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An efficient *Chlorella* sp.-*Cupriavidus necator* consortium for phenol degradation and its symbiosis mechanism

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Abstract

A *Chlorella* sp.-*Cupriavidus necator* (*C. necator*) consortium was artificially established for phenol degradation. The synergistic relationship between *Chlorella* sp. and *C. necator* was initially demonstrated, and then the effects of *Chlorella* sp./*C. necator* inoculation ratio, light intensity, temperature and pH on the performance of this consortium were systematically evaluated and optimized. The optimal conditions for phenol degradation were as follows: a *Chlorella* sp./*C. necator* inoculation ratio of 1:1, a light intensity of 110 μmol m^{-2} s^{-1}, a temperature in the range of 25-32 ℃ and a pH in the range of 5.5-7.5. Under optimal conditions, this consortium could degrade phenol with a maximum concentration of 1200 mg L^{-1} within 60 h. Comparative transcriptomic analysis was conducted to discuss the symbiosis mechanism of this consortium subject to high phenol concentrations. The up-regulation of genes involved in photosynthesis and carbon fixation of *Chlorella* sp. demonstrated the CO_2 and O_2 exchange between Chlorella sp. and *C. necato*r, which, we suggest, may be the main reason for their mutual promotion of growth and phenol degradation.

Keywords: Phenol degradation; Microalgae-bacteria consortium; Synergistic relationship; Transcriptomic analysis

1 Introduction

Phenol and its derivatives often occur as hazardous pollutants in industrial effluents such as petroleum refineries, plastic, paper & pulp, pharmaceuticals and coal processing (Mohammadi et al., 2015), which can lead to acute environmental impacts even at low concentrations. The removal of phenol and its derivatives from industrial effluents is of great importance for wastewater treatment.

Conventional physical-chemical methods for phenol treatment include solvent extraction, adsorption, coagulation and chemical oxidation (Mohammadi et al., 2015). However, most of these methods yield by-products which create secondary pollution and lead to additional costs for treatment (Senthivelan et al., 2014). Compared with these physical-chemical treatment methods, biological treatment which may involve microalgae, bacteria, fungi and their consortia (Bhattacharya et al., 2018) may be an eco-friendly alternative for phenol removal. Compared to

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individual microalgae or bacteria, a microalgae-bacteria consortium shows many advantages such as cost-efficient aeration, efficient removal of pollutants and mitigation of greenhouse gas emissions (Su et al., 2012). Moreover, microalgae can concomitantly remove other nutrients and pathogens (including viruses) (Muñoz et al., 2003), and microalgal biomass provides a potential valuable by-product, which can offset a portion of plant operating costs.

Borde et al. (2003) reported for the first time that a consortium of *C. sorokiniana* and *A. haemolyticus* degraded 89 % of 200 mg L^{-1} phenol at 48 h under an Ar atmosphere. The consortium formed by *Chlorella vulgaris* MM1 and *Pseudomonas* MT1 was able to completely remove 433 mg L^{-1} of phenol from the mixtures of phenol and pyridine with a hydraulic retention time (HRT) of 2.7 days (Essam et al., 2013). However, the phenol removal rate decreased to 78% when the phenol concentration was increased to 500 mg L^{-1}. Maza-Márquez et al. (2017) developed a consortium using two microalgae strains (*C. vulgaris* and *S. obliquus*) and two bacteria strains (*P. agglomerans* and *R. terrigena*) to treat olive washing wastewater effluent that had undergone active carbon pretreatment. The results of this experiment showed that 90.3±11.4% of the total phenolic compounds (185.8±12.7 mg L^{-1}) was removed at an HRT of 3 days. A consortium consisting of *Chlorella vulgaris* and *Alcaligenes* sp. was established by Tamer et al. (2006) to biologically treat coking factory wastewater in Egypt with a phenol concentration of 135 mg L^{-1}. The results showed that the phenol removal rate only was 16 % after 7 days. However, pretreatment of the wastewater with activated carbon or UV radiation subsequently allowed almost complete removal of phenol by the consortium after 3-4 days of inoculation. Although the feasibility of removing phenol from wastewater by microalgae-bacteria consortium was demonstrated by the previous studies, the low tolerance to phenol, long degradation duration and additional physical or chemical pretreatment steps restricted its widespread application. To overcome these constraints we suggest that the development of more efficient microalgae-bacteria consortia have the potential to enhance the degradation efficiency of phenol at high concentrations.

Symbiotic interactions between microalgae and bacteria have been widely investigated. Carbon and macro- and micro-nutrients seem to play a central role. Studies show micro-nutrients like vitamins (Croft et al., 2005; Kuo et al., 2013; Teplitski et al., 2011), macronutrients like nitrogen and carbon (Bolch et al., 2011; Kazamia et al., 2012; Teplitski et al., 2011; Kim et al., 2014) and phytohormones (Teplitski et al., 2011) are exchanged between microalgae and bacteria. Although previous studies have explored the symbiotic mechanisms of microalgae and bacteria consortia at the physiological, biochemical and molecular levels, the understanding of the mechanisms is far from complete and requires further dedicated research (Perera et al., 2019).

In this study, a *Chlorella* sp.-*Cupriavidus necator* (*C. necator*) consortium was artificially established for phenol degradation. The controlled experimental conditions were systematically studied, which included *Chlorella* sp./*C. necator* inoculation ratio, light intensity, temperature and pH. The degradation performance of this consortium applied to a substrate containing 600-1200 mg L^{-1} of phenol was investigated under optimal conditions. Finally, the interactions between *Chlorella*

sp. and *C. necator* were discussed through comparative analysis in order to provide deep insight into the physiology of the consortium.

