

# Study on the isoprene-producing co-culture system of *Synechococcus elongatus*- *Escherichia coli* through omics analysis

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## Research

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# Abstract

**Background:** The majority of microbial fermentations are currently performed in the batch or fed-batch manner with the high process complexity, huge water consumption and so on. The continuous microbial production can contribute to the green sustainable development of the fermentation industry. The co-culture systems of photo-autotrophic and heterotrophic species can play important roles in establishing the continuous fermentation mode for the bio-based chemicals production.

**Results:** In the present paper, the co-culture system of *Synechococcus elongatus*-*Escherichia coli* was established and put into operation stably for isoprene production. Compared with the axenic culture, the fermentation period of time was extended from 100h to 400h in the co-culture and the isoprene production was increased to 8-fold. For in depth understanding this novel system, the differential omics profiles were analyzed. The responses of BL21(DE3) to *S. elongatus* PCC 7942 were triggered by the oxidative pressure through the Fenton reaction and all these changes were linked with one another at different spatial and temporal scales. The oxidative stress mitigation pathways might contribute to the long-lasting fermentation process. The performance of this co-culture system can be further improved according to the fundamental rules discovered by the omics analysis.

**Conclusions:** The isoprene-producing co-culture system of *S. elongatus*-*E. coli* was established and then analyzed by the omics methods. This study on the co-culture system of the model *S. elongatus*-*E. coli* is of significance to reveal the common interactions between photo-autotrophic and heterotrophic species without natural symbiotic relation, which could provide the scientific basis for rational design of microbial community.

## 1. Background

With the depletion of the nonrenewable petrochemical resources, many biofuels and platform chemicals have been industrially produced by fermentation to substitute the petroleum-based counterparts. However, the majority of microbial fermentations are currently performed within a short period of time in the batch or fed-batch manner, which means the high process complexity, huge water consumption, high production costs and large fixed capital investment. In China the fermentation industry discharges about 8 billion tons of wastewater per year, accounting for 10% of the total industrial emissions [1]. Therefore, the green sustainable development of the fermentation industry appears to be very important, which can benefit from the continuous microbial production running stably for a long period of time like the industrial chemo-process.

The co-culture systems of photo-autotrophic and heterotrophic species can play a key role in establishing the continuous fermentation mode for the biobased chemicals production. Hays *et al.* described that model cyanobacterium *S. elongatus* PCC 7942 could co-culture with *Bacillus subtilis*, *E. coli* or *Saccharomyces cerevisiae* for a long period of time [2]. The previous study on the co-culture of algae and bacteria suggested that the niche complementarity and functional redundancy led to high efficiency and

stability of the co-culture systems in the face of biotic or abiotic disturbances [3]. The continuous and symbiotic growth was achieved and maintained for 3.5 years by co-culturing algal species *Scenedesmus obliquus*, *Scenedesmus* sp. D202, aerobic bacterial species *Bacillus* sp. D320 and *Rhodobacter sphaeroides*, and diazotrophic bacterial species *Methanobacteria* sp. D422 and *Spirulina* sp. D11 [4]. Cong *et al.* artificially constructed a co-culture system between *Candida tropicalis* and *S. obliquus*. Compared with the axenic culture system, the biomass and photosynthetic activity of *S. obliquus* were increased by 30.3% and 61% respectively [5]. The co-culture of *Synechocystis* PCC6803 with *Pseudomonas*-related GM41 strain resulted in 8-fold increase in the cyanobacterial biomass [6]. Zhang *et al.* reported that compared with axenic culture, co-culture of microalgae *Chlorella vulgaris* and yeast *Rhodotorula glutinis* could increase biomass by 17.3% and lipid yields by 70.9% [7]. Shu *et al.* enhanced CO<sub>2</sub> fixation and oil production by co-culturing *Chlorella* and *S. cerevisiae* [8]. Therefore, the co-culture systems of photo-autotrophic and heterotrophic species can contribute to the efficient and stable fermentation with high productivity and yield.

Although some co-culture systems of photo-autotrophic and heterotrophic species were successfully established, only a few efforts have been devoted to investigating the interaction mechanisms between photo-autotrophic and heterotrophic species at multi-level of RNA, protein and metabolite. The transcriptomics, proteomics and metabolomics are critical to study the interaction mechanisms of the co-culture systems. Due to the incompleteness and complementarity of these different methods, the multi-omics analysis can obtain a "panorama" of cells in the co-culture systems and demonstrate novel insights into the biological mechanisms. Amin *et al.* investigated the signaling and interaction between cosmopolitan phytoplankton and associated bacteria through transcriptomic and targeted metabolite analyses, which is the milestone in the analysis of the interaction between algae and bacteria. Tryptophan and indole-3-acetic acid were determined to be the key signalling molecules, which were part of a complex metabolites exchange including bacterial-excreted ammonia and diatom-excreted organosulfur molecules [9]. Therefore the methods of transcriptome, proteome and metabolome should be adopted to analyze microbial interactions in the co-culture systems, which might finally contribute to the technology breakthrough of the continuous fermentation mode.

Isoprene is an ideal raw material for synthetic rubber, and 95% of isoprene production is used for the production of synthetic rubber. With the depletion of the nonrenewable petrochemical resources and the increasing global environmental concerns, bio-isoprene will be a promising alternative towards the petroleum-isoprene. A renewable and reliable source of isoprene is imperative to economic competitiveness and national security [10]. So far, some progresses in axenic culture have been made for the biosynthesis of isoprene using various microorganisms such as *S. cerevisiae*, *Synechocystis*, *B. subtilis* and *E. coli* [11–16]. The breakthrough has been made by Genencor, which developed an *E. coli*-based system with the ability to produce ~60 g/L of isoprene [10]. However, there are no reports for the biosynthesis of isoprene by co-culture fermentation yet.

Although cyanobacteria can have the symbiosis with bacteria and fungi in natural environments, no natural symbiosis relations between cyanobacteria and heterotrophic specie *E. coli* are reported so far.

The synthetic consortium of the sucrose-secreting cyanobacterium *S. elongatus* PCC 7942 and *E. coli* was constructed recently [2]. However, this novel consortium is a complete light-driven system with low cell density, which constrained the possibly wide application in the fermentation industry. In the present paper, a novel co-culture fermentation system of model microorganisms *S. elongatus* PCC 7942 and *E. coli* BL21(DE3) was established for the production of isoprene with high cell concentration. The effects of *S. elongatus* PCC 7942 on *E. coli* BL21(DE3) were also investigated using methods of omics analysis.

## 2. Materials And Methods

### 2.1 Strains, plasmids and media

Strains and plasmids were listed in Table 1. *S. elongatus* PCC 7942 was generously provided by Dr. Xuefeng Lv's laboratory [17]. BL21 (DE3) was used as the host for isoprene production. The isoprene producer (IP) harboring plasmid pYJM14 with mevalonate pyrophosphate decarboxylase gene *ERG19*, mevalonate kinase gene *ERG12*, phosphomevalonate kinase gene *ERG8* and IPP isomerase gene *IDI1*, and plasmid pYJM20 with isoprene synthase gene *ispS*, HMG-CoA synthase gene *mvaS* and acetyl-CoA acetyltransferase/ hydroxymethylglutaryl-CoA (HMG-CoA) reductase gene *mvaE* [18].

