Construction and analysis of an artificial consortium based on fast-growing cyanobacterium *Synechococcus elongatus* UTEX 2973 to produce platform chemical 3-hydroxypropionic acid from CO_2

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Research

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Abstract

Background: Cyanobacterial carbohydrates, such as sucrose, have been considered as a potential renewable feedstock to support the production of fuels and chemicals. However, the separation and purification processes of these carbohydrates will increase the production cost of chemicals. It has been proposed that co-culture fermentation is an efficient and economical way to utilize these cyanobacterial carbohydrates. However, studies about the application of co-culture system to achieve the green biosynthesis of platform chemicals are still rare.

Results: In this study, we successfully achieved one-step conversion of sucrose derived from cyanobacteria to fine chemicals by constructing a microbial consortium consisted of fast-growing cyanobacterium *Synechococcus elongatus* UTEX 2973 and *Escherichia coli* to sequentially produce sucrose and then platform chemical 3-hydroxypropionic acid (3-HP) from CO_2 under photoautotrophic growth condition. First, efforts were made to overexpress sucrose permease coding gene *cscB* under strong promoter P_{cpc560} in *S. elongatus* UTEX 2973 for efficient sucrose secretion. Second, sucrose catabolic pathway and malonyl CoA-dependent 3-HP biosynthetic pathway were introduced into *E. coli* BL21(DE3) for heterologous biosynthesis of 3-HP from sucrose. By optimizing the cultivation temperature from 37℃ to 30℃, a stable artificial consortium system was constructed with the capability of producing 3-HP up to 68.29 mg/L directly from CO_2. In addition, cell growth of *S. elongatus* UTEX 2973 in the consortium was enhanced, probably due to the quick quenching of reactive oxygen species (ROS) in the system by *E. coli*, which in turn improved photosynthesis of cyanobacteria.

Conclusion: The study demonstrated the feasibility of achieving the one-step conversion of sucrose to fine chemicals using an artificial consortium system. The study also confirmed that the heterotrophic bacteria could promote cell growth of cyanobacteria by relieving oxidative stress in this microbial consortium, which further suggests the potential values of this system for the future industrial application.

Background

Cyanobacteria are capable of producing organic matters from inorganic carbon (CO_2) by using solar energy. Due to the challenges associated with global climate change and sustainable energy supply, cyanobacteria has recently attracted significant attention as environmental-friendly and sustainable “microbial cell factory” to produce biofuels and valuable chemicals directly from CO_2 [1]. In addition, cyanobacteria were also considered as a mean of production of carbohydrate feedstocks to support industrial fermentative processes [2]. Moreover, it has been reported that several cyanobacterial species are capable of synthetizing and secreting sucrose as an osmolyte under appropriate environmental stimulus, such as osmotic pressure [3], and this production can be sustained over long time periods and higher than that from plant-based feedstocks like sugarcane and beet [4, 5]. As sucrose is a easily

fermentable feedstock for many microorganisms [6, 7], significant efforts have been made to improve the production of extracellular sucrose in cyanobacteria [8]. For example, Du et al. achieved sucrose productivity at 1.43 mg/L/h in the wild-type Synechocystis sp. PCC 6803 under 600 mM NaCl stress in bioreactor, and doubled the productivity to 3.13 mg/L/h by co-overexpressing key genes related to sucrose synthesis sps (slr0045), spp (slr0953) and ugp (slr0207), and deleting glucosylglycerolphosphate synthase gene ggpS (sll1566) [9]. In another study, Ducat et al. integrated a cscB gene encoding sucrose permease from Escherichia coli W [10, 11] into the Synechococcus elongatus PCC 7942 genome, meanwhile silenced the carbon competing pathway by knockout invertase invA and ADP-glucose pyrophosphorylase glgC, to achieve sucrose secretion at rate of 36.1 mg/L/h [12]. Recently, a fast growing cyanobacterium Synechococcus elongatus UTEX 2973 (hereafter S. elongatus UTEX 2973) with the growth rate similar to that of yeast was identified [13], and a extracellular sucrose productivity at 35.5 mg/L/h was demonstrated in a engineered S. elongatus UTEX 2973 carrying the sucrose transporter cscB in a bioreactor experiment [14]. Lately, the sucrose productivity was further increased to 79.2 mg/L/h by up-regulated sps which codes sucrose-phosphate synthase enzyme and sucrose synthesis genes S. elongatus UTEX 2973 [15]. Besides the high rate of sucrose secretion and growth, this strain exhibits high tolerance to high-temperature (41℃) and high-lights (500 μmol photons•m^{-2}•s^{-1}), suggesting significant advantages for outdoor cultivation in the future [13]. However, as purification of sucrose from culture supernatant is costly and the system was easily contaminated when sucrose is produced at a large scale [16], alternative ways of utilizing sucrose produced by cyanobacteria need to be developed for potential biotechnological applications.

In nature, microorganisms typically live and interact with other microbes by establishing a stable interchange of substance in complicated communities [17, 18]. Inspired by the commonly found symbiotic relationship of various microbes in nature, researches have been conducted to simulate symbiotic systems by designing artificial route for interchange of substance [19, 20]. Very recently, Ducat et al. constructed a co-culture system with a cyanobacterium S. elongatus PCC 7942 and a heterotrophic bacterium Halomonas boliviensis, in which the growth of H. boliviensis was supported by sucrose produced by S. elongatus PCC 7942 [21], and Li et al. constructed a co-culture system consisted with the sucrose secretory cyanobacteria S. elongatus PCC 7942 and three different yeasts to mimic lichen and research the interaction between the autotropic and heterotrophic strains [22]. Although these studies have established new alternatives for the utilization of sucrose derived from cyanobacteria, the applications of using co-culture system to achieve one-step conversion of sucrose to fine chemicals are still rare [23]. In addition, comparing with axenic culture of cyanobacteria, this autotrophic-heterotrophic symbiotic systems have been found resist contamination effectively and exhibit good robustness in fluctuating environments [24, 25].

3-hydroxypropionic acid (3-HP, $C_3H_6O_3$) as an important platform chemical is widely used for production of many chemicals, such as acrylic acid, malonic acid and biodegradable plastic poly-3-hydroxypropionic acid, and can also be used as food additives or preservatives as well [26]. As chemical synthesis of 3-HP causes serious environmental pollution [27], biosynthesis of 3-HP has attracted significant attention

recently. Several 3-HP biosynthetic pathways have been reported, and at least four substrates have been used to produce 3-HP, including β-alanine [28], lactate [29], malonyl-CoA [30] and glycerol [31]. Among them, the malonyl-CoA-dependent pathway, which employs acetyl-CoA carboxylase to convert precursor acetyl-CoA into malonyl-CoA and malonyl-CoA reductase to convert malonyl-CoA into 3-HP, respectively [32, 33], was reported with some distinct advantages compared to others, such as a broad feedstock spectrum, thermodynamic feasibility, and redox neutrality [34]. To date, the malonyl-CoA-dependent pathway has been engineered in E. coli [35], Saccharomyces cerevisiae [36], Synechocystis sp. PCC 6803 [37] and S. elongates PCC 7942 [38] for both heterotrophic and photoautotrophic production of 3-HP [35]. However, until now, no study about biosynthesis of 3-HP by co-culture system has been reported.

In this study, we reported the construction of an artificial consortium system consisted of fast growth cyanobacterium S. elongatus UTEX 2973 and an engineered E. coli BL21(DE3) to produce 3-HP under photoautotrophic condition. In the consortium system, E. coli BL21(DE3) was genetically modified to synthesize 3-HP using sucrose produced by the engineered S. elongatus UTEX 2973 (Fig. 1). With the application of this co-culture system, the final production of 3-HP was about 68.29 mg/L which is comparable to that in E. coli when only malonyl-CoA reductase was overexpressed [30]. In addition to the relationship that S. elongatus provide sucrose as carbon source for growth and 3-HP production in E. coli, the study also found that increased gene expression of reactive oxygen species (ROS) quenching genes in E. coli may promote cyanobacterial growth by relieving oxidative stress in the environments.