2 Experiment

2.1 Organisms and culture conditions

The green microalgae *Chlorella* sp. was separated from a water sample collected locally (Shanghai), and maintained in petri dishes using BG11 solid medium. *Chlorella* sp. cells were successively transferred from petri dishes to 250 mL flasks, and then cultivated in 400 mL bubble column photobioreactors with 1% CO_2 under 120 μmol m^{-2} s^{-1} and 25 ℃ conditions. Phenol-degrading bacteria *C. necator* (formerly *Ralstonia eutropha*, Martinez et al., 2015) was purchased from the China General Microbiological Culture Collection Center (No. 1.7092) and maintained in petri dishes using a nutrient broth solid medium consisting of: 10 g L^{-1} of peptone, 3 g L^{-1} of beef extract, 5 g L^{-1} of sodium chloride and 15 g L^{-1} of agar. *C. necator* cells were successively transferred from petri dishes to 250 mL flasks, and then incubated at 30 ℃ on a rotary shaker (150 rpm). The cells of those microorganisms, *Chlorella* sp. and *C. necator*, were harvested during their logarithmic growth phase by centrifugation. All the harvested cells were resuspended into the simulation phenol wastewater with the required biomass density and used in the following experiments.

2.2 Feasibility of *Chlorella* sp.-*C. necator* consortium for phenol degradation

The stock solution of phenol (2000 mg L^{-1}) was prepared by dissolving the requisite amount of phenol in sterilized BG11 medium. The solution of a required concentration of phenol was prepared by diluting the stock solution with the sterilized BG11 medium.

In order to study the feasibility of the *Chlorella* sp.-*C. necator* consortium for phenol degradation, three experiments including *Chlorella* sp. monoculture, *C. necator* monoculture and the co-culture of *Chlorella* sp. and *C. necator* were designed for phenol degradation. The three experiments were conducted in 250 mL conical flasks (working volume 100 mL) with breathable sealing membranes, and incubated on a rotary shaker (150 rpm) under continuous illumination (110 μmol m^{-2} s^{-1}) and a constant temperature of 25 ℃. The initial concentrations of phenol, *Chlorella* sp. and *C. necator* were 500 mg L^{-1}, 0.2 g L^{-1} and 0.2 g L^{-1}, respectively. All tests were performed in duplicate.

2.3 Optimization of operating conditions

An evaluation of the influence of operating conditions (*Chlorella* sp./*C. necator* inoculation ratio, pH, light intensity and temperature) on the performance of the *Chlorella* sp.-*C. necator* consortium was conducted in 250 mL conical flasks (working volume 100 mL) with breathable sealing membranes. The culture parameters at different operating conditions are shown in Table 1. The initial phenol and *Chlorella* sp. inoculation concentrations were kept at 500 mg L^{-1} and 0.2 g L^{-1}, respectively. Different concentrations of *C. necator* (0.05, 0.1, 0.2 and 0.3 g L^{-1}) were inoculated to regulate the *Chlorella* sp./*C. necator* inoculation ratios in a range between 4:1 and 2:3. The initial pH was adjusted using HCl (1 mol L^{-1}) or NaOH (1 mol L^{-1}) solution to 5.5, 7.5, 9.5 and 11.5, respectively. The light intensities of 110, 240 and 580 μmol m^{-2} s^{-1} were adjusted by changing the

distances between the flasks and the LED (maximum light intensity around 2000 μmol m^{-2} s^{-1}). The dark condition (0 μmol m^{-2} s^{-1}) was achieved by covering the flask with aluminum-foil. The temperature was regulated using a constant temperature water bath and set at 17, 25, 32 and 40℃, respectively. All tests were performed in duplicate.

2.4 Performance of *Chlorella* sp.-*C. necator* consortium for phenol degradation under optimal conditions.

The degradation performance of the *Chlorella* sp.-*C. necator* consortium was investigated with different initial phenol concentrations under optimal conditions. The initial phenol concentrations were adjusted using stock phenol solution to 600, 800, 1000 and 1200 mg L^{-1}, respectively. All the experiments were conducted in 500 mL conical flasks (working volume 300 mL) with breathable sealing membranes on a rotary shaker (150 rpm) under 110 μmol m^{-2} s^{-1} and 25℃ conditions. The initial concentrations of *Chlorella* sp. and *C. necator* were 0.2 and 0.2 g L^{-1}, respectively. The initial pH was adjusted to 7.5 using NaOH solution (1 mol L^{-1}). All tests were performed in duplicate.

2.5 Comparative transcriptomic analysis

Comparative transcriptomic analysis was used to investigate the potential symbiotic mechanism between *Chlorella* sp. and *C. necator* at a molecular level in the process of degrading high concentrations of phenol. However, the direct co-culture could not be used for the comparative transcriptomic analysis because the microarray only quantifies target sequences of individuals. In this experiment, *Chlorella* sp. were co-cultivated with the immobilized *C. necator* beads and then separated for the comparative transcriptomic analysis.

Immobilized *C. necator* beads were prepared using polyvinyl alcohol (PVA) and sodium alginate (SA). The preparation process was conducted as follows: first, PVA (6 wt%) and SA (3 wt%) were added to deionized water and autoclaved for 30 min at 121 ℃. The obtained gel was cooled at room temperature and then mixed with *C. necator* (0.2 g L^{-1}). Then the resulting mixture was added drop-wise into a calcium chloride solution (2 wt %) with a peristaltic pump and maintained at 4 ℃ for 24 h to form gel beads. Finally, the beads were washed with deionized water five times for removing the adhered *C. necator* on the surface.