*S. elongatus* PCC 7942 was propagated in BG11 medium. The isoprene producer was propagated in LB medium. The co-culture medium consisted of 0.75 g/L NaNO<sub>3</sub>, 0.014 g/L CaCl<sub>2</sub>, 0.01 g/L NaCO<sub>3</sub>, 0.5 mg/L Na<sub>2</sub>EDTA·2H<sub>2</sub>O, 4.9 g/L K<sub>2</sub>HPO<sub>4</sub>·3H<sub>2</sub>O, 1 g/L Citric acid·H<sub>2</sub>O, 0.15 g/L Ferric Ammonium Citrate, 1 g/L (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.21g/L MgSO<sub>4</sub>, 5 g/L glucose, 3.4 µg/ml chloramphenicol, 10 µg/mL ampicillin and 0.5 ml/L trace elements (each 100 ml solution containing 0.25 g CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.37 g (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>·4H<sub>2</sub>O, 2.47 g H<sub>3</sub>BO<sub>3</sub>, 0.29 g ZnSO<sub>4</sub>·7H<sub>2</sub>O, 1.58 g MnCl<sub>2</sub>·4H<sub>2</sub>O).

### 2.2 Co-culture for the isoprene production

The seed culture of *S. elongatus* PCC 7942 was prepared in BG-11 medium for ~7 days at 28 °C. The seed culture of IP-strain was prepared in LB medium supplemented with 100 µg/mL ampicillin and 34 µg/ml chloramphenicol for ~12 h at 37 °C. The total 12 ml seed cultures of *S. elongatus* and IP-strain in the ratio of 1:1 or 1:4 (V/V) were used to inoculate a 250-mL bioreactor (Applikon Biotechnology, Netherland) containing 150 mL co-culture medium in light (30 µmol m<sup>-2</sup> s<sup>-1</sup>). The chloramphenicol (3.4 µg/ml) and ampicillin (10 µg/ml) were added to the co-culture medium. The cultivation temperature was maintained at 28 °C, the pH was controlled at 7.0 by automatic feeding concentrated glucose or 25 % (w/w) KOH solution, the aeration rate was 10 vvm, and the stirring rate was maintained at 150 rpm. The cells were induced at OD<sub>600</sub> 5 by the addition of 0.1 mM IPTG. The cell growth of IP-strain was assayed by plating dilution series on LB media to count colony forming units (CFU). The cell growth of *S. elongatus* PCC 7942 was measured by plating dilution series on BG-11 media to count CFU. Samples were collected for isoprene analysis at certain intervals. The co-culture for the isoprene production was carried out for three biological replicates.

## 2.3 GC analysis of isoprene

1 ml of off-gas samples from the bioreactor were analyzed as described earlier [14] using a GC (Agilent 7890A, America) equipped with a HP-AL/S column (25 m × 320 μm × 8 μm) and FID. Nitrogen was used as carrier gas with a linear velocity of 1 ml/min. The product was characterized by comparison with standard isoprene (TCI-EP, Tokyo, Japan). The peak area was converted to isoprene concentration by a standard curve plotted with a set of known concentration of isoprene.

## 2.4 RNA isolation and differential transcriptome analysis

Total RNA was extracted by the Easyspin RNA reagent (Aidlab, Beijing, China) and treated with RNase-free DNase I (Takara, Dalian, China) to remove genomic DNA contamination. RNA concentration was measured by Qubit® RNA Assay Kit in Qubit® 2.0 Fluorometer (Life Technologies, CA, USA). RNA purity was checked by the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). The library preparation, sequencing and data analysis were accomplished by Novogene, Inc. Differential expression analysis between two samples was carried out by the DESeq R package. Genes with an adjusted P-value  $\leq 0.05$  found by DESeq were assigned as differentially expressed genes. Gene Ontology (GO) enrichment analysis of differential expression genes was performed by the GOseq R package. GO terms with corrected P-value less than 0.05 were considered to be significantly enriched. KOBAS software was employed for the statistical enrichment of differential expression genes in KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways. The significant differential pathways were defined as those with an FDR value of  $\leq 0.05$ .

## 2.5 Total Protein Extraction and differential proteome analysis

Samples were minced individually with liquid nitrogen and lysed in lysis buffer containing 6 M Urea, 100 mM  $\text{NH}_4\text{HCO}_3$  (pH 8) and 0.2 % SDS, followed by 5 min of ultrasonication on ice. The lysate was centrifuged at 12000 g for 15 min at 4 °C and the supernatant was transferred to a clean tube. Extracts from each sample were reduced with 2mM DTT for 1 h at 56 °C, and subsequently alkylated with sufficient iodoacetamide for 1 h in the dark at room temperature. The samples were mixed with 4 times volume of precooled acetone and incubated at -20 °C for at least 2 h. Samples were then centrifuged and the precipitation was collected. The pellet was dissolved by dissolution buffer containing 0.1 M triethylammonium bicarbonate (TEAB, pH 8.5) and 6 M urea after twice washing with precooled acetone. The peptide preparation, TMT labeling, HPLC fractionation, LC-MS/MS analysis and data analysis were accomplished by Novogene, Inc. The protein with at least 1 unique peptide was identified at FDR less than 1.0 % on peptide and protein level, respectively. The protein quantitation results were statistically analyzed by Mann-Whitney Test, the significant ratios, defined as  $p < 0.05$  and  $|\log_2\text{FC}| > 0.58$  ( $\text{FC} > 1.5$  or  $\text{FC} < 0.66$  [fold change, FC]), were used to screen the differential expression proteins. GO analysis were conducted using the interproscan-5 program against the databases COG (Clusters of Orthologous Groups) and the non-redundant protein database (including Pfam, PRINTS, ProDom, SMART,

ProSiteProfiles, PANTHER), and KEGG were employed to analyze the protein family and pathway. The enrichment pipeline was used to perform the enrichment analysis of GO and KEGG, respectively.