Results

Growth of *S. elongatus cscB*$^+$ 2973 and sucrose secretion

elongatus UTEX 2973 was engineered to secrete sucrose by expressing sucrose permease coding gene *cscB* under strong promoter P_{cpc560}. Sucrose secretion from *S. elongatus cscB*$^+$ 2973 is mainly dependent upon the pH and the NaCl concentration of the medium, and alkaline environment was previously reported beneficial to the sucrose secretion from cyanobacterial cells [39]. We used an alkaline environment (pH≈8.3) with 150 mM NaCl (37℃) to ensure the production and secretion of sucrose from *S. elongatus cscB*$^+$ 2973 [14, 22]. Sucrose yield and the growth of *S. elongatus cscB*$^+$ 2973 in different culture media were compared in **Fig. 2**. The results showed that no sucrose was produced from *S. elongatus cscB*$^+$ 2973 cells without NaCl in the culture medium. However, sustainable production and secretion of sucrose could be observed for six days when 150 mM NaCl was added, and productivity of 612.0 mg/L and 576.5 mg/L sucrose was achieved when *S. elongatus cscB*$^+$ 2973 was grown in BG-11 and CoBG-11, respectively. In addition, slightly slow biomass accumulation of *S. elongatus cscB*$^+$ 2973 under salt stress condition was observed when compared with that under normal condition, which may be due to the fact that the *S. elongatus cscB*$^+$ 2973 cells used more carbon energy sources for sucrose synthesis rather than growth in order to resist the osmotic stress. In order to maintain the normal growth of *E. coli* in co-culture medium, the effect of different salt concentrations on its growth was also examined (**Suppl. Fig. S1**), and the results showed that *E. coli* was able to grow normally under the tested

range of salt concentration. Under the optimized condition, a sucrose productivity of 576.5-612.0 mg/L (4.00-4.25 mg/L/h) was achieved by *S. elongatus cscB*$^{+}$ 2973 cells for six days, which is at the compatible level of the similar studies conducted previously (**Table 2**). For example, although no CO_2 aeration during *S. elongatus* cultivation, sucrose secretion in this study was still higher than 2.2 mg/L/h reported in a previous study [22].

Growth of engineered *E. coli* mutant in co-culture medium

In order to ensure *E. coli* BL21 utilize sucrose as sole carbon source, we cloned and expressed the essential genes for sucrose metabolism, *cscB*, *cscK* and *cscA* (*ECW_m2596*), into *E. coli* BL21 to generate an engineered strain *E. coli* cscN. In addition, to synthesis 3-HP, malonyl-CoA reductase coding gene *mcr* was introduced into *E. coli* cscN, resulting engineered strain *E. coli* ABKm. In a previous study, the artificial consortium was constructed by inoculating heterotrophic bacterium inoculate into a *S. elongatus* culture with OD_{750} = 0.5 [25]. In our study, sucrose yield of *S. elongatus cscB*$^{+}$ 2973 was ~200 mg/L when cells reach to OD_{750} = 0.5. So, we selected four concentration 50, 100, 150, 200 mg/L, to examine whether *E. coli* ABKm could be stably maintained in the system using those levels of sucrose as the sole carbon source in M9 and CoBG-11 media, respectively (**Fig. 3A and 3B**). The results showed that the minimal sucrose concentration required to support normal growth of *E. coli* ABKm is 200 mg/L. This concentration (200 mg/L) can be achieved when *S. elongatus cscB*$^{+}$ 2973 was grown in CoBG-11 medium for approximately 2 days. A previous study showed *E. coli* Δ*cscR* strain required a minimal sucrose concentration of 1.2 g/L for growth [25], which is approximate six-fold higher than our result for strain ABKm, suggesting that after expressing *cscA*, *cscB* and *cscK*, the efficiency of sucrose utilization might have been improved in strain ABKm [25, 40]. Meanwhile, we constructed this pathway into *E. coli* MG1655 and BW25113 and the similar result was observed (data not shown). Additionally, we also determined the 3-HP yield in strain ABKm with lower concentration levels of sucrose (i.e. 50~200 mg/L) in CoBG-11, and the results showed that even with lower concentration of sucrose in the medium, strain ABKm was still able to produce 3-HP (**Fig. 3C**).

Establishing stable artificial consortium to produce 3-HP

Since the optimal growth temperatures for both *E. coli* and *S. elongatus* UTEX 2973 are both 37°C, we initially set it as the incubation temperature for co-culture system. However, the analysis showed that strain ABKm grew poorly after 1~2 days in this system compared with the previous study [25] (**Suppl. Fig. S2**). The analysis suggested that it may be due to the fast utilization of the carbon source and the rapid growth of *E. coli* at this temperature, which could destroy the balance between sucrose-secretion and sucrose-utilization. To confirm this hypothesis, we determined the rates of sucrose-secretion and sucrose-utilization in *S. elongatus* UTEX 2973 and *E. coli*, respectively. The results showed that the sucrose-

utilizing rate of *E. coli* strain ABKm was increased gradually with the increase of initial sucrose concentration, reaching ~4.20 mg/L/h at initial sucrose concentration of 200 mg/L at 37℃ during 48 h (**Fig. 4B**). Although sucrose secretion rate of *S. elongatus cscB*$^+$ can be up to ~4.11 mg/L/h, we speculated that with the accumulation of *E. coli* biomass, sucrose consumption rate of ABKm could be faster than sucrose secretion rate of *S. elongatus cscB*$^+$ at 37℃. So the balance of "producing-consuming" was broken, leading to collapse of the consortium. These results led us to adjust the cultivation temperature from 37℃ to 30℃, aiming to slow down the consumption of *E. coli* and achieve balanced growth of *S. elongatus* UTEX 2973 and *E. coli* in the system. The growth of strain ABKm under 30℃ was then exhibited (**Fig. 4A)**, and sucrose-utilizing rate of this strain was determined as ~2.00 mg/L/h at 30℃ in 48 h (**Fig. 4B**). Interestingly, although temperature was decreased to 30℃, sucrose secretion by *S. elongatus cscB*$^+$ 2973 was not affected (**Fig. 4C and 4D**). As a result, the artificial consortium with *S. elongatus cscB*$^+$ 2973 and *E. coli* strain ABKm was successfully constructed, which can be maintained stably for at least 7 days at 30℃ (**Fig. 4E**).

To evaluate the production capacity of the artificial consortium, the yield of 3-HP by *E. coli* strain ABKm was analyzed. As shown in **Fig. 5A**, 3-HP production reached up to ~68.29 mg/L in 7 days. In parallel, we also detected the 3-HP production in *E. coli* strain ABKm under pure culture condition with continuous supplementation of sucrose according to the calculated sucrose-secreting rate of *S. elongatus cscB*$^+$ 2973 (**Fig. 5B**), and the results showed that the 3-HP yield under pure condition was slightly lower than that in the consortium. Besides, we also observed that *S. elongatus cscB*$^+$ 2973 cultivated in the consortium grew better than them in pure-culture condition (**Fig. 6A**), consistent with the previous findings [25]. Together, we concluded that *S. elongatus cscB*$^+$ 2973 could generate more sucrose to support ABKm for 3-HP production under co-culture condition because of the better growth. In addition, free sucrose in co-cultures medium was completely consumed, indicating that the *E. coli* ABKm strain was able to utilize the cyanobacterium-derived carbohydrates efficiently to accumulate desired product.