The phenol degradation experiment for comparative transcriptomic analysis was designed to observe two groups: *Chlorella* sp. monoculture (denoted as Alg), *Chlorella* sp. and immobilized *C. necator* co-culture (denoted as Alg_Co). The inoculation concentrations of phenol, *Chlorella* sp. and immobilized *C. necator* were 800 mg L^{-1}, 0.2 g L^{-1} and 0.2 g L^{-1}, respectively. The other experimental conditions were similar to those described in Section 2.2. The *Chlorella sp.* cells cultivated in Alg and Alg_Co were collected after 12 h had elapsed. Each sample of Alg and Alg_Co was analyzed in triplicate. The sample treatment, transcriptomic determination, gene annotation and the related bioinformatics analysis were performed as described in previous studies [Cheng et al.,2019a; Zhou et al., 2017]. False discovery rate (FDR) and Log2 fold change (LFC) were selected to investigate the target genes. If the FDR is less than 0.05 and the absolute value of LFC is not less than 1, the gene is regarded as being significantly expressed.

2.6 Analysis methods

10 mL of sample was periodically withdrawn from each flask to measure the residual phenol concentration, growth of *Chlorella* sp. and *C. necator* and pH of the culture medium. The pH of the sample was measured using a Five Easy pH meter (METTLER TOLEDO) immediately after the sample was harvested. The maximum quantum yield of photosystem II was determined using 2 mL of sample. The Fv/Fm value was measured using a fluorescence monitoring system (FMS2, Lufthansa Scientific Instruments Co., Ltd., UK) after the sample had been stored in dark conditions for 30 min. 1 mL of sample was centrifuged at 6000 rpm for 10 min to obtain the supernatant. The concentration of residual phenol in the supernatant was measured by the colorimetric assay 4-amino antipyrine method (Zhou et al., 2017).

The biomass concentrations of *Chlorella* sp. and *C. necator* in monoculture were determined gravimetrically. Generally, 5 mL of sample was filtered using a pre-dried and pre-weighed cellulose membrane (0.45 μm pore size), washed with deionized water, dried for 24 h at 105 ℃, cooled in a desiccator and then weighed again. The dry weight of the blank filter was subtracted from that of the loaded filter to obtain the dry weight.

The biomass concentration of *Chlorella* sp. in the consortium was determined indirectly by measuring the chlorophyll a and b (Chl *a*+*b*) concentrations in the consortium according to the method of Mohammad et al. (2020). Generally, 0.5 mL of consortium sample was centrifuged at 13,400 rpm for 10 min and the supernatant was discarded. Chlorophyll a and b were extracted from the pellets using methanol (1.5 mL) and quantified as described in Pruvost et al (2011). The concentrations of Chl *a*+*b* (mg L^{-1}) were calculated using equation (1-3):

$$\text{Chl } a = [-8.0962 \times (OD_{652} - OD_{750}) + 16.5169 \times (OD_{665} - OD_{750})] \times 3 \quad (1)$$

$$\text{Chl } b = [27.4405 \times (OD_{652} - OD_{750}) - 12.1688 \times (OD_{665} - OD_{750})] \times 3 \quad (2)$$

$$\text{Chl } a + b = \text{Chl } a + \text{Chl } b \quad (3)$$

Where, Chl *a*, Chl *b and* Chl *a*+*b* are the concentrations of chlorophyll a (mg L^{-1}), chlorophyll b (mg L^{-1}) and chlorophyll a and b (mg L^{-1}), respectively. OD_{652}, OD_{665} and OD_{750} are the optical densities of the extraction solution at wavelengths of 652, 665 and 750 nm, respectively.

A standard curve was prepared for measuring biomass concentrations using Chl *a*+*b* concentrations in a series of *Chlorella* sp. suspensions. Chl *a*+*b* concentrations and biomass concentrations of *Chlorella* sp. were correlated according to the following equation (4):

$$Y = 0.0223X - 0.0056 \; [R^2 = 0.997] \quad (4)$$

Where, *Y* and *X* are biomass concentrations (g L^{-1}) and Chl *a*+*b* concentrations (mg L^{-1}) of *Chlorella* sp., respectively.

The biomass concentration of *Chlorella* sp. in the consortium was calculated using equation (4) after measuring the concentrations of Chl *a*+*b* in the consortium. The total biomass concentration of the *Chlorella* sp.-*C. necator* consortium was determined similarly to the biomass of the monoculture. The biomass concentration of *C. necator*. in the consortium was obtained by subtracting the biomass concentration of *Chlorella* sp. from the total biomass of the consortium.

3 Results and discussion

3.1 Feasibility of *Chlorella* sp.-*C. necator* consortium for phenol degradation

In order to determine the effectiveness of the artificial *Chlorella* sp.-*C. necator* consortium for improving phenol degradation, degradation trials using the *Chlorella* sp. monoculture, *C. necator* monoculture and the co-culture of *Chlorella* sp. and *C. necator* were conducted in batch reactors with 500 mg L^{-1} of phenol. As shown in Fig. 1a, the residual phenol concentration in *Chlorella* sp. monoculture remained almost the same as the initial concentration after *Chlorella* sp. inoculation after 24 h. However, *C. necator* was observed to rapidly degrade the phenol after a lag phase of 6 h, and completely degraded the initial 500 mg L^{-1} of phenol after 18 h. The decrease of pH in the *C. necator* monoculture further demonstrated the degradation of phenol, which could be ascribed to the generation of CO_2 in the process of phenol degradation by *C. necator* (Fig. 1d). Compared with the *Chlorella* sp. *and C. necator* monoculture, no significant lag period was observed in their consortium, and complete phenol removal was observed at 12 h (Fig. 1a). The above results suggest that *C. necator* is largely responsible for phenol degradation, and the presence of *Chlorella* sp. enhances the phenol degradation rate..