## 2.6 Metabolites Extraction, UHPLC-MS/MS Analysis and data analysis

The samples (100 mg) were individually grounded with liquid nitrogen and the homogenate was resuspended with cold methanol and 0.1 % formic acid by well vortexing. The samples were incubated on ice for 5 min and then were centrifuged at 15000 rpm, 4°C for 5 min. The supernatant was diluted to final concentration containing 60 % methanol by LC-MS grade water. The samples were subsequently transferred to a fresh Eppendorf tube with 0.22 µm filter and then were centrifuged at 15000 g, 4 °C for 10 min. Finally, the filtrate was injected into the UHPLC-MS/MS analysis. Metabolites Extraction, UHPLC-MS/MS analysis and data analysis were accomplished by Novogene, Inc. The raw data files generated by UHPLC-MS/MS were processed using the Compound Discoverer 3.0 (CD 3.0, Thermo Fisher) to perform peak alignment, peak picking, and quantitation for each metabolite. The main parameters were set as follows: retention time tolerance, 0.2 minutes; actual mass tolerance, 5 ppm; signal intensity tolerance, 30 %; signal/noise ratio, 3; and minimum intensity, 100000. After that, peak intensities were normalized to the total spectral intensity. The normalized data was used to predict the molecular formula based on molecular ion peaks, additive ions and fragment ions. And then peaks were matched with ChemSpider (<http://www.chemspider.com/>) database and mzCloud (<https://www.mzcloud.org/>) to obtain the accurate qualitative and relative quantitative results. The significant ratios, defined as  $p < 0.05$  and  $|\log_2FC| > 1$  ( $FC > 2$  or  $FC < 0.5$ ), were used to screen the differential metabolites.

## 2.7 Statistical analysis

Experiments were performed in triplicate, and the values are presented as the mean  $\pm$  standard deviation.  $P < 0.05$  was taken to indicate statistical significance.

# 3. Results

## 3.1 Establishment of the isoprene-producing co-culture system of *S. elongatus*-*E. coli*

In order to establish the isoprene-producing co-culture system of *S. elongatus*-*E. coli*, the medium was designed by mixing isoprene fermentation medium [19] and BG11 culture medium at the ratio of 1:1 in volume. *S. elongatus* PCC 7942 and BL21 (DE3) were introduced into the co-culture system at the inoculation ratio of 1:1 and 1:4 (*S. elongatus* PCC 7942 vs BL21 (DE3), v/v) respectively. The chloramphenicol (3.4 µg/ml) and ampicillin (10 µg/ml) were added to the co-culture medium to reduce the plasmids loss of the engineered *E. coli* and increase the stability of the heterogeneous mevalonate (MVA) pathway genes. Although the low levels of chloramphenicol and ampicillin were not fatal for the cyanobacteria [20, 21], the antibiotics addition completely inhibited the growth of *S. elongatus* PCC 7942 in the axenic culture and only the axenic culture of BL21 (DE3) was used as the control.

The time courses of biomass, isoprene titer and yield in the axenic culture and co-cultures at the different inoculation ratios were determined respectively. As shown in Fig. 1A, the cell concentration of BL21 (DE3) in the co-culture was higher than the control, indicating that the presence of *S. elongatus* PCC 7942 promoted the growth of BL21 (DE3). The improved growth of *S. elongatus* PCC 7942 in the co-culture suggested that the inhibition of antibiotics on *S. elongatus* PCC 7942 was released by BL21 (DE3) in the co-culture system. As shown in Fig. 1B, by increasing the inoculation ratio from 1:4 in coculture1 to 1:1 in coculture2, the isoprene fermentation period of time was extended from 100 h to 400 h. The isoprene titer in co-culture was also increased 7-fold to 0.4 g/L. The longer fermentation time and higher isoprene titer in the co-culture process indicated that the presence of *S. elongatus* PCC 7942 promoted the isoprene synthesis. As shown in Fig. 1C, the maximum yield of isoprene was all close to 0.5 % during the processes of co-culture and axenic culture. Therefore the novel co-culture system of *S. elongatus*-*E. coli* was well established.

### 3.2 Differential omics analysis of *E. coli*/BL21(DE3) between the co-culture system and the control

In order to study the cellular response of *E. coli* BL21(DE3) to the addition of *S. elongatus* PCC 7942, the samples (EC1, EC2 and EC3) of co-culture2 for the differential omics analysis were taken at 18 h, 250 h and 500 h, respectively. The samples (E1) of axenic culture of BL21 (DE3) were taken at 18 h as the control. Because the antibiotics addition completely inhibited the growth of *S. elongatus* PCC 7942 in the axenic culture, the differential omics analysis was not performed for the *S. elongatus* PCC 7942.

The differential profiles of gene expression, proteins and metabolites in *E. coli* BL21(DE3) were identified by pairwise comparisons of E1, EC1, EC2 and EC3 (Table 2). In pairs of EC1 vs. E1, 374 genes were differentially expressed, including 212 up-regulated genes and 162 down-regulated genes. 359 proteins were differentially expressed, including 210 up-regulated proteins and 149 down-regulated proteins. There were 83 differential metabolites (55 up-regulations and 28 down-regulations) in positive mode. There were 105 differential metabolites (78 up-regulations and 27 down-regulations) in negative mode. In pairs of EC2 vs. EC1, 856 genes were differentially expressed, including 397 up-regulated genes and 459 down-regulated genes. 70 proteins were differentially expressed, including 52 up-regulated proteins and 18 down-regulated proteins. There were 141 differential metabolites (110 up-regulations and 31 down-regulations) in positive mode. There were 97 different metabolites (64 up-regulations and 33 down-regulations) in negative mode. In pairs of EC3 vs. EC2, 224 genes were differentially expressed, including 54 up-regulated genes and 170 down-regulated genes. 20 proteins were differentially expressed, including 17 up-regulated proteins and 3 down-regulated proteins. There were 181 differential metabolites (43 up-regulations and 138 down-regulations). There were 105 different metabolites (42 up-regulations and 63 down-regulations) in negative mode.

### 3.3 Functional analysis of differential transcriptome, proteome and metabolome

As shown in Fig. 2, KEGG enrichment analysis of transcriptome and proteome suggested that the flagellar assembly and thiamine metabolism in EC1 were significantly down-regulated while ribosome was up-regulated compared to E1. Dozens of differential transcripts and proteins were assigned to the

tryptophan metabolism, sulfur metabolism, pyrimidine and purine metabolisms, arginine and proline metabolisms, branched chain amino acid metabolisms, pantothenate and CoA metabolism, biotin metabolisms, lipoic acid metabolism, homologous recombination, DNA replication and base excision repair. GO enrichment analysis of transcriptome and proteome also showed that compared to E1, flagella-dependent cell motility was significantly down-regulated, and the processes and molecular functions involved with ribosome were mainly up-regulated in EC1. Both KEGG and GO enrichment analyses indicated that in EC1 the flagellum-dependent motility of *E. coli* strains decreased significantly, but the metabolic activity increased significantly.

As shown in Fig. 2, KEGG enrichment analysis of transcriptome and metabolome suggested that compared to the control E1, thiamine metabolism, arginine synthesis and metabolism, sulfur metabolism, lysine synthesis and degradation, TCA, ubiquinone synthesis, riboflavin metabolism, tyrosine metabolism, glutamic acid metabolism, purine metabolism and aromatic compound degradation were significantly changed in EC1.

As shown in Fig. 2, KEGG enrichment analysis of proteome and metabolome showed that compared to the control E1, tryptophan metabolism, thiamine metabolism, sulfur metabolism, arginine metabolism, purine metabolism, pantothenic acid and coenzyme A metabolism, biotin metabolism, phenylalanine metabolism, benzoic acid metabolism, glyoxylate metabolism and branched chain amino acid synthesis significantly varied in EC1.

