic Biotechnology Innovation Capacity Improvement Project (no. TSBICIP-KJGG-007).

Availability of supporting data

All data generated or analyzed during this study are included in this published article and its additional files.

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors have agreed to the publication of this manuscript.

Competing interests

The authors declare no competing financial interests.

References

1. Quintana, N.; Van der Kooy, F.; Van de Rhee, M. D.; Voshol, G. P.; Verpoorte, R., Renewable energy from cyanobacteria: energy production optimization by metabolic pathway engineering. Appl Microbiol Biotechnol **2011,** 91 (3), 471-90.
2. Hays, S. G.; Ducat, D. C., Engineering cyanobacteria as photosynthetic feedstock factories. Photosynth Res **2015,** 123 (3), 285-295.
3. Hagemann, M., Molecular biology of cyanobacterial salt acclimation. FEMS Microbiol Rev **2011,** 35 (1), 87-123.
4. Niederholtmeyer, H.; Wolfstadter, B. T.; Savage, D. F.; Silver, P. A.; Way, J. C., Engineering cyanobacteria to synthesize and export hydrophilic products. Appl Environ Microbiol **2010,** 76 (11), 3462-6.
5. Han, Y.; Watson, M., Production of microbial levan from sucrose, sugarcane juice and beet molasses. J Ind Microbiology **1992,** 9 (3), 257-260.
6. Chisti, Y., Constraints to commercialization of algal fuels. J Biotechnol **2013,** 167 (3), 201-14.

7. Luan, G.; Lu, X., Tailoring cyanobacterial cell factory for improved industrial properties. Biotechnol Adv **2018,** 36 (2), 430-442.
8. Beliaev, A. S.; Romine, M. F.; Serres, M.; Bernstein, H. C.; Linggi, B. E.; Markillie, L. M.; Isern, N. G.; Chrisler, W. B.; Kucek, L. A.; Hill, E. A., Inference of interactions in cyanobacterial–heterotrophic co-cultures via transcriptome sequencing. ISME J **2014,** 8 (11), 2243-2255.
9. Giri, S.; Ona, L.; Waschina, S.; Shitut, S.; Yousif, G.; Kaleta, C.; Kost, C., Metabolic dissimilarity determines the establishment of cross-feeding interactions in bacteria. Curr Biol **2021**.
10. Paerl, H. W.; Pinckney, J., A mini-review of microbial consortia: their roles in aquatic production and biogeochemical cycling. Microbial Ecology **1996,** 31 (3), 225-247.
11. Bruckner, C. G.; Rehm, C.; Grossart, H. P.; Kroth, P. G., Growth and release of extracellular organic compounds by benthic diatoms depend on interactions with bacteria. Environ Microbiol **2011,** 13 (4), 1052-63.
12. Seymour, J. R.; Ahmed, T.; Durham, W. M.; Stocker, R., Chemotactic response of marine bacteria to the extracellular products of *Synechococcus* and *Prochlorococcus*. Aquat Microb Ecol **2010,** 59 (2), 161-168.
13. Li, T.; Li, C. T.; Butler, K.; Hays, S. G.; Guarnieri, M. T.; Oyler, G. A.; Betenbaugh, M. J., Mimicking lichens: incorporation of yeast strains together with sucrose-secreting cyanobacteria improves survival, growth, ROS removal, and lipid production in a stable mutualistic co-culture production platform. Biotechnol Biofuels **2017,** 10, 55.
14. Morris, J. J.; Kirkegaard, R.; Szul, M. J.; Johnson, Z. I.; Zinser, E. R., Facilitation of robust growth of *Prochlorococcus* colonies and dilute liquid cultures by "helper" heterotrophic bacteria. Appl Environ Microbiol **2008,** 74 (14), 4530-4.
15. Brenner, K.; You, L.; Arnold, F. H., Engineering microbial consortia: a new frontier in synthetic biology. Trends Biotechnol **2008,** 26 (9), 483-9.
16. Fredrickson, J. K., ECOLOGY. Ecological communities by design. Science **2015,** 348 (6242), 1425-7.
17. Hays, S. G.; Yan, L. L. W.; Silver, P. A.; Ducat, D. C., Synthetic photosynthetic consortia define interactions leading to robustness and photoproduction. J Biol Eng **2017,** 11, 4.
18. Weiss, T. L.; Young, E. J.; Ducat, D. C., A synthetic, light-driven consortium of cyanobacteria and heterotrophic bacteria enables stable polyhydroxybutyrate production. Metab Eng **2017,** 44, 236-245.
19. Liu, H.; Cao, Y.; Guo, J.; Xu, X.; Long, Q.; Song, L.; Xian, M., Study on the isoprene-producing co-culture system of *Synechococcus elongates-Escherichia coli* through omics analysis. Microb Cell Fact **2021,** 20 (1), 6.
20. Yu, J. J.; Liberton, M.; Cliften, P. F.; Head, R. D.; Jacobs, J. M.; Smith, R. D.; Koppenaal, D. W.; Brand, J. J.; Pakrasi, H. B., *Synechococcus elongat*us UTEX 2973, a fast growing cyanobacterial chassis for biosynthesis using light and CO_2. Sci Rep-Uk **2015,** 5.
21. Zhang, L.; Chen, L.; Diao, J.; Song, X.; Shi, M.; Zhang, W., Construction and analysis of an artificial consortium based on the fast-growing cyanobacterium *Synechococcus elongatus* UTEX 2973 to