Similarly, as shown in Fig. 1b, the biomass concentrations of *Chlorella* sp. and *C. necator* in consortium were much higher than that of the corresponding monoculture. In this case, the Fv/Fm values, the maximum quantum yield of photosystem II, of *Chlorella* sp. in consortium, were much higher than that of *Chlorella* sp. in monoculture (Fig. 1c). Hence, the enhancement of photosynthesis could induce more O_2 generation, which is important for aerobic degradation of phenol by *C. necator* (Maza-Márquez et al., 2014). The results demonstrated that *Chlorella* sp. and *C. necator* in the co-culture system promoted mutual growth, which might be facilitated by the exchange of CO_2 and O_2 between *Chlorella* sp. and *C. necator* (Borde et al., 2003).

Thus, based on the enhanced mutual growth and the improvement of phenol degradation, the hypothesized synergetic relationship between *Chlorella* sp. *and C. necator* and the application feasibility of this consortium appears confirmed.

3.2 Optimization of operating conditions for phenol degradation by *Chlorella* sp.-*C. necator* consortium

In order to investigate the maximum phenol degradation performance of this consortium, operating parameters (*Chlorella* sp./*C. necator* inoculation ratio, pH, light intensity and temperature) were studied and optimized. The experimental results for a number of operational parameters are presented and discussed below.

3.2.1 *Chlorella* sp./*C. necator* inoculation ratio

The initial ratio of microalgae to bacteria is regarded as one of the most important factors for determining the rate of phenol degradation and the growth of microalgae and bacteria (Maza-Márquez et al., 2014; Guieysse et al., 2002:). To further investigate these factors, the *Chlorella* sp./*C. necator* inoculation ratios were adjusted to 4:1, 2:1, 1:1 and 2:3 by mixing 0.2 g L^{-1} of *Chlorella* sp. with 0.05, 0.1, 0.2 and 0.3 g L^{-1} of *C. necator*, respectively. Fig.2 shows the effect of different inoculation ratios on the phenol degradation rate and the growth of *Chlorella* sp. and *C. necator*. As shown in Fig. 2a, the consortia with the 2:3 and 1:1 ratios degraded the initial concentration of 500

mg L^{-1} phenol completely within 12 h, and showed the best phenol degradation performance. The consortium with the 2:1 ratio degraded phenol more slowly with complete removal after 18 h. The residual phenol concentration of the consortium with a 4:1 ratio was similar to the initial phenol concentration after 24 h indicating minimal degradation. The results of this experiment suggest that phenol removal is likely enhanced by increasing the *C. necator* inoculation concentration. Meanwhile, *C. necator* growth (Fig. 2b) was also synchronous with phenol removal, which further supports the hyphothesis that phenol removal is likely dominated by metabolic degradation of *C. necator*.

In general, high concentrations of phenol exhibit a strong toxicity to microalgae, which seriously affects the growth of microalgae and even leads to death (Cheng et al. 2017; Priyadharshini et al., 2017; Xiao et al., 2019). In this experiment, the toxicity of phenol to *Chlorella* sp. appeared to be diminished by the addition of *C. necator* except when the ratio of *Chlorella* sp. to *C. necator* was increased to a level of 4:1. As shown in Fig. 2c and 2d, the biomass concentrations and Fv/Fm values of *Chlorella* sp. in consortia with 2:1, 1:1 and 2:3 ratios were enhanced relative to those of *Chlorella* sp. in monoculture (Fig. 2b and 2c). However, no obvious improvement in the consortium with the 4:1 ratio was observed, which was due to minimal growth of *C. necator* and degradation of phenol. For the consortium with 4:1 ratio, the low inoculation concentration of *C. necator* (0.05g L^{-1}) most likely resulted in *C. necator* taking more incubation time to adjust to the stress created by the high phenol concentration. In addition, the pH of the culture medium continued to increase with the passage of time due to the photosynthesis of *Chlorella* sp. (Fig. 2d), which increased to 8.41 and 9.08 after 6 h and 24 h (Fig. S1a), respectively. As shown in Fig. 3 a and b, a high pH (pH 9.5) inhibited the growth of bacteria and caused phenol degradation to cease. Therefore, the delay of phenol degradation caused by low inoculation concentration of *C. necator* and the inhibition of *C. necator* growth caused by high pH most likely led to the lack of phenol degradation for the consortium with the 4:1 ratio. Conversely, the pH of the consortia with the 2:1, 1:1 and 2:3 ratios decreased after 6 h and then increased thereafter (Fig. S1a). For the three consortia, the pH is dictated by the acidification process resulting from CO_2 release via phenol degradation of *C. necator* and by the alkalization process resulting from CO_2 uptake via the photosynthesis of *Chlorella* sp. (Fig. 1d). Up until the elapsed time of 6 h, *C. necator* degraded phenol and released CO_2 into the growth medium, which resulted in a decrease in pH (Fig. S1a). Thereafter *Chlorella* sp. appeared to recover and photosynthesis was enhanced (Fig. 2d) which led to a resultant increase in pH.

These experiments suggest that the consortia with the 2:3 and 2:1 ratios exhibited the best performance. Compared to the ratio of 2:3, the ratio of 1:1 had a similar phenol removal rate and a low inoculation concentration of *C. necator*. Therefore, the ratio of 1:1 was selected as the optimal *Chlorella* sp./*C. necator* ratio and was utilized in the subsequent experiments.