As shown in Fig. 3, KEGG enrichment analysis of transcriptome and proteome suggested that the folate synthesis, tryptophan metabolism and histamine metabolism, homologous recombination, and base excision repair took significant changes in EC2 compared to EC1. The GO enrichment analysis of transcriptome and proteome showed that folate synthesis also changed significantly. KEGG and GO enrichment analyses of transcriptome showed that compared to the control EC1, ribosomes and the metabolism and degradation of fatty acids were significantly changed in EC2.

As shown in Fig. 3, KEGG enrichment analysis of transcriptome and metabolome showed that compared to the control EC1, the fatty acid synthesis, the synthesis of unsaturated fatty acids, tryptophan metabolism, arginine synthesis and metabolism, ubiquinone synthesis, phenylalanine metabolism, the degradation of aromatic compounds, pyrimidine metabolism, pantothenic acid metabolism, biotin metabolism, and CoA metabolism, TCA significantly varied in EC2.

As shown in Fig. 3, KEGG enrichment analysis of proteome and metabolome showed that compared to the control EC1, the histidine metabolism, quorum sensing, TCA, PPP, ubiquinone synthesis, glutamate metabolism and glyoxylic acid had significant changes in EC2.

As shown in Fig. 4, KEGG enrichment analysis of transcriptome and proteome suggested that the biofilm formation, TCA cycle, lysine synthesis and degradation, fatty acid synthesis, metabolism and degradation were significantly changed in EC3 compared to EC2. The GO enrichment analysis of transcriptome and proteome showed that the redox process and redox enzymes were mainly up-regulated at the protein level

while were mainly down-regulated at the transcription level in EC3 compared to EC2, indicating that the metabolic activity of *E. coli* strains in EC3 was changing from prosperity to decline.

As shown in Fig. 4, KEGG enrichment analysis of transcriptome and metabolome showed that compared to EC2, the synthesis of ubiquinone, arginine and proline metabolisms, tyrosine metabolism, phenylalanine metabolism, aromatic compounds degradation, TCA, glyoxylic acid metabolism, purine and pyrimidine metabolisms, biotin metabolism, lysine synthesis and degradation, sulfur metabolism, tryptophan metabolism, fatty acid synthesis and degradation significantly varied in EC3

As shown in Fig. 4, KEGG enrichment analysis of proteome and metabolome showed that compared to EC2, fatty acid metabolism and degradation, terpene synthesis, histidine metabolism, oxidative phosphorylation, two-component system, TCA, tryptophan metabolism, pyruvate metabolism, propionic acid metabolism, glyoxylate metabolism had significant changes in EC3.

### **3.4 Correlation analysis of differential transcriptome, proteome and metabolome**

The response of BL21(DE3) to the *S. elongatus* PCC 7942 was investigated through the omics correlation analysis. A series of changes at the levels of transcription, protein, and metabolism were detected (Fig. 5 and 6). Firstly, BFR for binding ferrous ion was up-regulated and the YifE for repairing iron-sulfur clusters was up-regulated. The thiol cysteine was converted to cystine. The downstream thiamine synthesis was subsequently down-regulated. Secondly, the toxic proteins ea8.5 and CspD and the phage shock proteins PspB, PspD and PspE were up-regulated to cope with the oxidative pressure. Thirdly, the flagella synthesis and assembly (FlagE, FlagI, FlagH, FlagN) were decreased. The protein YciG, relevant to the motility of cells and horizontal gene transfer, was upregulated. Fourthly, the expression of NADH: ubiquinone oxidoreductase I in the respiratory chain of *E. coli* strains was inhibited by CpxR due to the oxidative pressure or the physical contact of *S. elongatus* PCC 7942, which led to the up-regulation of CusF for strengthening the efflux of  $\text{Cu}^{2+}$  and the up-regulation of YaiF for the degradation of ubiquinone. MenE was also up-regulated to make up the loss of the ubiquinone after the release of the  $\text{Cu}^{2+}$  toxicity. The expressions of menE and yaiA were all subsequently decreased in EC2 compared to EC1. Fifthly, many aromatic compounds such as indole and ubiquinone increased, sharing the common precursor chorismate in the biosynthesis pathway. The stress-responding protein YcfR was upregulated to increase the indole production for the inhibition of the biofilm formation and the strengthening of arginine catabolism. The differential expressions of proteins AstBD implied that the glutamate could be recycled through arginine catabolism.

As shown in Fig. 6 and 7, the overexpression of protein puts pressure on the host after the addition of IPTG, leading to up-regulation of cold-shock protein. Both the mRNA and protein of the isoprene synthase were detected to be up-regulated in EC2 and kept unchanged in EC3. In contrast, MvaE were gradually up-regulated and kept increasing in EC3. The intermediate metabolites (MVA and DMAPP) in MVA pathway were also observed to be accumulated. The pyrophosphohydrolase NudB was up-regulated to release the accumulation of toxic DMAPP. In addition, the fatty acid biosynthesis was increased.

## 4 Discussion

### 4.1 Response of *E. coli* BL21(DE3) to *S. elongatus* PCC 7942 in co-culture system

The response of BL21(DE3) to *S. elongatus* PCC 7942 in this co-culture system was actually triggered by the oxidative pressure from photosynthesis. The previous studies also described that the products of photosynthetic reactions (e.g., reactive oxygen species and O<sub>2</sub>) inhibited the growth of heterotrophs when the heterotrophs were exposed to high densities of cyanobacteria in the light [2, 22]. However, the influences of photosynthesis on cellular response of heterotrophs have not been investigated in details previously. In the present paper, we discovered that the *S. elongatus* PCC 7942 led to oxidative pressure on the *E. coli* strains through the Fenton reaction within the cell membrane, which triggered a series of changes at the levels of transcription, protein, and metabolism. All the changes were linked with one another at different spatial and temporal scale. Firstly, in order to stop the Fenton reaction, BFR was up-regulated to decrease the ferrous ion concentration and the thiol cysteine was converted to cystine to reduce H<sub>2</sub>O<sub>2</sub>. The downstream thiamine synthesis was subsequently down-regulated to support the production of cysteine. The Fe-S proteins on the cell membrane were damaged by the Fenton reaction and the YifE was up-regulated for the repair of damaged proteins. Secondly, the toxic proteins ea8.5 and CspD were upregulated to change the cell cycle and improve the cell tolerance, which could contribute to the long-lasting fermentation process. Thirdly, the phage shock proteins PspB, PspD and PspE were up-regulated to cope with the oxidative pressure. Fourthly, the flagella synthesis and assembly were decreased. The protein YciG, relevant to the motility of cells and horizontal gene transfer, was upregulated. Thus the swimming and swarming mobility of *E. coli* BL21(DE3) strains were weakened. Fifthly, the expression of NADH: ubiquinone oxidoreductase I in the respiratory chain of *E. coli* strains was inhibited by CpxR due to the oxidative pressure. The NADH consumption through the oxidative phosphorylation was reduced and the NADH pool was rebalanced through the glutamate biosynthesis and metabolism. The differential proteins AstBD implied that the glutamate could be recycled through arginine catabolism. High level of Cu<sup>2+</sup> coexisting with ubiquinone is toxic to the strains because of the thiol depletion in EC2, which triggered the up-regulation of YifE for repairing iron-sulfur clusters, the up-regulation of CusF for strengthening the efflux of Cu<sup>2+</sup> and the up-regulation of YaiF for the degradation of ubiquinone. MenE was also up-regulated to make up the loss of the ubiquinone after the release of the Cu<sup>2+</sup> toxicity. In order to keep the balance of ubiquinone pool, the expressions of menE and yaiA were all decreased in EC2 compared to EC1. Sixthly, the stress-responding protein YcfR was upregulated to increase the indole production for the inhibition of the biofilm formation and the strengthening of arginine catabolism. Finally, the aromatic compounds such as indole and ubiquinone increased, sharing the common precursor chorismate in the biosynthesis pathway. Therefore, the regulation of photosynthesis is the key influencing factor of co-culture.