Analysis of oxidative stress to cyanobacteria in artificial consortium system

ROS are common byproducts of aerobic metabolism such as photoreaction and respiration in oxygenic photosynthetic organisms [41], and ROS accumulation in cells could cause oxidative damage to cyanobacterial cells. In addition, previous studies have found that organic buffers in culture media may also contribute to the generation of H_2O_2 [42]. For example, the concentration of 1~10 mM 4-(2-hydroxyethyl)1-piperazineethanesulfonic acid (HEPES) in culture medium could produce enough H_2O_2 to kill *Prochlorococcus* [43]. Since there is also organic buffer (TES) used to maintain pH in our study, to clarify whether this organic buffer generates H_2O_2, we determined the titer of H_2O_2 in blank CoBG-11under the same culture condition. The results showed that no H_2O_2 was detected in blank culture medium, suggesting that H_2O_2 in culture medium was mostly synthesized from the living cells. Next, we examined the impact of *E. coli* co-cultivation on H_2O_2 production, and the results showed that the H_2O_2

content was significantly reduced when the heterotrophic partner of *E. coli* was included in the system (**Fig. 6B**), consistent with the previous study [22].

To further understand this phenomenon at the molecular level, expression level of several ROS quenching genes in *E. coli* ABKm strain under pure and co-culture conditions was comparatively analyzed by qRT-PCR (**Suppl. Table. S1**). It is well known that *E. coli* contains three types of catalases: hydroperoxidase I (HPI) (*katG*), hydroperoxidase II (HPII) (*katF*), and hydroperoxidase III (HPIII) (*katE*) [44-46]. In addition, the synthesis of HPII often increases markedly when cells enter the stationary phase [47, 48]. The transcriptional expression of these three genes was determined. As shown in **Fig. 7**, relative expression level of *katG*, *katF* and *katE* in the *E. coli* ABKm strain was dramatically up-regulated in CoBG-11 under continuous supplementation of sucrose according to the calculated sucrose-secreting rate of *S. elongatus cscB*$^{+}$ 2973, comparing with them in the *E. coli* pure culture, suggesting that *E. coli* might be able to remove ROS when co-cultivated with cyanobacterial partner and thus possibly alleviate the overall oxidative stress in the consortium system.

Discussion

Photosynthetic cyanobacteria have been considered as one important alternative for providing sustainable feedstock, and cyanobacterial carbohydrates have been considered as a potential renewable feedstock to support the production of fuels and chemicals. However, since axenic cultures of cyanobacteria are vulnerable to contamination [49] and it is expensive to separate and purify these products from culture medium [50], their application is limited. To address the issues, synthetic consortia of cyanobacteria paired with specific heterotrophic partner have been proposed. A fast-growing *S. elongatus* UTEX 2973 reported recently exhibits high tolerance to high-temperature and high-lights [13]. In this study, we reported the construction of an artificial co-culture system utilizing *S. elongatus* UTEX 2973 and *E. coli* BL21 to produce heterologous chemical 3-HP directly from CO_2. In addition, the analysis of the mechanism underlaid the co-culture systems also provided a fundamental basis for the further optimization of artificial consortium systems via metabolic engineering.

Sucrose is a commonly used carbon source for industrially microbial fermentation [51, 52]. In this study, we transformed a sucrose transporter *cscB* to a fast growth cyanobacterium *S. elongatus* UTEX 2973 and achieved a sucrose-secrete *S. elongatus cscB*$^{+}$ 2973 with a productivity of 612.0 mg/L BG-11 for six days. To construct artificial consortium using sucrose as sole carbon source, sucrose-utilizing ability of *E. coli* was also improved. It is previously shown that the ability of sucrose utilization was endowed in *E. coli* strain Δ*cscR* by introducing *cscA* and *cscB* genes from *Pseudomonas putida* [23] [40]. To achieve the growth of *E. coli* BL21 in designed co-culture system, genes related to sucrose utilization, *cscA*, *cscB* and *cscK*, were introduced [53]. The results showed that the engineered *E. coli* strain ABKm was able to grow well when sucrose concentration was as low as 200 mg/L (**Fig. 3**), which presents the highest sucrose utilization efficiency in engineered *E. coli* reported so far [40].

It was previously reported that ROS accumulated in the culture medium severely inhibited axenic growth of cyanobacteria; however, it was efficiently alleviated through co-culture with *Rhodotorula glutinis* [22]. We also found a better growth of *S. elongatus* UTEX 2973 in the co-culture system (**Fig. 6A**). The content of H_2O_2 in the consortium system was found to be lower than the axenic culture, suggesting that *E. coli* might contribute to the alleviation of the ROS stress and thus promote growth of cyanobacteria in the artificial consortium, which was further confirmed by the up-regulation of the ROS quenching relevant genes in *E. coli* strain ABKm (**Fig. 7**). The up-regulation of ROS quenching capability could be important as ROS may deleteriously affect cellular metabolism such as nutrient bioavailability, photosynthesis and carbon fluxes in plants [54]. With the decreased H_2O_2 content and the increased cell growth of *S. elongatus* UTEX 2973, we also evaluated the expression levels of several genes involved in photosynthesis including carbon dioxide concentrating mechanism protein coding gene *ccmM* [55], Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) coding gene *rbcL* [56], PSII related subunits coding genes *cp43* and *cp47*, PSI essential subunits of reaction center coding genes *psaA* and *psaB* [57], and chlorophyll a synthesis related genes *chlaA* and *pcrA* [58] (**Suppl. Table. S1**), as expected, all these genes were up-regulated (**Suppl. Fig. S3**). Besides ROS quenching, several studies have reported that heterotrophic partner can provide the necessary inorganic carbon by decomposing organic matter or growth factor such as vitamin B_{12} to cyanobacteria in the natural and artificial consortium [18, 59, 60], which we will also analyze in the future work. Further analysis of interaction in the consortium could provide the necessary theoretical basis for potential application of artificial co-culture systems in many areas, such as controlling blooms [61], degradation of pollutant [62-64], and soil remediation [65-67].

Although the artificial consortium system was successfully established and desired product was achieved in this study, there are still many areas that need to be improved in the future, such as the yield of 3-HP. Towards the goal of increasing the yield of target product, on the one hand, we could enhance the supply of carbon source. Besides silenced the competing consuming pathway [12], Qiao et al. reported that sucrose yield in *S. elongatus* PCC 7942 can be enhanced from 6.5 mg/L/h to 8.0 mg/L/h by overexpressing of sucrose-phosphate synthase (*sps*) and glucose-1-phosphate adenylyltransferase (*glgC*) at the same time [68]. The research also suggested that glycogen could serve as a supporting rather than a competitive carbon pool for sucrose synthesis. In addition, Weiss et al. used alginate to encapsulate *S. elongatus* and enhanced sucrose yield rates ~2-fold within 66 h [21]. On the other hand, heterotrophic partner also needs to be improved. Chelladurai et al. developed a variety of recombinant *E. coli* strains by expressing heterologous gene *mcr* and overexpressing endogenous acetyl-CoA carboxylase and biotinilase encoding genes *accADBCb* and nicotinamide nucleotide transhydrogenase encoding gene *pntAB* that converts NADH to NADPH in *E. coli*. In addition, several deletion mutations in phosphotransacetylase (*pta*) acetate kinase (*ackA*) and lactate dehydrogenase (*ldhA*), or α-ketoglutarate dehydrogenase complex (*sucAB*) were carried out with the recombinant strains. The final 3-HP titer enhanced about 3-fold from 0.71 to 2.14 mM [30]. Moreover, Cheng et al. reported that overexpressing heterogeneous acetyl-CoA carboxylase (from *Corynebacterium glutamicum*) and codon-optimized *mcr* in *E. coli* BL21, and three types of modified *E. coli* strains with different host-vector systems were constructed and investigated, and the results showed that the combination of *E. coli* BL21 and pET28a

was the most efficient host-vector system for 3-HP production. And the concentration of 3-HP was enhanced from 0.68 g/L to 1.80 g/L in shake flask cultivation [69]. These studies provide valuable guidance for further metabolic engineering in *E. coli*.