produce the platform chemical 3-hydroxypropionic acid from CO_2. Biotechnol Biofuels **2020,** 13, 82.

22. Bernstein, H. C.; McClure, R. S.; Thiel, V.; Sadler, N. C.; Kim, Y. M.; Chrisler, W. B.; Hill, E. A.; Bryant, D. A.; Romine, M. F.; Jansson, J. K.; Fredrickson, J. K.; Beliaev, A. S., Indirect interspecies regulation: Transcriptional and physiological responses of a cyanobacterium to heterotrophic partnership. mSystems **2017,** 2 (2).
23. Bobadilla Fazzini, R. A.; Preto, M. J.; Quintas, A. C.; Bielecka, A.; Dos Santos, V. A., Consortia modulation of the stress response: proteomic analysis of single strain versus mixed culture. Environ Microbiol **2010,** 12 (9), 2436-49.
24. Nouaille, S.; Even, S.; Charlier, C.; Le Loir, Y.; Cocaign-Bousquet, M.; Loubiere, P., Transcriptomic response of Lactococcus lactis in mixed culture with Staphylococcus aureus. Appl Environ Microbiol **2009,** 75 (13), 4473-82.
25. Liu, H.; Cao, Y.; Guo, J.; Xu, X.; Long, Q.; Song, L.; Xian, M., Study on the isoprene-producing co-culture system of *Synechococcus elongates-Escherichia coli* through omics analysis. Microb Cell Fact **2021,** 20 (1), 1-18.
26. Amin, S.; Hmelo, L.; Van Tol, H.; Durham, B.; Carlson, L.; Heal, K.; Morales, R.; Berthiaume, C.; Parker, M.; Djunaedi, B., Interaction and signalling between a cosmopolitan phytoplankton and associated bacteria. Nature **2015,** 522 (7554), 98-101.
27. Stanier, R. Y.; Kunisawa, R.; Mandel, M.; Cohen-Bazire, G., Purification and properties of unicellular blue-green algae (order *Chroococcales*). Bacteriol Rev **1971,** 35 (2), 171-205.
28. Jeong, S. Y.; Kim, T. G., Development of a novel methanotrophic process with the helper micro-organism Hyphomicrobium sp. NM3. J Appl Microbiol **2019,** 126 (2), 534-544.
29. Song, X.; Diao, J.; Yao, J.; Cui, J.; Sun, T.; Chen, L.; Zhang, W., Engineering a central carbon metabolism pathway to increase the intracellular acetyl-CoA pool in *Synechocystis* sp. PCC 6803 grown under photomixotrophic conditions. ACS Synth Biol **2021,** 10 (4), 836-846.
30. Gao, W.; Zhang, W.; Meldrum, D. R., RT-qPCR based quantitative analysis of gene expression in single bacterial cells. J Microbiol Methods **2011,** 85 (3), 221-7.
31. Livak, K. J.; Schmittgen, T. D., Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods **2001,** 25 (4), 402-8.
32. Fischer, B. B.; Hideg, E.; Krieger-Liszkay, A., Production, detection, and signaling of singlet oxygen in photosynthetic organisms. Antioxid Redox Signal **2013,** 18 (16), 2145-62.
33. Chen, L.; Xie, M.; Bi, Y.; Wang, G.; Deng, S.; Liu, Y., The combined effects of UV-B radiation and herbicides on photosynthesis, antioxidant enzymes and DNA damage in two bloom-forming cyanobacteria. Ecotoxicol Environ Saf **2012,** 80, 224-30.
34. Singh, S. P.; Montgomery, B. L., Reactive oxygen species are involved in the morphology-determining mechanism of *Fremyella diplosiphon* cells during complementary chromatic adaptation. Microbiol (Reading) **2012,** 158 (Pt 9), 2235-2245.