### 4.2 Effects of induction on the isoprene-producing *E. coli* strain

The effects of IPTG induction on the *E. coli* strains were investigated. The overexpression of protein puts pressure on the host, leading to up-regulation of cold-shock protein. The intermediate metabolites (MVA

and DMAPP) in MVA pathway were also observed to be accumulated. The accumulation of intermediate metabolites had great effects on the endogenous metabolism of *E. coli* strains. Firstly, the pyrophosphohydrolase NudB was up-regulated to release the accumulation of toxic DMAPP. Secondly, the fatty acid biosynthesis was increased as described previously, which showed that isoprenoids-producing *E. coli* strains suffered from the disturbance of fatty acid biosynthesis [23]. It might be the differential expressions of key enzymes in MVA pathway that resulted in the imbalance of the isoprene biosynthesis pathway and the accumulation of the intermediate metabolites. Both the mRNA and protein of the isoprene synthase were detected to be up-regulated in EC2 and kept unchanged in EC3. In contrast, MvaE were gradually up-regulated and kept increasing in EC3.

#### **4.3 Potential of the co-culture system of *S. elongatus*PCC 7942 and *E. coli*BL21(DE3) for isoprene production**

Although this co-culture system had a long fermentation period of time for isoprene production, the isoprene titer was relatively lower than the previous reports. Yang et al. reported that the isoprene production of 6.3 g/L was achieved in a fed-batch culture by *E. coli* using hybrid MVA pathway [18]. Liu et al. established a two-step process with the final isoprene production of 11.0 g/L by dividing the MVA pathway into the upstream process from sugar to MVA, and the downstream process from MVA to isoprene [14]. Genencor developed an *E. coli*-based production system with the ability to produce  $\approx 60$  g/L of isoprene [10]. In spite of this great gap, the isoprene production in the co-culture could be further improved by process control optimizations and strain improvements according to the fundamental rules discovered in the present paper.

Process control optimizations can improve the performance of co-culture systems of *S. elongatus*-*E. coli* by regulating the light availability, essential nutrient supplementation, induction mode and using the isoprene-producing engineered *S. elongatus* PCC 7942 in place of the wild type. Firstly, the light availability may influence the growth of Cyanobacteria in any algal systems through photosynthesis [24]. The light availability may further influence the cross-feeding of metabolites between *S. elongatus* PCC 7942 and *E. coli* BL21(DE3) including oxygen and carbon dioxide through photosynthesis and respiration. The light availability may also affect the oxidative pressure on the *E. coli* strains due to the photosynthesis. Therefore, the optimal light conditions can finally contribute to the high cell density co-culture with proper ratio of *S. elongatus* PCC 7942 and *E. coli* BL21(DE3). Secondly, the glucose and nitrogen limitation, the induction mode and betaine supplementation were found to be the key factors for isoprene production through the systematical optimization of fermentation conditions [14]. Thirdly, the photosynthetic isoprene production by cyanobacteria from CO<sub>2</sub> has been proved to be feasible previously [15, 16]. So *S. elongatus* PCC 7942 could be engineered for isoprene production to improve the performance of this co-culture system. The inhibition of antibiotics can also be completely released by replacing the wild type with the isoprene-producing engineered *S. elongatus* PCC 7942 resistant to the antibiotics.

When performing strain improvements, the rational design of *E. coli* strain should be derived from the omics correlation analysis of this co-culture system. Firstly, the *E. coli* strains could be engineered to be more suitable for the co-culture systems by coping with the oxidative pressure from photosynthesis. The engineered *E. coli* strains with improved oxidative stress mitigation pathways could contribute to the stability and high efficiency of synthetic consortia. It is interesting that the natural consortia also have similar mechanisms to mitigate oxidative stress of reactive oxygen species (ROS) or elevated local oxygen levels by the heterotrophs, i.e., catalase-dependent ROS scavenging by *Prochlorococcus* [25, 26]. Secondly, the *E. coli* strains could be improved to reduce the accumulation of MVA and isopentenyl diphosphate (IPP) by employing the balanced MVA pathway. The balanced biosynthetic pathway can keep the key enzyme stable, avoid the disturbance to overall cell system and maximize metabolic flux to the isoprene. The careful managements of MVA and IPP levels have been described previously in details through the evaluation on the promoters, ribosome binding sites, copy number of genes, key enzymes, novel IPP-bypass pathway and novel MVA-mediated pathway [10, 27-30]. All of the strategies can be applied to balance MVA for the isoprene production.

#### **4.4 Co-culture system and the common interaction mechanisms of the model algae and bacteria without natural symbiotic relation**

The study on the co-culture system of the model algae and bacteria without natural symbiotic relation is of significance to reveal the common interactions between photo-autotrophic and heterotrophic species. The natural consortia such as the unicyanobacterial consortium (UCC) are usually pervasive and robust. In UCC the cyanobacterium supplies photosynthetically-derived oxygen and carbon to the heterotrophs, while the heterotrophs can improve the growth of cyanobacterium by scavenging wastes and providing key metabolites [31, 32]. Although the natural symbiotic relation is often regarded as the prerequisite for the establishment of co-culture, the artificial synthetic consortia have been constructed by engineering the microbes interacting with one another through molecular signals or metabolic intermediates (i.e., complementary auxotrophs, quorum sensing) [33-36]. Furthermore, some attempts have recently been devoted to the design and construction of co-culture systems composed of model photo-autotrophic and heterotrophic species without natural symbiotic relation. Cong *et al.* artificially constructed a co-culture system between the model microorganisms *C. tropicalis* and *S. obliquus* [5]. Zhang *et al.* described a co-culture system of *C. vulgaris* and oleaginous yeast *Rhodotorula glutinis* [7]. Shu *et al.* enhanced CO<sub>2</sub> fixation and oil production by co-culturing the model Chlorella and *S. cerevisiae* [8]. Hays *et al.* designed the synthetic consortia composed of model cyanobacterium and heterotrophic species such as *B. subtilis*, *E. coli* and *S. cerevisiae* [2]. These studies including the co-culture of *S. elongatus* PCC 7942 and *E. coli* BL21(DE3) indicated that there might be a unspecific interaction between photo-autotrophic and heterotrophic species. More studies on the co-culture systems of the model photo-autotrophic and heterotrophic species are required to reveal the common interaction mechanism.