Conclusion

With defined composition and controllable functions, synthetic consortium holds great promises for diverse value-added productions, bioenergy and environmental applications. In this study, we demonstrated the feasibility of constructing an artificial consortium aims to achieve the one-step conversion of sucrose to platform chemical 3-HP directly from CO_2. With the application of this co-culture system, the final production of 3-HP was about 68.29 mg/L which is comparable to that in *E. coli* when only malonyl-CoA reductase was overexpressed. Meanwhile, this study also confirmed that in this microbial consortium, the heterotrophic bacteria could promote cell growth of cyanobacteria by relieving oxidative stress, which further demonstrate the potential values of this system for the green biosynthesis of chemicals in the future.

Material And Method

Strains, plasmids construction and culture condition

elongatus UTEX 2973 and *E. coli* BL21(DE3) were respectively engineered and applied to the construction of the artificial consortium system. The essential genes for sucrose metabolism, permease coding gene *cscB*, invertase coding gene *cscA* and fructokinase coding gene *cscK* were derived from *E. coli* W [53], while the malonyl-CoA reductase gene *mcr* was from *Chloroflexus aurantiacus* [70]. Super strong promoter P_{cpc560} was used to direct the gene expression in *S. elongatus* UTEX 2973 [71]. All plasmids are prepared in *E. coli* DH5α. Sucrose hydrolysis system was integrated into pET-30a in *E. coli* with kanamycin resistance, and *mcr* was integrated into pACYC184 under the constitutive promoter P_{J23100} in *E. coli* with spectinomycin resistance, respectively.

elongatus UTEX 2973 and resulting engineered strains were cultivated in BG-11 medium (pH7.5) under a light intensity of about 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 incubator (SPX-250B-G, Boxun, Shanghai, China) [72]. In order to maintain the stable phenotype of sucrose secretion, appropriate antibiotic(s) were added when necessary. *E. coli* strains were grown on LB medium or agar plates with appropriate antibiotic(s) added to maintain plasmids at 37°C in shaking incubator (HNY-100B, Honour, Tianjin, China) at 200 rpm or in an incubator, respectively. All strains used in this study were listed in **Table 1**.

Conjugation of *S. elongatus* UTEX 2973

Constructs were delivered into *S. elongatus* UTEX 2973 through conjugation [73]. *E. coli* HB101 harboring pRL443 and pRL623 (named “helper”) and *E. coli* DH5α harboring the plasmid with target gene were cultivated overnight and then transferred separately into fresh liquid LB medium with appropriate antibiotic(s) at 1:50 ratio. When both strains grew to exponential phase (OD_{600}=0.3~0.5), 10 mL cells of each strain were collected by centrifugation and washed with fresh LB medium three times to remove all the antibiotics. Then 0.1 mL fresh LB was used to re-suspend each strain, and they were mixed together and incubated at 37℃ for 30 min. During this time, 10 mL of the *S. elongatus* UTEX 2973 cells at exponential phase (OD_{750}≈1) were collected by centrifugation and re-suspended in 0.2 mL fresh BG11 medium. *S. elongatus* UTEX 2973 cells was mixed with the *E. coli* mixture mentioned above and incubated at 37℃ under light for 30 min. Then, the mixture was spread on BG11 agar plates which was then covered by sterile cellulose filters (0.45 μm pore size). The plates were incubated under light at intensity of approximately 100 μmol photons $m^{-2}s^{-1}$ for 24 h, and then the cellulose filters were transferred onto new BG11 agar plates with appreciate antibiotics [74].

Construction of artificial consortium system

Co-culture medium (named CoBG-11) was designed based on BG-11 medium and optimized for *E. coli* growth by supplementing 150 mM NaCl, 4 mM NH_4Cl and 3 g/L 2-[[1,3-dihydroxy-2-(hydroxymethyl) propan-2-yl] amino] ethanesulfonic acid (TES). The pH value is adjusted with NaOH to 8.3. NaCl and NH_4Cl were used to maintain the cell survival of *E. coli*, and NaCl was used as a stress inducer for sucrose accumulation in *S. elongatus* UTEX 2973, respectively.

Before two strains were cultivated together, *S. elongatus* UTEX 2973 was propagated in BG-11 at 37°C with appropriate antibiotics to exponential phase (OD_{750}≈1.0), and then collected by centrifugation and inoculated into 25 mL CoBG-11 medium and grown at 30℃ for 48 h to OD_{750} of 0.5. *E. coli* was incubated in CoBG-11 with 1 g/L sucrose for 48 h and then collected by centrifugation, re-suspended with deionized water and inoculated into the 25 mL *S. elongatus* culture above at initial OD_{600} of 0.01.

Quantification of cyanobacteria and *E. coli*

For pure culture of *S. elongatus* UTEX 2973 and *E. coli*, cell density was measured at OD_{750} and OD_{600} using a UV-1750 spectrophotometer (Shimadzu, Kyoto, Japan), respectively. For co-culture, serial dilutions were made and solid LB agar plates were used to determine *E. coli* viability and cell number by colony-forming units (CFU) after 24 h incubation at 37°C. Cell number of *S. elongatus* UTEX 2973 was determined by hemocytometer under microscopy (BX43, Olympus, Shinjuku, Tokyo, Japan).

Determination of extracellular sucrose content

Supernatant of *S. elongatus* UTEX 2973 pure culture was collected and analyzed for sucrose content *via* a colorimetric Glucose-Sucrose Assay (Megazyme, Ireland) that employs high purity glucose oxidase, peroxidase and β-fructosidase (invertase). At pH 4.6, sucrose is hydrolyzed by invertase to D-glucose and D-fructose, and then free D-glucose is determined by converting to a red colored quinoneimine dye compound through the action of glucose oxidase and peroxidase at pH 7.4, and employing *p*-hydroxybenzoic and 4-aminoantipyrine. Measurements were conducted at 510 nm.

Quantification of 3-HP

3-HP concentration was quantified according to a previous method [37]. 3-HP standard of analytical purity was purchased from Tokyo Chemical Industry (Tokyo, Japan). The supernatant containing 3-HP was collected from the co-culture medium by centrifuging at 12,000 rpm for 2 min at room temperature (Eppendorf 5430R, Hamburg, Germany) and used for 3-HP analysis. Sample derivatization was carried out according to the two stage technique described previously [75]. GC-MS analysis was conducted on a GC-MS system-GC 7890 coupled to an MSD 5975 (Agilent Technologies, Inc., Santa Clara, CA) equipped with a HP-5MS capillary column (30 mm×250 mm id).

Quantitative real-time RT-PCR analysis

Approximately 4×10^6 cells of *S. elongatus* UTEX 2973 cells of pure or co-culture were collected by centrifugation at 12,000 rpm, 4°C for 1 min. The supernatant was removed and the cells were used for RNA extraction and RT-qPCR analysis using methods described previously [76]. The relative abundance of different mRNA molecules could be estimated using $2^{-\triangle\triangle CT}$ [77].

Analysis of hydrogen peroxide (H_2O_2) concentration

H_2O_2 content in supernatant was analyzed *via* H_2O_2 Quantitative Assay Kit (Sangon Biotech, Shanghai, China). In the reaction, Fe^{2+} is oxidized to Fe^{3+} by H_2O_2 when pH is less than 7.0, and then Fe^{3+} generated combines with dye molecules to form claret-colored Fe^{3+}-dye complex with maximum absorption wavelength at 560 nm or 595 nm, and the absorption value is directly proportional to the concentration of hydrogen H_2O_2 in cells.

Declarations

Abbreviations

GC-MS gas chromatography-mass; WT, wild type.

The authors declare that they have no competing interests.

Ethical Approval and Consent to participate

Not applicable.

Consent for publication

All authors agree to publication.

Availability of supporting data

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

Competing interests

The authors declare no competing financial interests.

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Authors’ contributions

XYS and WZ designed the research. LZ performed the major experiments and wrote the draft manuscript; JJD, XYS helped with some of the experiments; MLS helped with the GC-MS analysis; LZ, LC, XYS and WZ analyzed data, drafted and revised the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1. Plasmids and strains used in this study.