35. Gao, L.; Pan, X.; Zhang, D.; Mu, S.; Lee, D. J.; Halik, U., Extracellular polymeric substances buffer against the biocidal effect of H_2O_2 on the bloom-forming cyanobacterium *Microcystis aeruginosa*. Water Res **2015,** 69, 51-58.
36. Wang, Y.; Chen, L.; Zhang, W., Proteomic and metabolomic analyses reveal metabolic responses to 3-hydroxypropionic acid synthesized internally in cyanobacterium *Synechocystis* sp. PCC 6803. Biotechnol Biofuels **2016,** 9, 209.
37. Shu, C.-H.; Tsai, C.-C.; Chen, K.-Y.; Liao, W.-H.; Huang, H.-C., Enhancing high quality oil accumulation and carbon dioxide fixation by a mixed culture of *Chlorella* sp. and *Saccharomyces cerevisiae*. J Taiwan Inst Chem Eng **2013,** 44 (6), 936-942.
38. Lin, P. C.; Zhang, F.; Pakrasi, H. B., Enhanced production of sucrose in the fast-growing cyanobacterium *Synechococcus elongatus* UTEX 2973. Sci Rep **2020,** 10 (1), 390.
39. Beliaev, A. S.; Romine, M. F.; Serres, M.; Bernstein, H. C.; Linggi, B. E.; Markillie, L. M.; Isern, N. G.; Chrisler, W. B.; Kucek, L. A.; Hill, E. A.; Pinchuk, G. E.; Bryant, D. A.; Wiley, H. S.; Fredrickson, J. K.; Konopka, A., Inference of interactions in cyanobacterial-heterotrophic co-cultures via transcriptome sequencing. ISME J **2014,** 8 (11), 2243-2255.
40. Merchant, S. S.; Helmann, J. D., Elemental economy: Microbial strategies for optimizing growth in the face of nutrient limitation. Adv Microb Physiol **2012,** 60, 91-210.
41. Zahalak, M.; Pratte, B.; Werth, K. J.; Thiel, T., Molybdate transport and its effect on nitrogen utilization in the cyanobacterium *Anabaena variabilis* ATCC 29413. Mol Microbiol **2004,** 51 (2), 539-49.
42. Varkey, D.; Mazard, S.; Ostrowski, M.; Tetu, S. G.; Haynes, P.; Paulsen, I. T., Effects of low temperature on tropical and temperate isolates of marine *Synechococcus*. ISME J **2016,** 10 (5), 1252-63.
43. Latifi, A.; Ruiz, M.; Zhang, C.-C., Oxidative stress in cyanobacteria. FEMS Microbiol Rev **2009,** 33 (2), 258-278.
44. He, Q. F.; Dolganov, N.; Bjorkman, O.; Grossman, A. R., The high light-inducible polypeptides in Synechocystis PCC6803 - Expression and function in high light. J Bio Chem **2001,** 276 (1), 306-314.
45. Cornejo, J.; Willows, R. D.; Beale, S. I., Phytobilin biosynthesis: cloning and expression of a gene encoding soluble ferredoxin-dependent heme oxygenase from *Synechocystis* sp. PCC 6803. Plant J **1998,** 15 (1), 99-107.
46. Cheng, D.; He, Q., PfsR is a key regulator of iron homeostasis in *Synechocystis* PCC 6803. PLoS One **2014,** 9 (7), e101743.
47. Vance, J. E., Phospholipid synthesis and transport in mammalian cells. Traffic **2015,** 16 (1), 1-18.
48. Frain, K. M.; Gangl, D.; Jones, A.; Zedler, J. A.; Robinson, C., Protein translocation and thylakoid biogenesis in cyanobacteria. Biochim Biophys Acta **2016,** 1857 (3), 266-73.
49. de Keyzer, J.; van der Does, C.; Driessen, A. J., The bacterial translocase: a dynamic protein channel complex. Cell Mol Life Sci **2003,** 60 (10), 2034-52.
50. Fulda, S.; Huang, F.; Nilsson, F.; Hagemann, M.; Norling, B., Proteomics of *Synechocystis* sp strain PCC 6803 - Identification of periplasmic proteins in cells grown at low and high salt concentrations. Eur J