## **Conclusions**

In the present paper, the isoprene-producing co-culture system of *S. elongatus*-*E. coli* was established in this study and the differential omics analysis was carried out for this novel system. Many changes were discovered to be linked with one another at different spatial and temporal scales due to the oxidative pressure on *E. coli* from *S. elongatus* through the Fenton reaction. According to the omics analysis, the performance of this co-culture system can be further improved. This study on the co-culture of the algae and bacteria without natural symbiotic relation is of significance to reveal the common interactions between photo-autotrophic and heterotrophic species.

## Declarations

### Acknowledgements

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### Author' contributions

HL conceived of the study, participated in its design, conducted the experiments and drafted the manuscript. YC, JG and XX participated in the coordination of this study and helped to draft the manuscript. QL and LS performed research. MX conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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### Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

### Ethics approval and consent to participation

Not applicable.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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## Tables

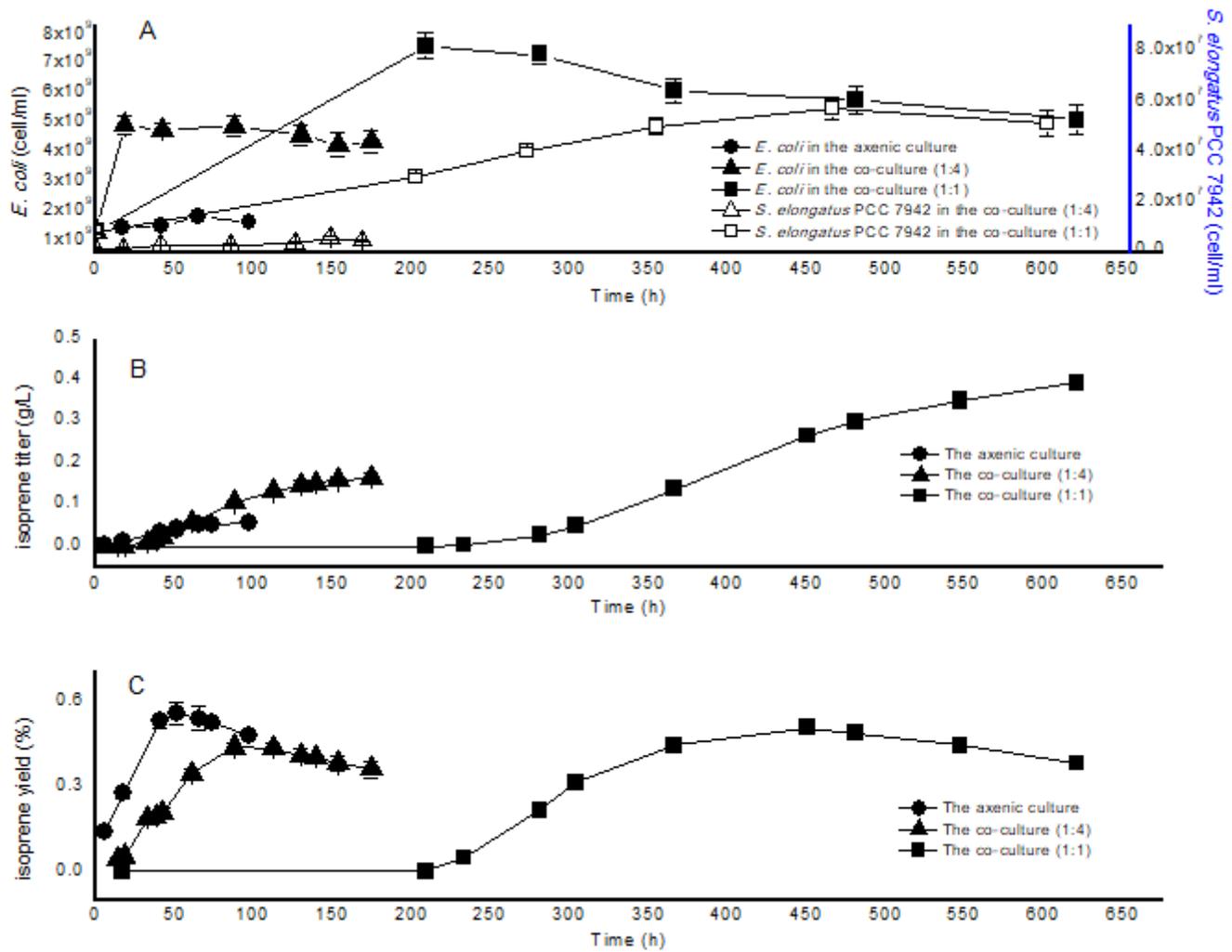
**Table 1** Strains and plasmids used in this study

Strain / plasmid	Relevant genotype / property	Source / reference
Strains		
<i>E. coli</i> BL21(DE3)	F <sup>-</sup> <i>ompT hsdS<sub>B</sub>(r<sub>B</sub><sup>-</sup>m<sub>B</sub><sup>-</sup>) gal dcm rne131(DE3)</i>	Invitrogen
<i>S. elongatus</i> PCC 7942	Wild type	[17]
Isoprene producer IP	BL21(DE3) / pYJM14 and pYJM20	[18]
Plasmids		
pYJM20	pACYCDuet-1 derivative carrying isoprene synthase gene <i>ispS</i> , HMG-CoA synthase gene <i>mvaS</i> and acetyl- CoA acetyltransferase/ hydroxymethylglutaryl-CoA (HMG- CoA) reductase gene <i>mvaE</i> , T7 promoter, Cm <sup>R</sup>	[18]
pYJM14	pTrcHis2B derivative carrying mevalonate pyrophosphate decarboxylase gene <i>ERG19</i> , mevalonate kinase gene <i>ERG12</i> , phosphomevalonate kinase gene <i>ERG8</i> and IPP isomerase gene <i>IDI1</i> , Trc promoter, Ap <sup>R</sup>	[18]

**Table 2** The differential profiles of gene expression, proteins and metabolites in *E. coli* BL21(DE3) in pairwise comparisons of E1, EC1, EC2 and EC3