3.2.2 pH

The pH of the growth medium affects many biological processes and strongly governs the growth of both microalgae and bacteria (Muñoz and Guieysse, 2006). The effect of pH on phenol degradation and the growth of *Chlorella* sp. and *C. necator* was studied at pH ranging from 5.5 to 11.5. As shown in Fig. 3a, the consortium appeared capable of degrading 500 mg L^{-1} of phenol completely at both pH 5.5 and 7.5 within 12 h. However, under alkaline conditions (pH 9.5 and 11.5), the consortium showed no marked phenol degradation activity. Similarly, the growth of *C.*

necator was correlated with phenol degradation. As shown in Fig. 3b, the biomass concentrations of *C. necator* reached the maximum at 12 h under pH 5.5 and 7.5 conditions. There was no significant growth of *C. necator* at pH 9.5 and 11.5 after incubation for 24 h. Most microorganisms cannot tolerate pH values below 4.0 or above 9.0 (Kim et al., 1981; Muñoz and Guieysse, 2006). At low (4.0) or high (9.0) pH values, acids or bases can penetrate microbe cells more readily because they tend to exist in an undissociated form under these conditions and electrostatic forces cannot prevent them from entering cells (Kim et al., 1981; Annadurai et al., 1999; Muñoz and Guieysse, 2006). Likewise for the consortia with 2:1, 1:1 and 2:3 ratios, the toxic effect of phenol on *Chlorella* sp. growth appeared to be diminished by *C. necator* via its phenol metabolism at pH 5.5 and 7.5. As shown in Fig. 3c and 3d, the *Chlorella* sp. in consortium showed higher biomass concentrations and Fv/Fm values at pH 5.5 and 7.5 than these at pH 9.5 and 11.5. The photosynthesis of *Chlorella* sp. caused the pH rise for the consortia cultivated at pH 5.5 and 7.5 (Fig. S1b). However, as shown in Fig. S1b, there was no significant pH change for the consortium cultivated at pH 9.5. The pH decrease observed for the consortium cultivated at pH 11.5 was attributed to the fact that the highly alkaline solution was able to readily absorb CO_2 from the atmosphere. The results of this experiment suggest an optimal pH for phenol removal to be in the range of 5.5 to 7.5.

3.2.3 Light intensity

Light illumination is one of the most limiting factors to be determined for microalga and bacteria partnerships for the treatment of wastewater (Mohammad et al., 2020). The effect of light intensity on phenol degradation and the growth of *Chlorella* sp. and *C. necator* was studied at light intensities of 0, 110, 240 and 580 μmol m^{-2} s^{-1}, respectively. As shown in Fig. 4a, the consortium degraded 500 mg L^{-1} of phenol completely at 18 h, 12 h and 18 h under 0, 110 and 240 μmol m^{-2} s^{-1}, respectively. However, the phenol degradation was almost inhibited at a high light intensity of 580 μmol m^{-2} s^{-1}. Under dark conditions (0 μmol m^{-2} s^{-1}), heterotrophic *C. necator* could mineralize phenol and grow (Fig. 4b). *Chlorella* sp., on the other hand, experienced growth inhibition because of the lack of light to enable photosynthesis (Fig. 4c). Both phenol degradation and *C. necator* growth in the consortium at a light intensity of 110 μmol m^{-2} s^{-1} were enhanced compared to dark conditions (Fig. 4 a and b). This result was ascribed to the oxygen released by *Chlorella* sp. photosynthesis under illumination (Borde et al. 2003). These experimental results further demonstrate the synergistic relationship between *Chlorella* sp. and *C. necator*. However, when the light intensity was increased to 240 and 580 μmol m^{-2} s^{-1}, the process of phenol removal was reduced and even completely inhibited. The result is consistent with the inhibition of growth or even death of *C. necator*., which may be related to cell stress response caused by reactive oxygen species (ROS) produced under the high light intensity and phenol concentration conditions (Khaengraeng and Reed, 2005). These results are also in agreement with Merbt et al. (2011) and Vergara et al. (2016), who have reported that high light intensity reduced bacterial nitrifying activity. Although the light intensity of 240 μmol m^{-2} s^{-1} had a negative effect on *C. necator* growth and phenol degradation, it enhanced the photosynthesis (Fig. 4d) and growth of *Chlorella* sp. (Fig. 4c), and resulted in a higher

pH at 24 h (Fig. S1c). However, the photosynthesis and growth of *Chlorella* sp. were completely inhibited at 580 μmol m^{-2} s^{-1} and high amounts of phenol were still present in growth medium. The results of this experiment suggest that low light intensity (100 μmol m^{-2} s^{-1}) is the optimal illumination condition for algal and bacterial phenol degradation.

3.2.4 Temperature

The temperature can strongly influence the growth and survival of microorganisms (Li et al, 2019). The effect of temperature on phenol removal and the growth of *Chlorella* sp. and *C. necator* was studied within a range of 17 ℃ and 40 ℃. As shown in Fig. 5a, the consortium could completely degrade 500 mg L^{-1} of phenol within 12 h at temperatures of 25 and 32 ℃. The phenol removal rates at 17 and 40 ℃ were much lower than those at 25 and 32 ℃. As shown in Fig. 5b, low and high temperatures below and above a threshold range appeared to negatively affect the growth of the bacteria and inhibit phenol degradation potential. High temperature most likely has a detrimental effect on the bacterial enzymes usually responsible for the benzene ring cleavage, which is the main process step in biological degradation (El-Naas et al., 2009). On the other hand, exposure to low temperature outside the optimal range is expected to slow bacterial activity. Meanwhile, this inhibitory effect is enhanced at high phenol concentrations (Onysko et al., 2000). Similarly, the phenol degradation improved the growth of *Chlorella* sp. at suitable temperature. As shown in Fig. 5c, d and Fig. S1d, the biomass concentration, Fv/Fm and pH of *Chlorella* sp. in the consortia maintained at 25 and 32℃ were enhanced compared to the same factors at 17 and 40 ℃. Therefore, the optimal temperature for phenol degradation and microalgae growth suggested by this experiment ranges from 25 to 32℃.