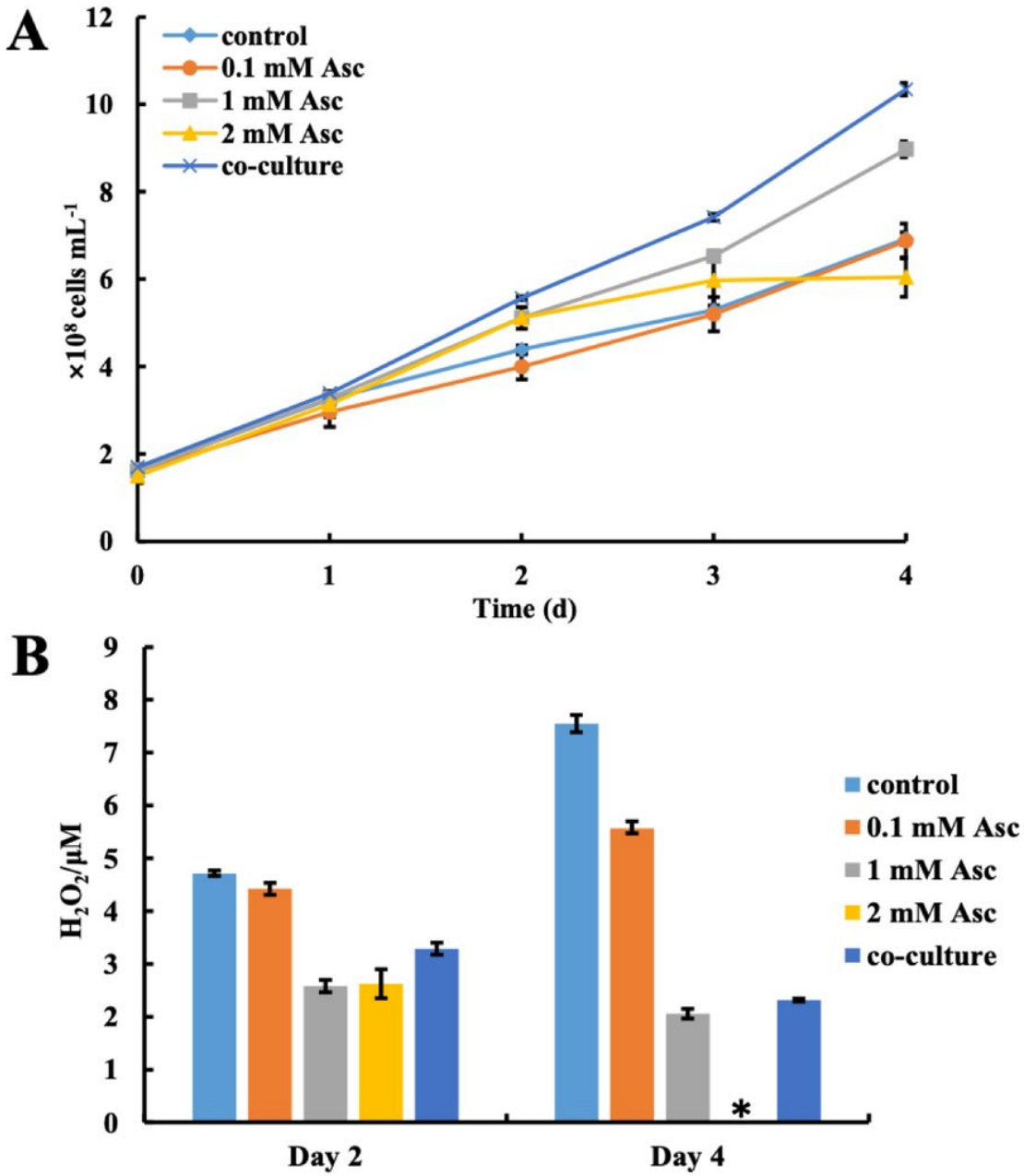
Biochem **2000,** 267 (19), 5900-5907.