Plasmids/Strains	Genotype	Source
E. coli strains		
DH5α	F⁻ *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20* φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17($r_K^- m_K^+$), λ⁻	TransGen Biotech
HB101	*supE*44, Δ(*mcrC-mrr*), *recA*13, *ara*-14, *proA*2, *lacY*1, *galK*2, *rpsL*20, *xyl*-5, *mtl*-1, *leuB*6, *thi*-1	Takara Bio
BL21(DE3)	F⁻ *ompT gal dcm lon hsdS*$_B$($r_B^- m_B^-$) λ(DE3)	TransGen Biotech
cscN	BL21(DE3)/pET30a	
ABKm	BL21(DE3)/pACYC184/pET30a	This study
Plasmids		
pACYC184	P_{J23100}-mcr-T_7; spe^R	This study
pET30a	*csc*A, *csc*B, *csc*K; kan^R	This study
pJA	P_{cpc560}-*csc*B-T_{rbcL}; spe^R, kan^R	This study
Cyanobacteria strains		
WT	wild type *Synechococcus elongatus* UTEX 2973	
cscB⁺ 2973	WT/pJA	This study

Table 2. Sucrose yield in different cyanobacteria strains under co-culture conditions.

Host strains	Genotype	Cultural condition	Cultural medium	Titer (mg/L)	Productivity (mg/L/h)	Source of value
Synechococcus elongatus PCC 7942	NS3::P_{lac}-*cscB*-Cm^r	30°C or 37°C [a], 2 % CO_2, 65 μE m^{-2} s^{-1} light, 150 mM NaCl for 2 days (baffled flasks)	^{CoY}BG-11 or ^{CoB}BG-11 [b]	156~625	3.25~13.02	[25]
	NS3::P_{lac}-*cscB*-Cm^r	28 °C [c], 1% CO_2, 65 μmol m^{-2} s^{-1} with 16:8 h light/dark cycle 100 mM NaCl For 4 days (baffled flasks)	BG-11 supplemented with 1 g/L HEPES (pH 8.9)	~210	~2.2	[22]
	NS3::P_{lac}-*cscB*-Cm^r	35°C, 2 % CO_2, 65 μE m^{-2} s^{-1} light, 140 mM NaCl for 3 days (bioreactor)	M1[d]	~1100	~15.27	[21]
Synechococcus elongatus UTEX 2973	pJA-*cscB*	37 °C, 100 μE m^{-2} s^{-1} light, 150 mM NaCl for 3 days (100 ml round flask)	CoBG-11	295.5	~4.11	This study
	pJA-*cscB*	30 °C, 100 μE m^{-2} s^{-1} light, 150 mM NaCl for 3 days (100 ml round flask)	CoBG-11	288	~4.00	This study

[a]: In co-culture system, 37°C for *E. coli* and *B. subtilis*, and 30°C for *S. cerevisiae*.

[b]: ^{CoB}BG-11 consists of BG-11 supplemented with 106 mM NaCl, 4 mM NH_4Cl and 25 mM HEPPSO, pH-8.3 KOH. ^{CoY}BG-11 consists of BG-11 supplemented with 0.36 g/L Yeast Nitrogen Base without amino acids (Sigma Aldrich), 106 mM NaCl, 25 mM HEPPSO, pH 8.3-KOH and 1 mM KPO_3.

[c]: In co-culture system, 28°C for three yeast strains.

[d]: M1 consists BG-11 medium was additionally supplemented with 15 mM $NaNO_3$, 4.5 mM K_2HPO_4 (phosphate buffering), 1.5 mM $MgSO_4$, 5 mM Na_2SO_4, 30 μM $FeCl_3$, 30 μM Na_2MoO_4, and 1x additional trace metals before the addition of KOH to pH 8.3.

Figures

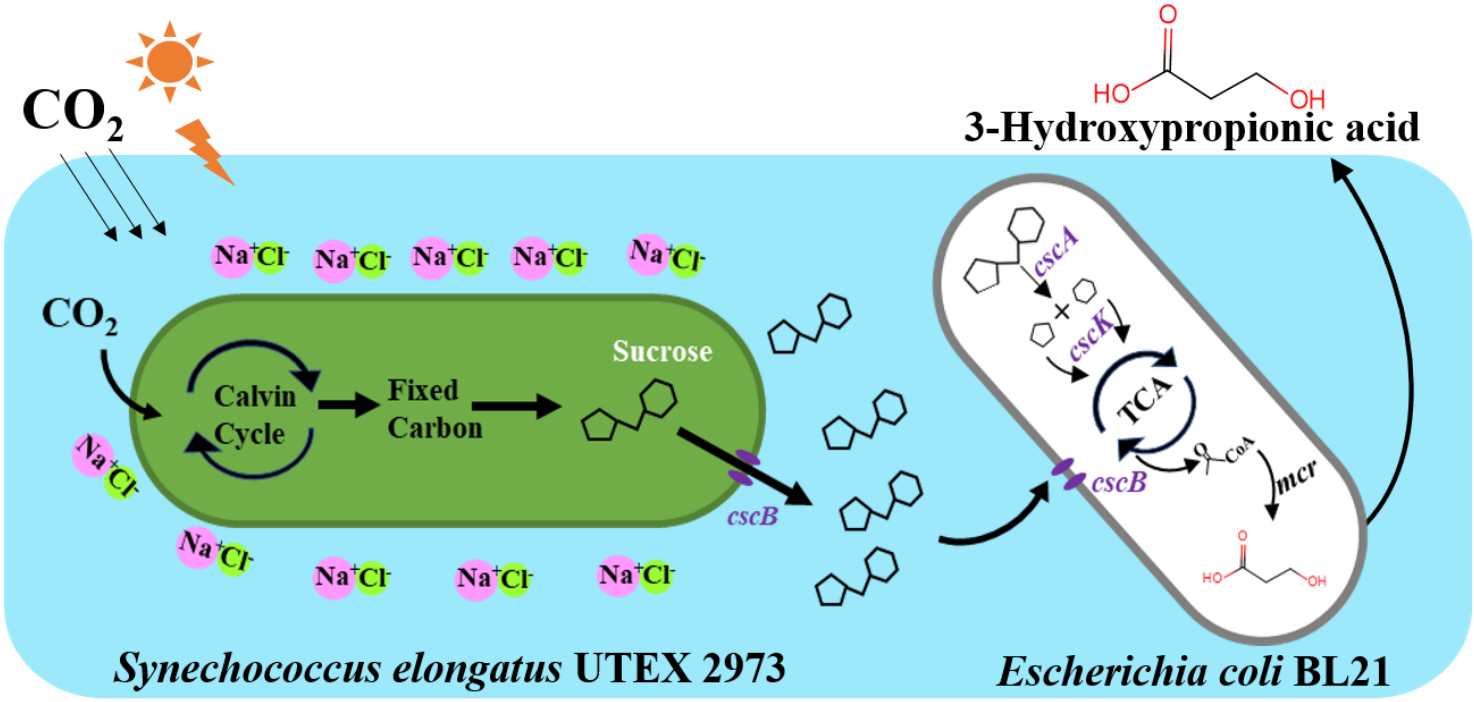

Figure 1

Schematic diagram of artificial consortium system. The engineered S. elongatus UTEX 2973 secreted sucrose under osmotic stress to support E. coli growth and synthesis of 3-HP under photoautotrophic growth condition.