3.3 Phenol degradation performance of *Chlorella* sp.-*C. necator* consortium under optimal conditions.

In order to investigate the maximum phenol degradation performance of the *Chlorella* sp.-*C. necator* consortium, the degradation of 600-1200 mg L^{-1} phenol was carried out under optimal operating conditions. As shown in Fig. 6a, 600, 800, 1000 and 1200 mg L^{-1} concentrations of phenol were completely degraded at 18, 24, 36 and 60 h, respectively. The required time for complete removal of phenol was positively correlated with the initial phenol concentration. During the process of phenol degradation, the phenol removal rate was relatively slow during the initial stage, the rate increased during the intermediate stage, and was the fastest during the final stage.

In the case of the highest initial phenol concentration of 1200 mg L^{-1}, the *Chlorella* sp.-*C. necator* consortium could not degrade phenol until 24 h had elapsed, and then began to degrade phenol slowly during the period from 24 h to 48 h. The consortium showed the fastest removal rate between 48 h and 60 h. Correspondingly, *C. necator* in the consortium showed a lag period of about 24 h (Fig. 6b), and then began to grow slowly between 24 and 30 h. *C. necator* exhibited exponential growth after 30 h. On the other hand, *Chlorella* sp. had a longer lag period of about 48h (Fig. 6c), then the culture began to grow after the phenol concentration dropped to 774 ±5.2 mg $L^{-1,}$ which was consistent with that of consortium cultivated at 800 mg L^{-1} of phenol. Finally, *Chlorella* sp.

grew exponentially in the period of 48 and 60 h. These results indicate that the growth of *Chlorella* sp. lags behind the phenol degradation and *C. necator* growth.

Under conditions of high phenol concentrations , the relationship between phenol degradation and microalgal and bacterial growth can be explained as follows. In the initial stage of phenol degradation, the high concentration of phenol most likely resulted in high stress to the microorganisms resulting in growth inhibition and low biomass concentrations of both *C. necator* and *Chlorella* sp. (Fig. 6 b and c). As *C. necator* slowly adapted to the high phenol stress conditions, growth increased and it began to degrade phenol. When the residual phenol concentration decreased to a more tolerable level for *Chlorella* sp., the microalgae began to grow more rapidly using CO_2, likely released from phenol degradation as a carbon source (Fig. 6 c). The Fv/Fm values of *Chlorella* sp. gradually recovered to a high level (Fig. 6 d). At the end stage, with lower phenol concentrations in the growth medium, both *Chlorella* sp. and *C. necator* grew exponentially and phenol exhibited rapid degradation. The synergistic effect was observed only in the final stage of phenol degradation. If the phenol tolerance of microalgae is enhanced, the early start of photosynthesis could increase the period of synergistic effect between microalgae and bacteria, which would speed up the phenol degradation rate.

This experiment shows that bacteria tolerance and degradation capability for phenol degradation were generally higher than that for microalgae (Maza-Márquez et al., 2018). Therefore, the maximum biodegradable phenol concentration of the consortium appears to be determined by the degradation performance of bacteria in the consortium. The phenol degradation rate is determined by the tolerance of microalgae, and, the earlier the synergistic effect appears, the faster the phenol degradation rate. Hence, microalgal tolerance of phenol may be more important than its capacity for phenol degradation and for the successful establishment of a microalga-bacteria consortium. These results suggest the importance of careful selection of microalga and bacteria strains prior to the artificial establishment of a microalga-bacteria consortium.

3.4 The symbiosis mechanism between *Chlorella* sp. and *C. necator*

The symbiotic mechanism between *Chlorella* sp. and *C. necator* was investigated by comparative transcriptomic analysis under a high phenol concentration. *Chlorella* sp. were co-cultivated with immobilized *C. necator* beads (Alg_Co) and then separated for the comparative transcriptomic analysis. *Chlorella* sp. were cultivated alone as the control (Alg). Immobilization can retard phenol transport and result in longer degradation time when compared to direct coculture, which leads to the release of *C. necator* and in turn results in the failure of comparative analysis. Therefore, 800 mg L^{-1} of phenol was selected in this experiment because of the relative shorter degradation time compared to 1000 and 1200 mg L^{-1}. As shown in Fig. S3 and S4, the phenol degradation rate of Alg_Co is slower than the direct coculture. Meanwhile, no *C. necator* cells were found to be released from the bead,s which are important for the successful application of transcriptomic analysis, after microscopic observation,.

For the two groups of microalgal cells 40,362 unigenes were detected. To identify genes that

displayed significant changes in expression under high phenol concentration stress, differentially expressed genes (DEGs) were analyzed by comparing the libraries for Alg_Co and Alg. Overall, a total of 3,529 unigenes in Alg_Co were significantly differentially-expressed compared with Alg, among which 2,166 and 1,363 genes were significantly up-regulated and down-regulated, respectively (Fig. S5). In order to determine signaling pathways involved in DEGs, DEGs were annotated using the KEGG database. An enriched bubble chart of the candidate genes provided a graphic display of top 20 most enriched pathways for DEGs. As shown in Fig. 7, photosynthesis-antenna proteins, photosynthesis, ribosome, DNA replication, carbon fixation in a photosynthetic organism ranked among the top 5 most enriched pathways, most of which are related to the photosynthesis of *Chlorella* sp.