51. Wang, P.; Dalbey, R. E., Inserting membrane proteins: The YidC/Oxa1/Alb3 machinery in bacteria, mitochondria, and chloroplasts. Bba-Biomembranes **2011,** 1808 (3), 866-875.
52. Edhofer, I.; Muhlbauer, S. K.; Eichacker, L. A., Light regulates the rate of translation elongation of chloroplast reaction center protein D1. Eur J Biochem **1998,** 257 (1), 78-84.
53. Nie, L.; Wu, G.; Culley, D. E.; Scholten, J. C.; Zhang, W., Integrative analysis of transcriptomic and proteomic data: challenges, solutions and applications. Crit Rev Biotechnol **2007,** 27 (2), 63-75.
54. Zhang, W. W.; Li, F.; Nie, L., Integrating multiple 'omics' analysis for microbial biology: application and methodologies. Microbiol-Sgm **2010,** 156, 287-301.
55. Nie, L.; Wu, G.; Zhang, W., Correlation of mRNA expression and protein abundance affected by multiple sequence features related to translational efficiency in *Desulfovibrio vulgaris*: a quantitative analysis. Genetics **2006,** 174 (4), 2229-43.
56. Ruffing, A. M., RNA-Seq analysis and targeted mutagenesis for improved free fatty acid production in an engineered cyanobacterium. Biotechnol Biofuels **2013,** 6.
57. Battchikova, N.; Aro, E. M., Cyanobacterial NDH-1 complexes: multiplicity in function and subunit composition. Physiol Plantarum **2007,** 131 (1), 22-32.
58. Corless, E. I.; Bennett, B.; Antony, E., Substrate recognition induces sequential electron transfer across subunits in the nitrogenase-like DPOR complex. J Biol Chem **2020,** 295 (39), 13630-13639.
59. Smith, S. R.; Dupont, C. L.; McCarthy, J. K.; Broddrick, J. T.; Obornik, M.; Horak, A.; Fussy, Z.; Cihlar, J.; Kleessen, S.; Zheng, H.; McCrow, J. P.; Hixson, K. K.; Araujo, W. L.; Nunes-Nesi, A.; Fernie, A.; Nikoloski, Z.; Palsson, B. O.; Allen, A. E., Evolution and regulation of nitrogen flux through compartmentalized metabolic networks in a marine diatom. Nat Commun **2019,** 10.
60. Lin, W.; Zhao, D.; Luo, J., Distribution of alkaline phosphatase genes in cyanobacteria and the role of alkaline phosphatase on the acquisition of phosphorus from dissolved organic phosphorus for cyanobacterial growth. J Appl Phycol **2018,** 30 (2), 839-850.
61. Glenn, K.; Smith, K. S., Allosteric regulation of *Lactobacillus* plantarum xylulose 5-phosphate/fructose 6-phosphate phosphoketolase (Xfp). J Bacteriol **2015,** 197 (7), 1157-63.
62. Kletzin, A.; Adams, M. W., Molecular and phylogenetic characterization of pyruvate and 2-ketoisovalerate ferredoxin oxidoreductases from *Pyrococcus furiosus* and pyruvate ferredoxin oxidoreductase from *Thermotoga maritima*. J Bacteriol **1996,** 178 (1), 248-57.
63. Omata, T.; Takahashi, Y.; Yamaguchi, O.; Nishimura, T., Structure, function and regulation of the cyanobacterial high-affinity bicarbonate transporter, BCT1. Funct Plant Biol **2002,** 29 (3), 151-159.
64. Nishimura, T.; Takahashi, Y.; Yamaguchi, O.; Suzuki, H.; Maeda, S.-i.; Omata, T., Mechanism of low CO_2-induced activation of the cmp bicarbonate transporter operon by a LysR family protein in the cyanobacterium *Synechococcus elongatus* strain PCC 7942. Mol Microbiol **2008,** 68 (1), 98-109.
65. Adhikari, P.; Berish, S. A.; Nowalk, A. J.; Veraldi, K. L.; Morse, S. A.; Mietzner, T. A., The fbpABC locus of Neisseria gonorrhoeae functions in the periplasm-to-cytosol transport of iron. J Bacteriol **1996,** 178

(7), 2145-9.

66. Mihara, H.; Esaki, N., Bacterial cysteine desulfurases: their function and mechanisms. Appl Microbiol Biotechnol **2002,** 60 (1-2), 12-23.
67. Johnson, D. C.; Dean, D. R.; Smith, A. D.; Johnson, M. K., Structure, function, and formation of biological iron-sulfur clusters. Annu Rev Biochem **2005,** 74, 247-81.
68. Pohl, M.; Sprenger, G. A.; Muller, M., A new perspective on thiamine catalysis. Curr Opin Biotechnol **2004,** 15 (4), 335-42.
69. Furt, F.; Oostende, C.; Widhalm, J. R.; Dale, M. A.; Wertz, J.; Basset, G. J., A bimodular oxidoreductase mediates the specific reduction of phylloquinone (vitamin K□) in chloroplasts. Plant J **2010,** 64 (1), 38-46.
70. Kaushik, M. S.; Singh, P.; Tiwari, B.; Mishra, A. K., Ferric uptake regulator (FUR) protein: properties and implications in cyanobacteria. Ann Microbiol **2016,** 66 (1), 61-75.
71. Pujol-Carrion, N.; de la Torre-Ruiz, M. A., Glutaredoxins Grx4 and Grx3 of *Saccharomyces cerevisiae* play a role in actin dynamics through their trx domains, which contributes to oxidative stress resistance. Appl Environ Microbiol **2010,** 76 (23), 7826-7835.
72. Lee, J. W.; Helmann, J. D., Functional specialization within the Fur family of metalloregulators. Biometals **2007,** 20 (3-4), 485-99.
73. Du, Y.; Zou, W.; Zhang, K.; Ye, G.; Yang, J., Advances and applications of clostridium co-culture systems in biotechnology. Front Microbiol **2020,** 11, 560223.
74. He, L.; He, T.; Farrar, S.; Ji, L.; Liu, T.; Ma, X., Antioxidants maintain cellular redox homeostasis by elimination of reactive oxygen species. Cell Physiol Biochem **2017,** 44 (2), 532-553.
75. Xue, S.; Su, Z.; Cong, W., Growth of Spirulina platensis enhanced under intermittent illumination. J Biotechnol **2011,** 151 (3), 271-7.
76. Bohmer, S.; Koninger, K.; Gomez-Baraibar, A.; Bojarra, S.; Mugge, C.; Schmidt, S.; Nowaczyk, M. M.; Kourist, R., Enzymatic oxyfunctionalization driven by photosynthetic water-splitting in the cyanobacterium Synechocystis sp PCC 6803. Catalysts **2017,** 7 (8).
77. Rinalducci, S.; Pedersen, J. Z.; Zolla, L., Generation of reactive oxygen species upon strong visible light irradiation of isolated phycobilisomes from *Synechocystis* PCC 6803. Bba-Bioenergetics **2008,** 1777 (5), 417-424.
78. Imlay, J. A., The molecular mechanisms and physiological consequences of oxidative stress: lessons from a model bacterium. Nat Rev Microbiol **2013,** 11 (7), 443-54.