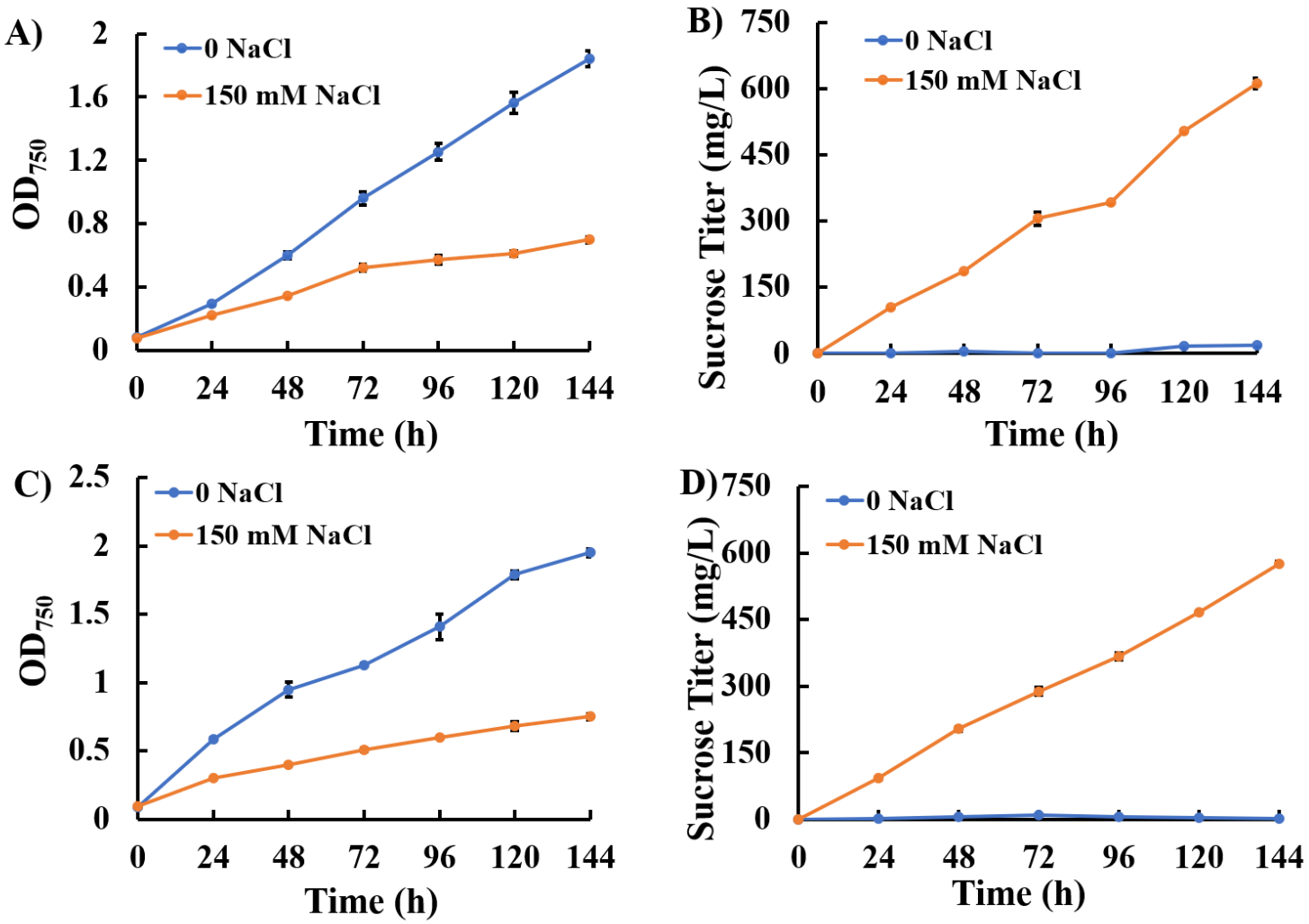

Figure 2

Effects of NaCl on growth and sucrose yield of S. elongatus cscB+. Growth of strain S. elongatus cscB+ in BG11 medium (A) and CoBG11 medium (C), respectively. Sucrose yield of strain S. elongatus cscB+ in BG11 medium (B) and CoBG-11 medium (D), respectively. The error bars represent the calculated standard deviation of the measurements of three biological replicates.

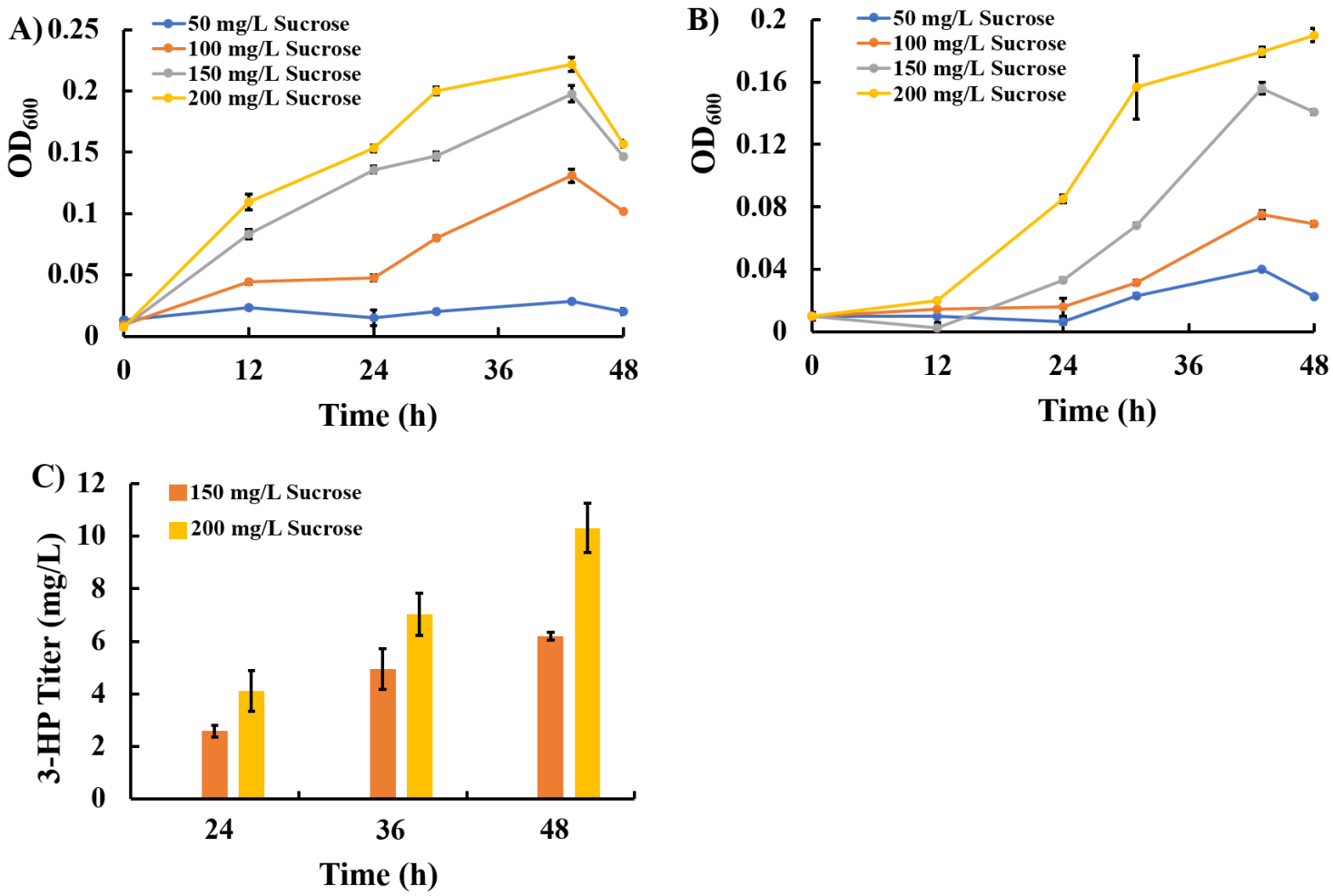

Figure 3

Growth and 3-HP production of E. coli ABKm pure culture under different sucrose concentrations. (A) Cultivated in M9 medium; (B) Cultivated in CoBG11 medium. The error bars represent the calculated standard deviation of the measurements of three biological replicates; (C) 3-HP production in E. coli ABKm strain under different initial sucrose concentration in co-culture medium. The error bars represent the calculated standard deviation of the measurements of three biological replicates.