Microalgae can fix CO_2 and produce O_2 through photosynthesis. As shown in Fig. 8a and Table S1, most of the detected genes in photosynthesis pathway were up-regulated, such as *PsaA*, *PsaB*, *PsaD*, *PsaE*, *PsaF PsaH*, *PsaK*, *PsaL, PsaN*, and *PsaO* in photosystem I, *PetE, PetH* and *PetJ* in photosynthetic electron transport, *PsbE*, *PsbP*, *PsbQ*, *Psb R*, *PsbW*, *PsbY* in photosystem II. Meanwhile, the antenna proteins such as *Lhca2-5*, *Lhcb1*, *Lhcb2*, *Lhcb4* and *Lhcb5* were also up-regulated (Fig. 8b and Table S2), which are important for photosynthetic light harvesting (Kim et al., 2010). Therefore, the expression of most genes involved in the light reactions and those encoding antenna proteins were up-regulated to different degrees when *Chlorella* sp. was cultivated with immobilized *C. necator*. This result implies that the photosynthesis of *Chlorella* sp. was enhanced. C*hlorella* sp. in Alg_Co produced more O_2 compared to that in Alg, which further enhanced the aerobic degradation of phenol by *C. necator.* Correspondingly, more CO_2 should be released during the biodegradation of phenol [Maza-Márquez et al., 2014]. As, The pathway of carbon fixation in photosynthetic organisms (the last step of photosynthesis) was also enriched. As shown in Fig. 9 and Table S3, most of the enzymes involved in C3 pathways were upregulated for *Chlorella* sp. in Alg_Co, which indicated that the carbon fixation process was enhanced. Ribulose-1,5-bisphosphate carboxylase-oxygenase (rubisco), the first and critical enzyme of the Calvin cycle (Spreitzer et al., 2002), is only activated when CO_2 concentrations are greater than its K_m for CO_2 (Cheng et al., 2013). The up-regulation of genes related with CO_2 fixation showed that the concentration of CO_2 increased in Alg_Co compared to Alg. O_2 and CO_2 are the key resources needed for the growth and metabolic activity of *C. necator* and *Chlorella* sp. Therefore, the up-regulation of genes involved in photosynthesis and carbon fixation implied that *C. necator* and *Chlorella* sp. build up a mutual partnership to exchange CO_2 and O_2, which resulted in the enhancement of phenol removal and the promotion of biomass accumulation.

Cell growth depends on protein synthesis by ribosomes, and both reductions in ribosome assembly and translation are often among the first and most drastic responses when cells experience environmental stress (Sanchez et al., 2016; Wang et al., 2019; Zhu et al., 2019). The expression patterns of microalgal ribosome genes in transcriptomic analysis were shown to be sensitive to environmental stress such as the introduction of phenolic compounds (Qian et al., 2011; Xiang et

al., 2018; Duan et al., 2019). Cheng et al. (2019b) reported that *Dictyosphaerium* sp. after exposure to nonylphenol (4 mg L^{-1}) resulted in having genes encode the ribosomal large subunit (*rpl3e*, *rpl7e*, *rpl14e*) and small subunit (*rps8e*, *rps30e*, *rpsAe*) that were completely inactivated compared to algae that experienced normal growthpatterns. In this study, the detailed enrichment analysis showed that ribosome pathways were among the most regulated pathways (Fig.7), and 128 unigenes were up-regulated and only two down-regulated in Alg_Co compared to Alg (Fig. 10; Table S4). The results also suggest that *Chlorella* sp. in Alg_Co is under less environmental stress, which is consistent with the resulting low concentration of residual phenol in the culture medium (Fig. S3). Meanwhile, as shown in Fig. S2, a low concentration of phenol (100-400 mg L^{-1}) could enhance the growth of *Chlorella* sp. Duan et al. (2019) reported that low concentration of BPA could enhance the growth of *Chlorella pyrenoidosa* and the expression of ribosomal genes up-regulated, which are supported by the results of this study. Low concentration of phenol appear to act as a hermetic substance to stimulate the growth of *Chlorella* sp..

4. Conclusions

In this study, a *Chlorella* sp.-*C. necator* consortium was artificially established for degradation of phenol at high concentrations. The efficacy of this technique was demonstrated after conducting experiments to determine optimal operating conditions that included the factors pH, light intensity, temperature. This consortium produced high phenol degradations rate for initial phenol concentrations of between 600 and 1200 mg L^{-1} under optimal conditions. The CO_2 and O_2 exchange mechanism between *Chlorella* sp. and *C. necator* were demonstrated observation of the up-regulation genes involved in photosynthesis and carbon fixation pathways through comparative transcriptomic analysis. This study suggests that this consortium has great potential for the bioremediation of phenol contaminants. Further research should be focused on the removal rate for other contaminants such as phenol derivates and the availability of nitrogen and phosphorous for the growth of microalgae and bacteria. Optimization and careful control of these factors are essential for efficient biological treatment the using microalga-bacteria consortia. .