Figures



1

Figure 1

Analysis of the effect of quenching ROS on cultivated cyanobacterial cell growth by adding ascorbic acid. Cell growth curves of Synechococcus cscB+ (A) and H2O2 content (B) in co-culture system and axenic culture with additional ascorbic acid.

Figure 2

Target metabolomics analysis of Synechococcus cscB+ under co-culture and axenic culture condition. (A) The metabolites change in central metabolic pathway in co-cultivated Synechococcus cscB+

compared with axenic culture condition; (B) Heatmaps of metabolomics profiles in Synechococcus cscB+ under co-culture and axenic culture condition. Each color on the heatmap corresponds to a concentration value. The higher the concentration, the darker the color (red represents the increase, and green represents the decrease).

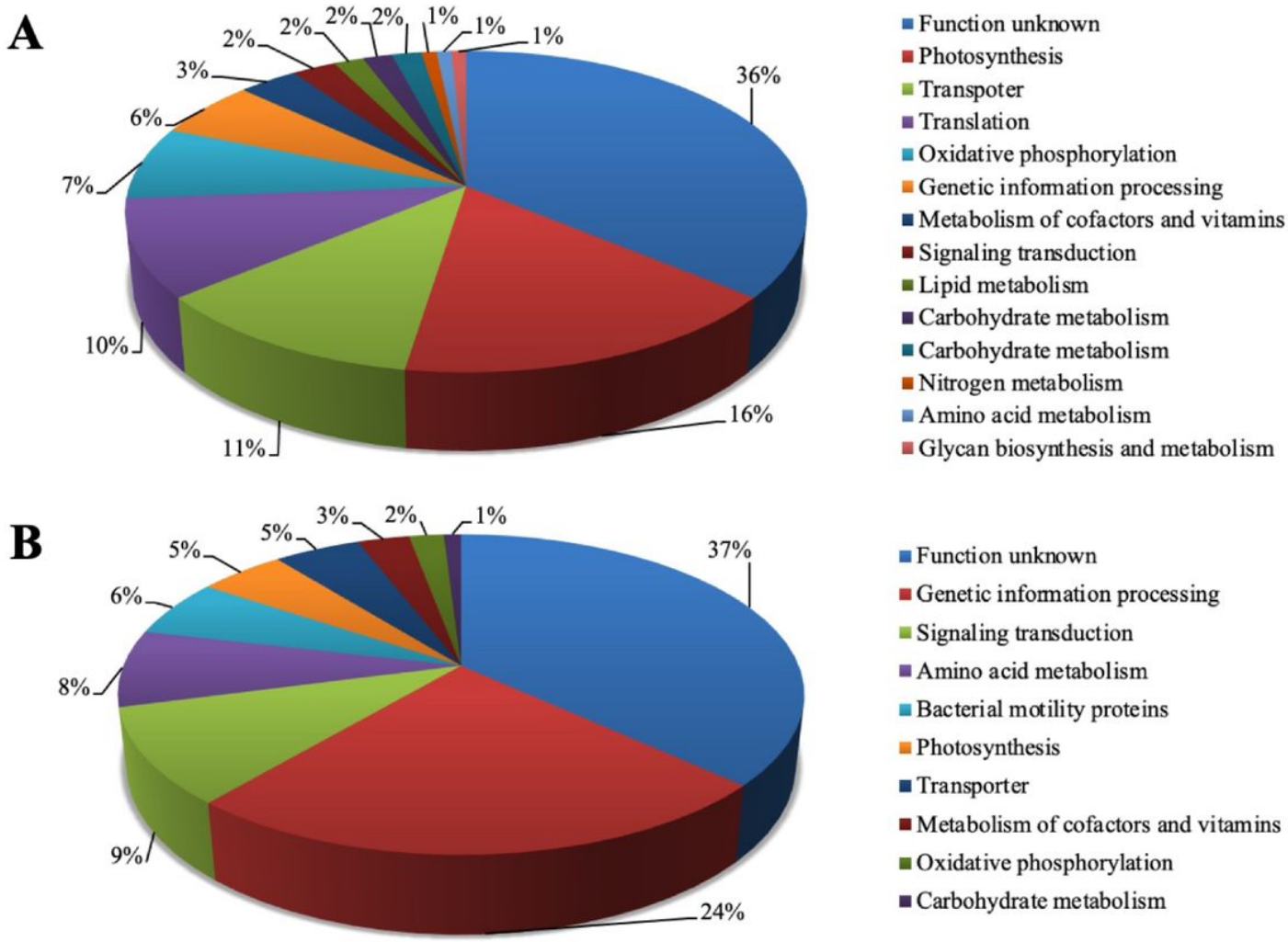


Figure 3

Pathway classification distribution of differentially expressed genes in co-cultivated Synechococcus cscB+ compared grew under pure culture condition. (A) Up-regulated genes KEGG pathway analysis; (B) Down-regulated genes KEGG pathway analysis. KEGG, Kyoto Encyclopedia of Genes and Genomes.

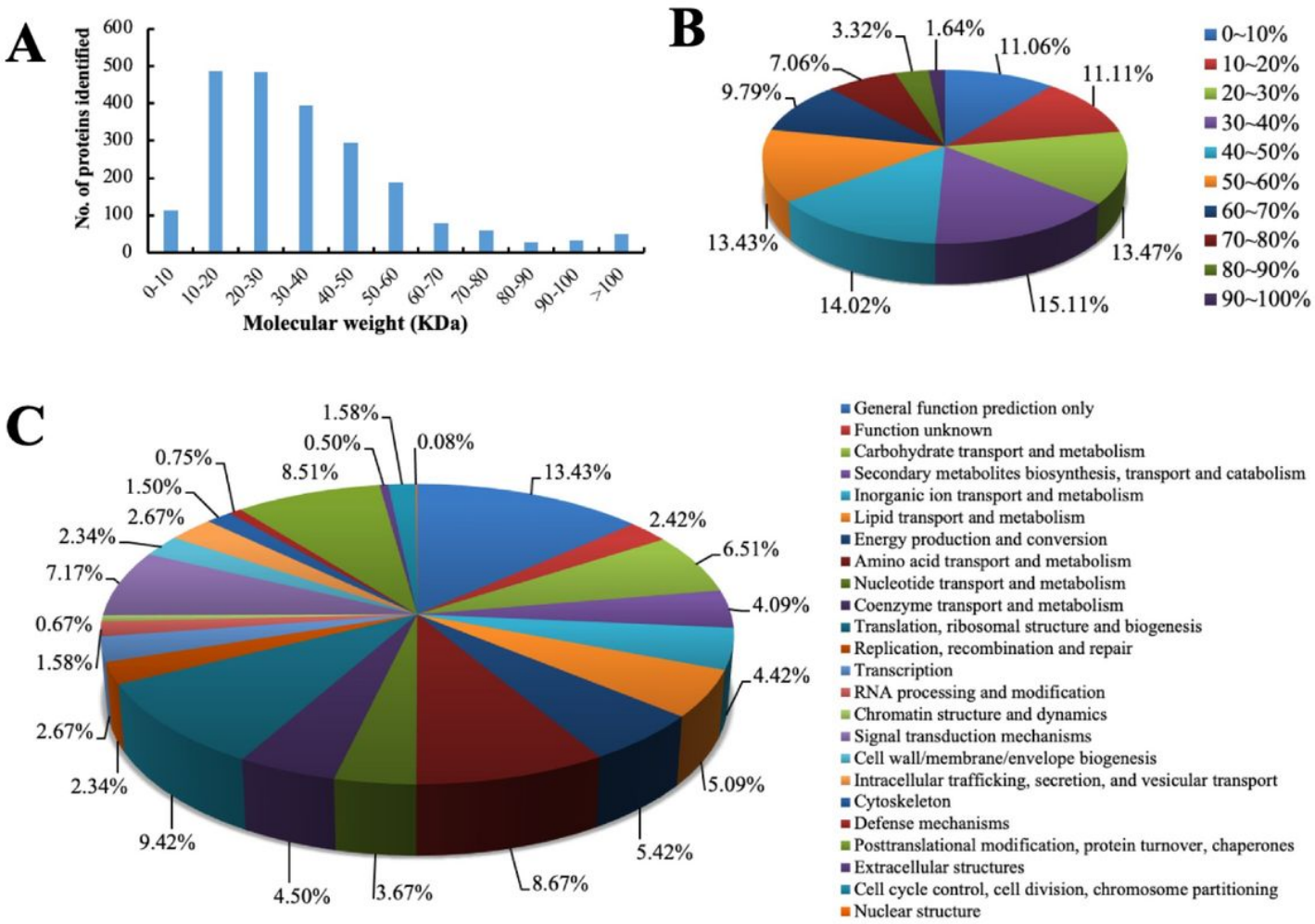


Figure 4

Distribution, coverage, and functional category of proteins identified in this study. (A) Distribution of protein identified among different molecular weights; (B) Coverage of proteins by the identified peptides; (C) Functional category coverage of the proteins identified.