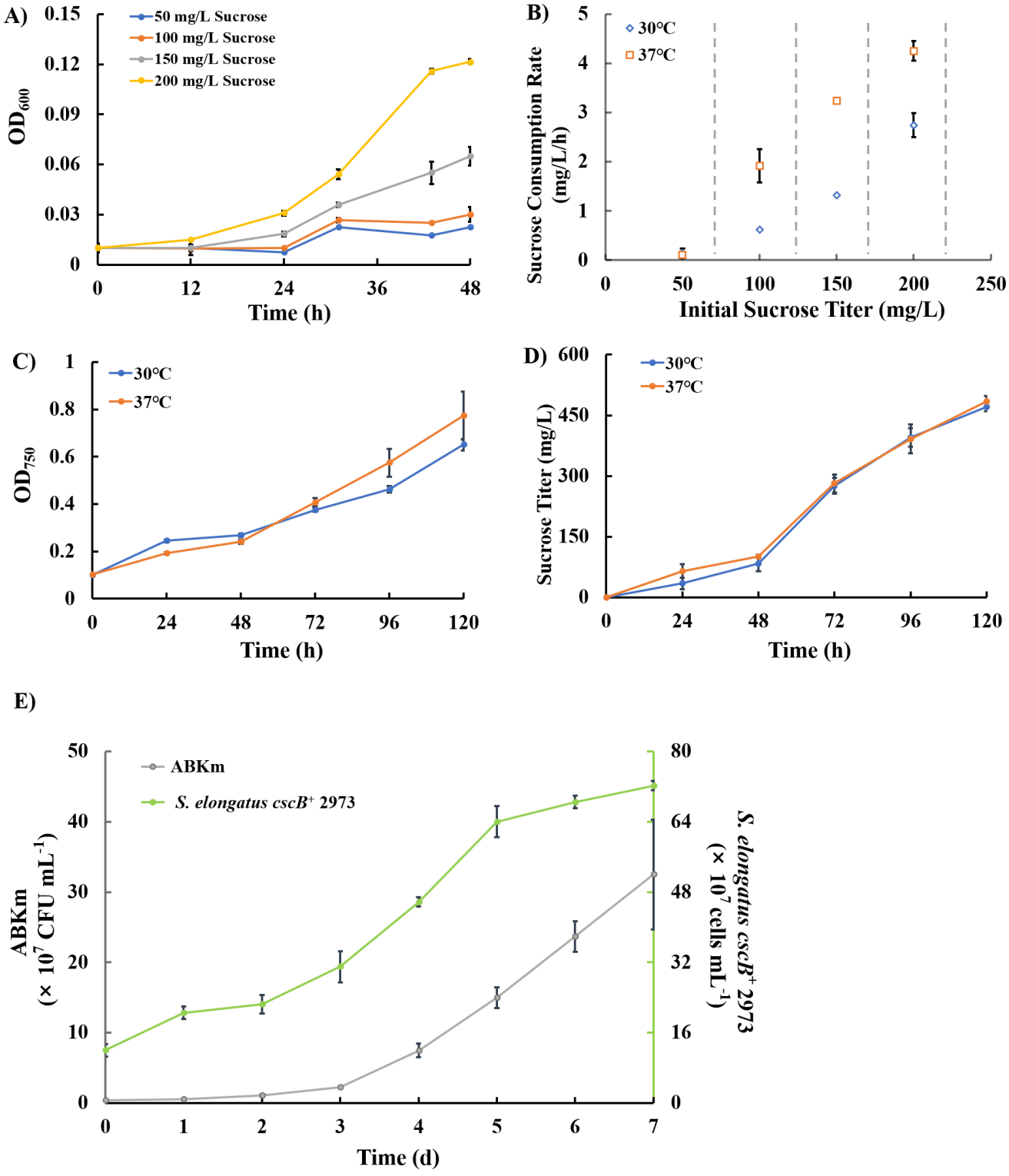

Figure 4

Construction of artificial consortium system with engineered S. elongatus UTEX 2973 and E. coli BL21. (A) Growth of pure culture ABKm strain in CoBG11 medium with different concentration of sucrose at 30°C; (B) Sucrose-utilizing rate of pure culture ABKm strain; (C) Growth of pure culture ABKm strain at 30°C and 37°C; (D) Sucrose yield of engineered S. elongatus cscB+; (E) Growth of two strains in artificial

consortium system at 30℃. The error bars represent the calculated standard deviation of the measurements of three biological replicates.

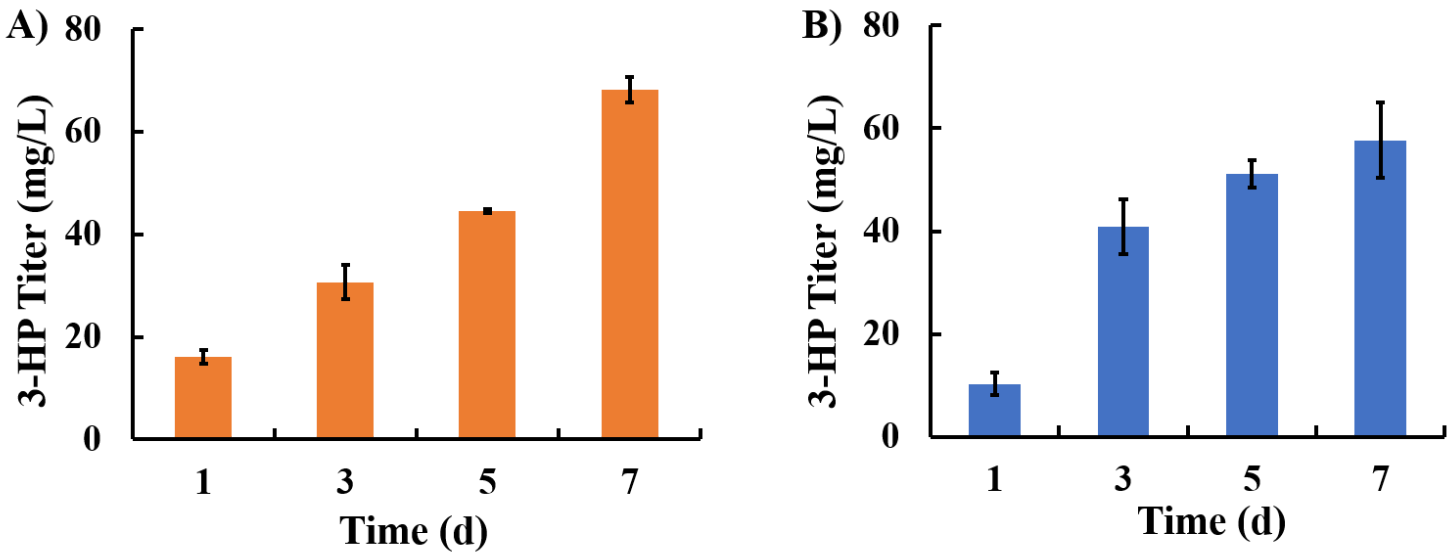

Figure 5

3-HP yield in different conditions. (A) 3-HP yield in artificial consortium system. (B) 3-HP production in strain ABKm under continuous supplementation of sucrose according to the calculated sucrose-secreting rate of S. elongatus cscB+. The error bars represent the calculated standard deviation of the measurements of three biological replicates.