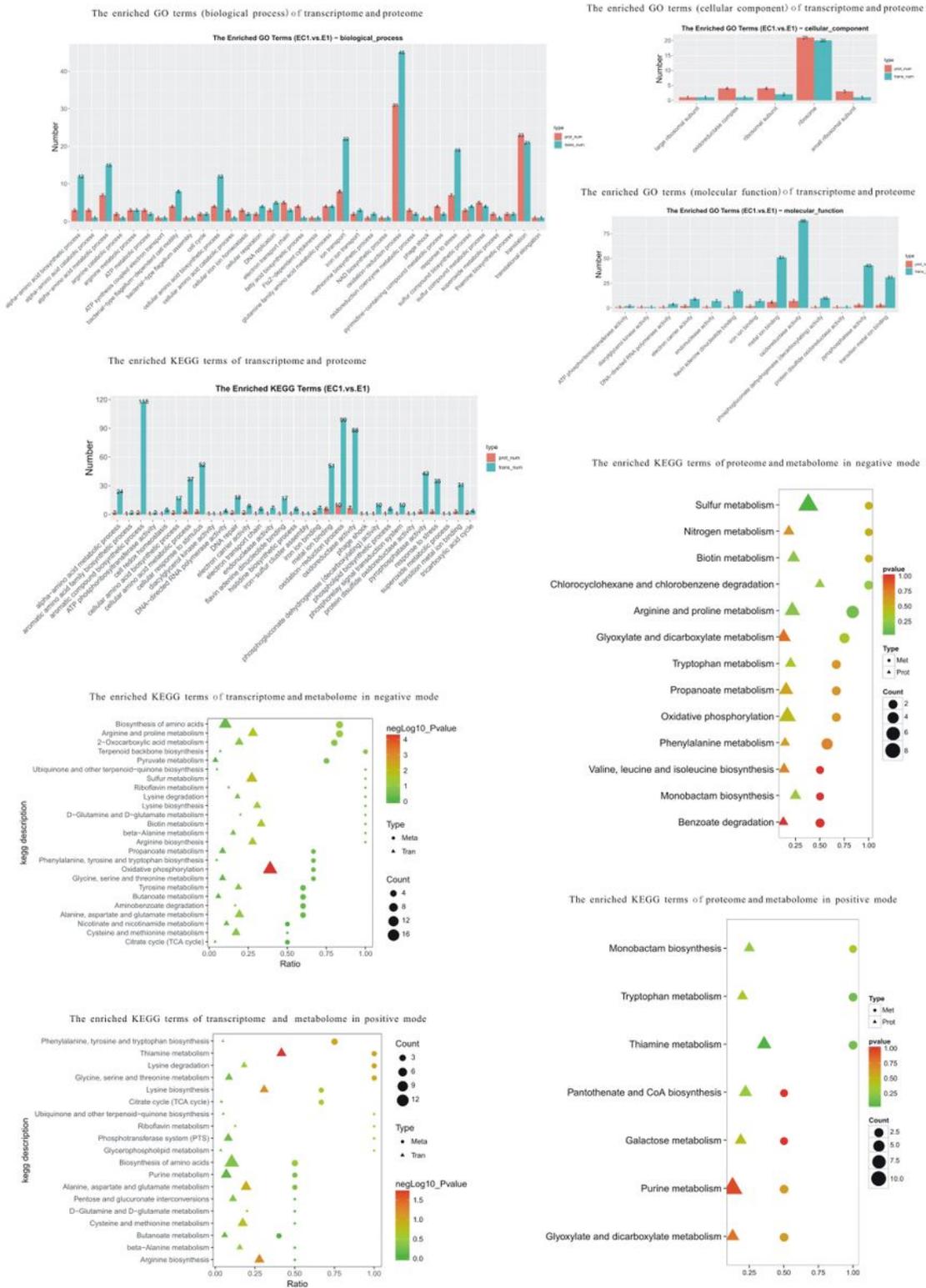
Samples		EC1.vs.E1		EC2.vs.EC1		EC3.vs.EC2	
		negative	positive	negative	positive	negative	positive
mRNAs	Total	374		856		224	
	Up	212		397		54	
	Down	162		459		170	
proteins	Total	359		70		20	
	Up	210		52		17	
	Down	149		18		3	
metabolites	Total	105	83	97	141	105	181
	Up	78	55	64	110	42	43
	Down	27	28	33	31	63	138

## Figures



**Figure 1**

Time courses of biomass, isoprene titer and yield in the axenic culture and co-culture. The axenic culture of BL21 (DE3) is the control. *S. elongatus* PCC 7942 and BL21 (DE3) were introduced into the co-culture system at the inoculation ratio of 1:4 and 1:1 respectively. The circles, triangles and squares indicate the control, the coculture1 (the inoculation ratio of 1:4) and coculture2 (the inoculation ratio of 1:1), respectively.



**Figure 2**

Functional analysis of differential transcriptome, proteome and metabolome in pairs of EC1 vs. E1. The GO and KEGG enrichment analyses were carried out for the differentially expressed proteins (genes) in proteome and transcriptome (Red represents up-regulation and blue represents down-regulation). The KEGG enrichment analysis was carried out for the differentially expressed profiles in proteome and metabolome. The circles and triangles represent the differential metabolites and proteins in the

corresponding pathway respectively. The KEGG enrichment analysis was also carried out for the differentially expressed profiles in transcriptome and metabolome. The circles and triangles represent the differential metabolites and transcripts in the corresponding pathway respectively. "Count" is the number of genes, metabolites or proteins enriched in the pathway. "Ratio" is the ratio of the number of differential genes, metabolites or proteins to the number of genes, metabolites or proteins annotated in the pathway. The colors of the circles and triangles represent the p-value of the hyper-geometric test.