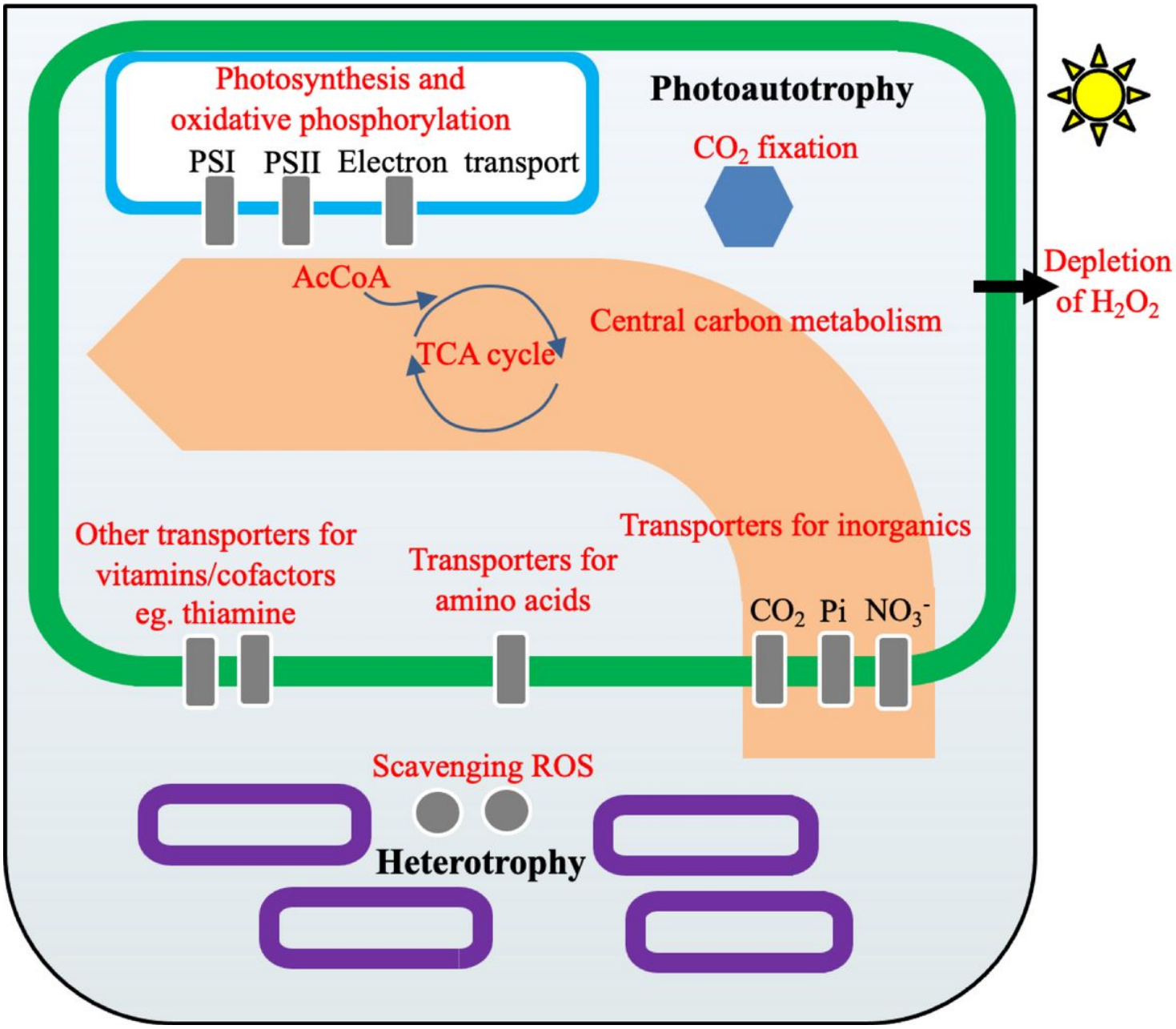


Figure 5

Schematic representation of the cross-feeding process from heterotrophic to photoautotrophic species occurred in the artificial co-culture system.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Fig.S1.jpg
- Fig.S2.jpg