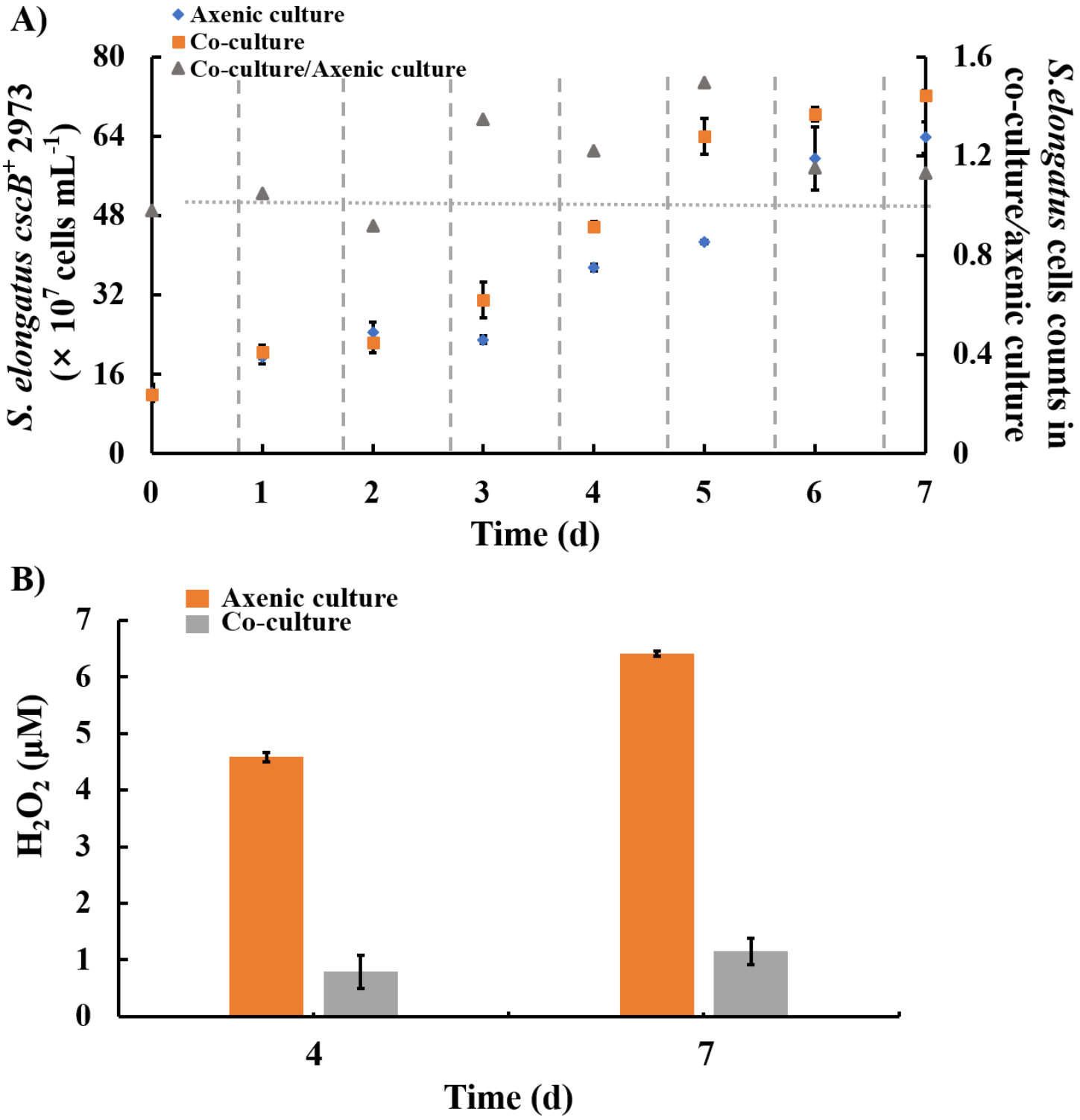

Figure 6

Growth of cyanobacteria and H2O2 content in different culture condition. (A) Growth difference of S. elongatus cscB+ in artificial consortium and axenic condition. Blue rhombus stands for the cell number in axenic, orange rectangle stands for the cell number in artificial consortium, and grey triangle stands for the ratio between the two conditions. (B) H2O2 content in blank CoBG-11 (control), axenic culture and co-

culture system under the same condition (30 °C, 100 μE m-2s-1 light, 150 mM NaCl). The error bars represent the calculated standard deviation of the measurements of three biological replicates.

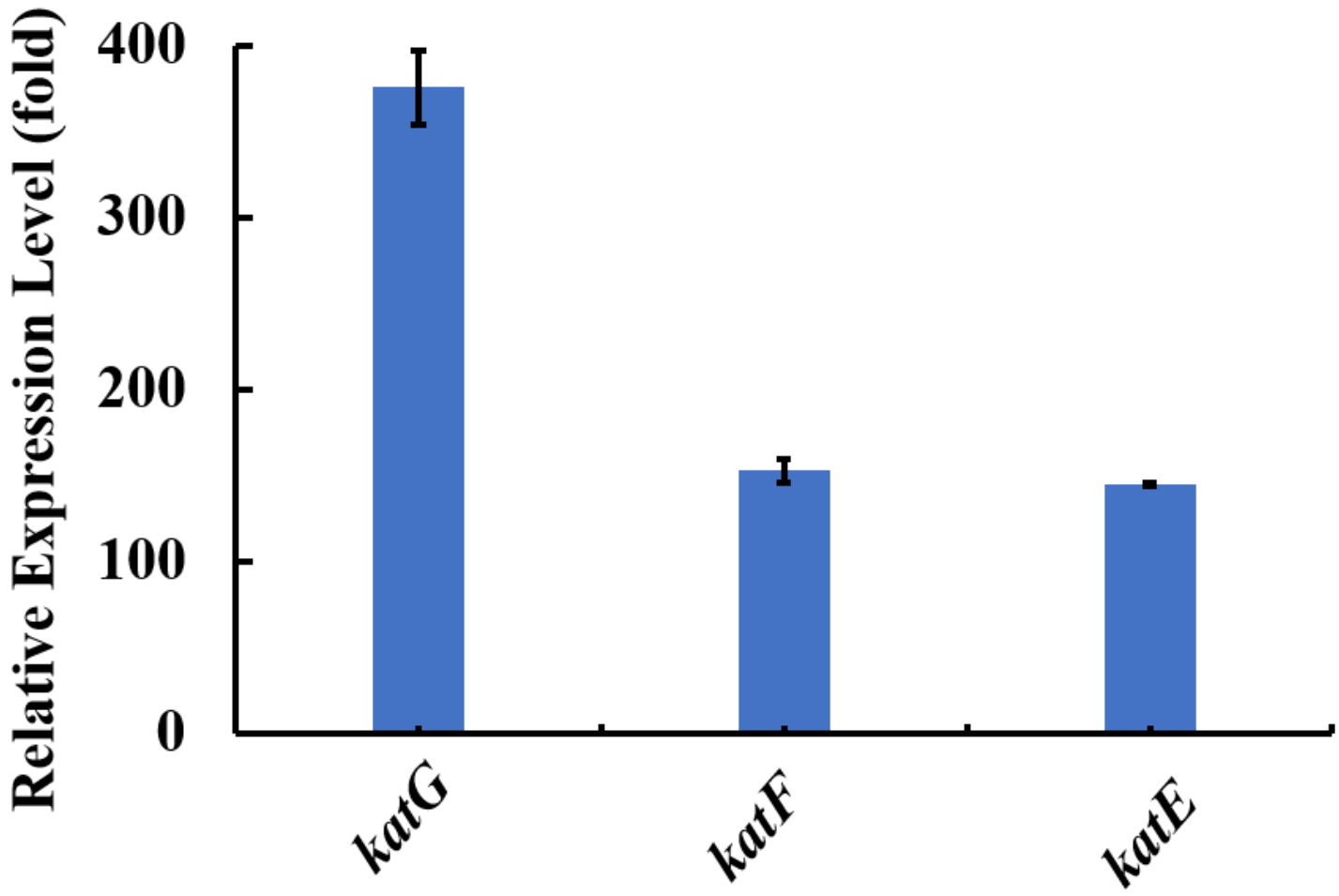

Figure 7

Expression level analysis of genes involved in ROS quenching. Genes expression analysis of katG, katF and katE in ABKm strain.

Supplementary Files

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