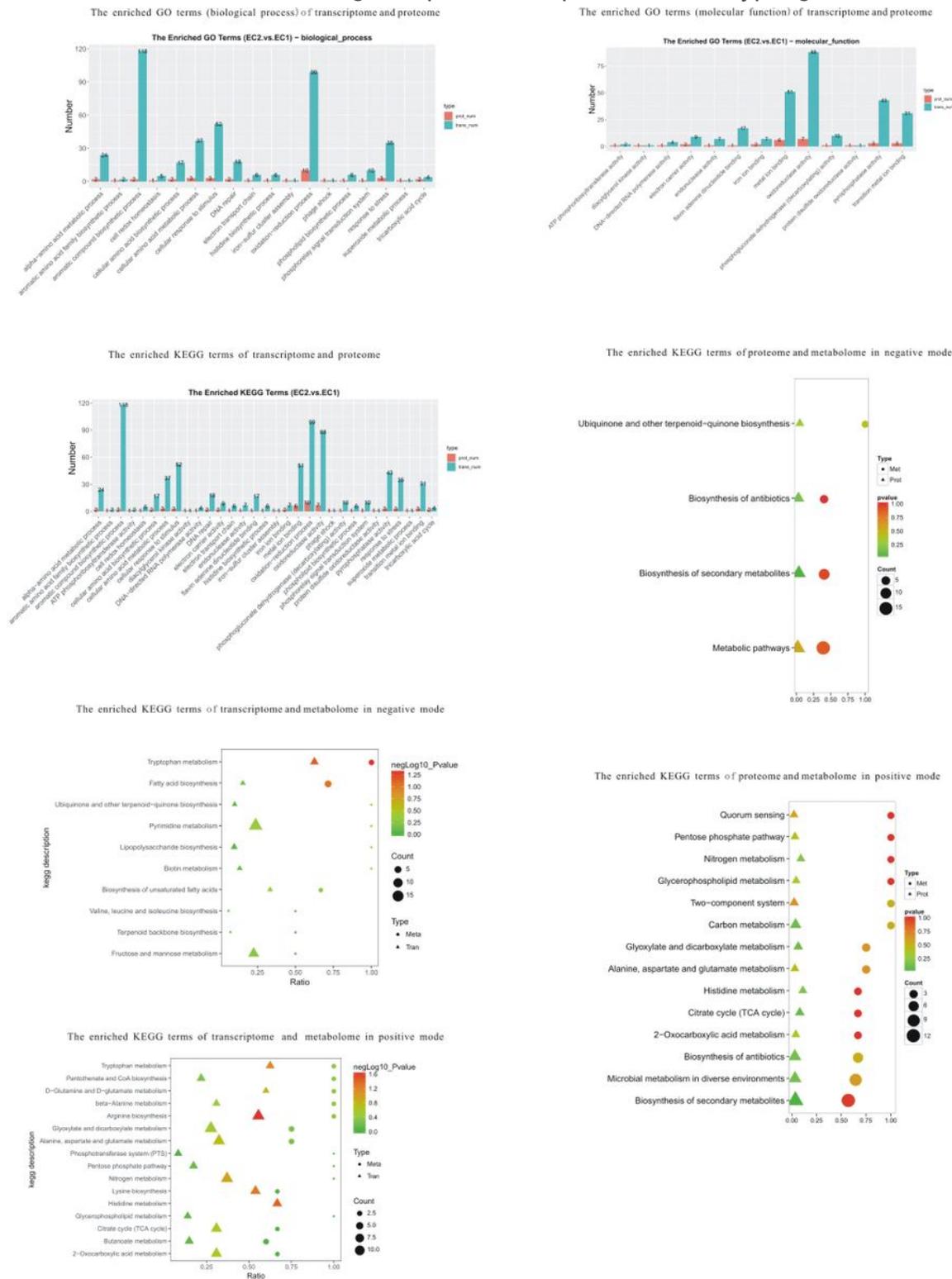


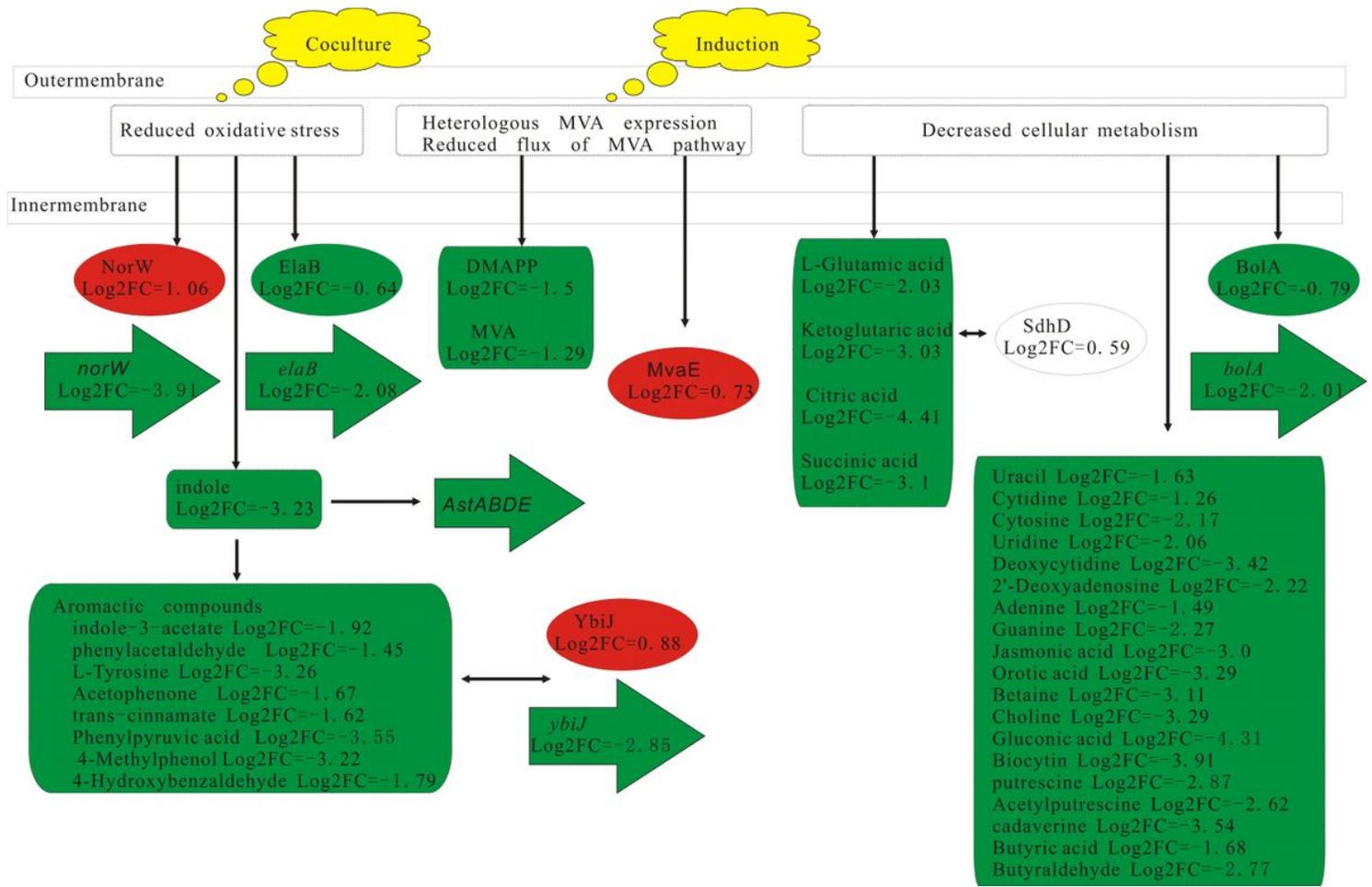
Figure 3

Functional analysis of differential transcriptome, proteome and metabolome in pairs of EC2 vs. EC1. The GO and KEGG enrichment analyses were carried out for the differentially expressed proteins (genes) in proteome and transcriptome (Red represents up-regulation and blue represents down-regulation). The KEGG enrichment analysis was carried out for the differentially expressed profiles in proteome and metabolome. The circles and triangles represent the differential metabolites and proteins in the corresponding pathway respectively. The KEGG enrichment analysis was also carried out for the differentially expressed profiles in transcriptome and metabolome. The circles and triangles represent the differential metabolites and transcripts in the corresponding pathway respectively. "Count" is the number of genes, metabolites or proteins enriched in the pathway. "Ratio" is the ratio of the number of differential genes, metabolites or proteins to the number of genes, metabolites or proteins annotated in the pathway. The colors of circles and triangles represent the p-value of the hyper-geometric test.









**Figure 7**

Correlation analysis of differential transcriptome, proteome and metabolome in pairs of EC3 vs. EC2. The red represents up-regulation and the green represents down-regulation.