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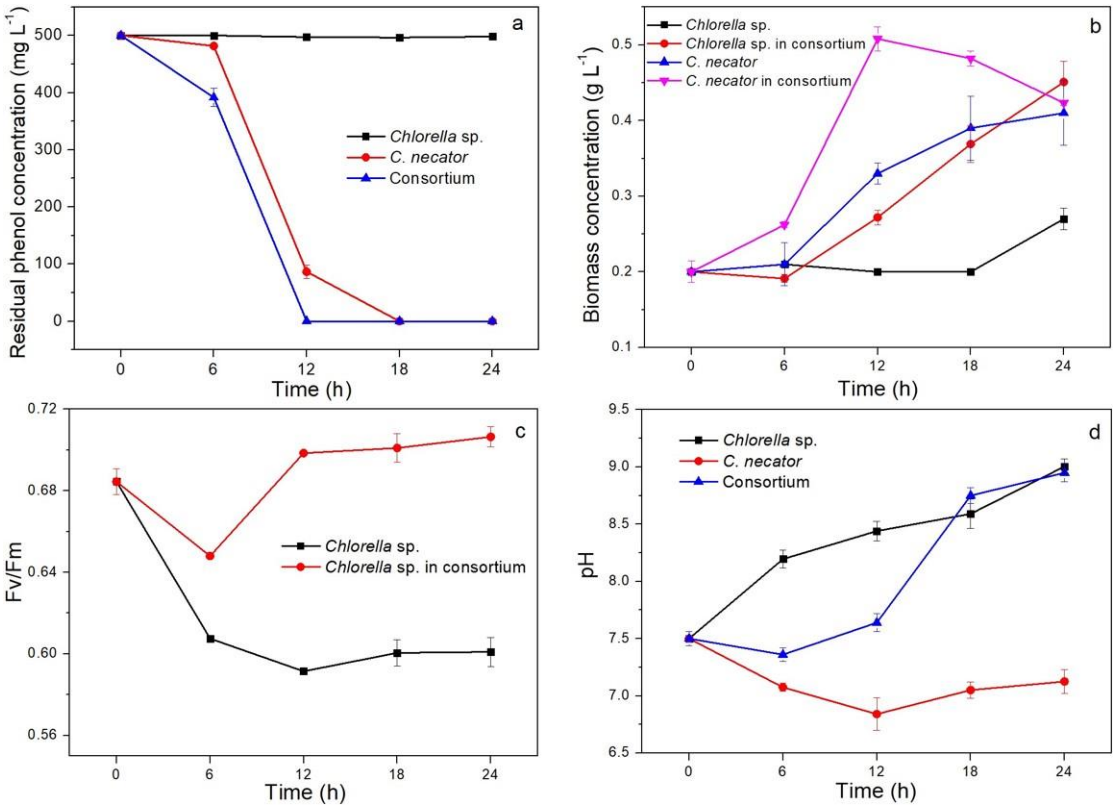


Fig. 1. Comparison of the residual phenol concentration (a), biomass concentration (b), Fv/Fm (c) and pH (d) of *Chlorella* sp., *C. necator* and their consortium.

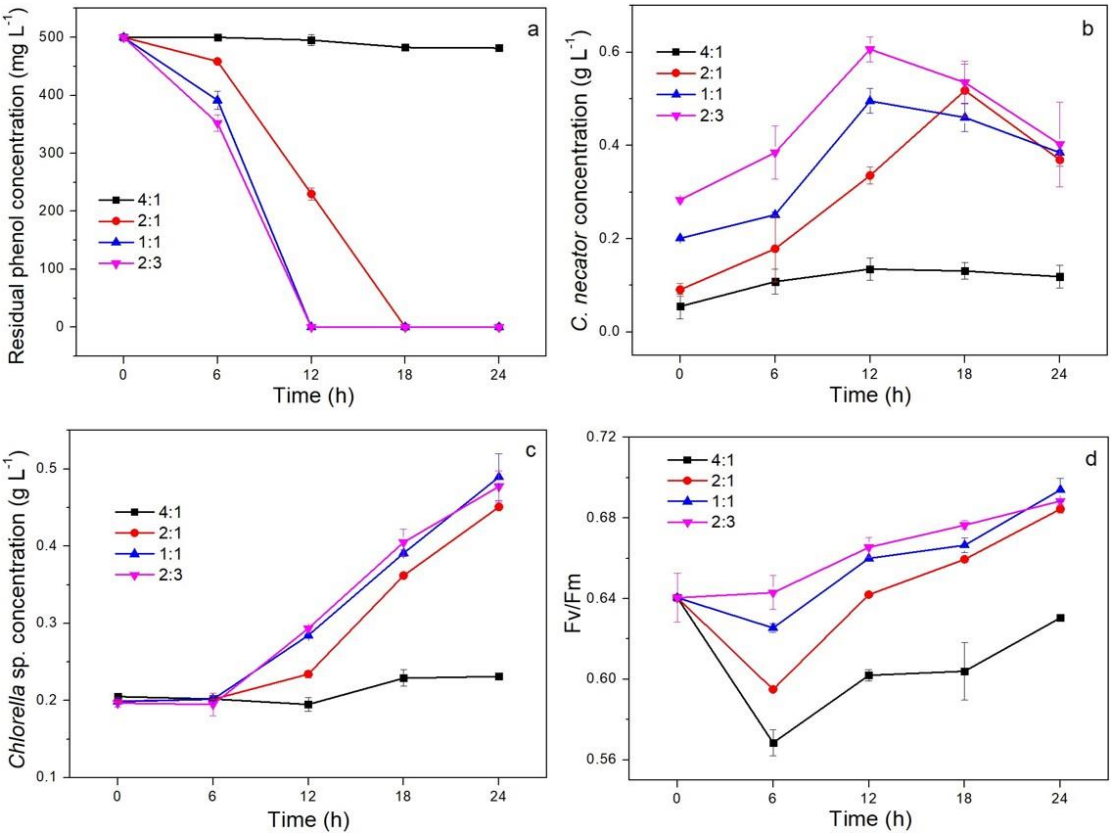
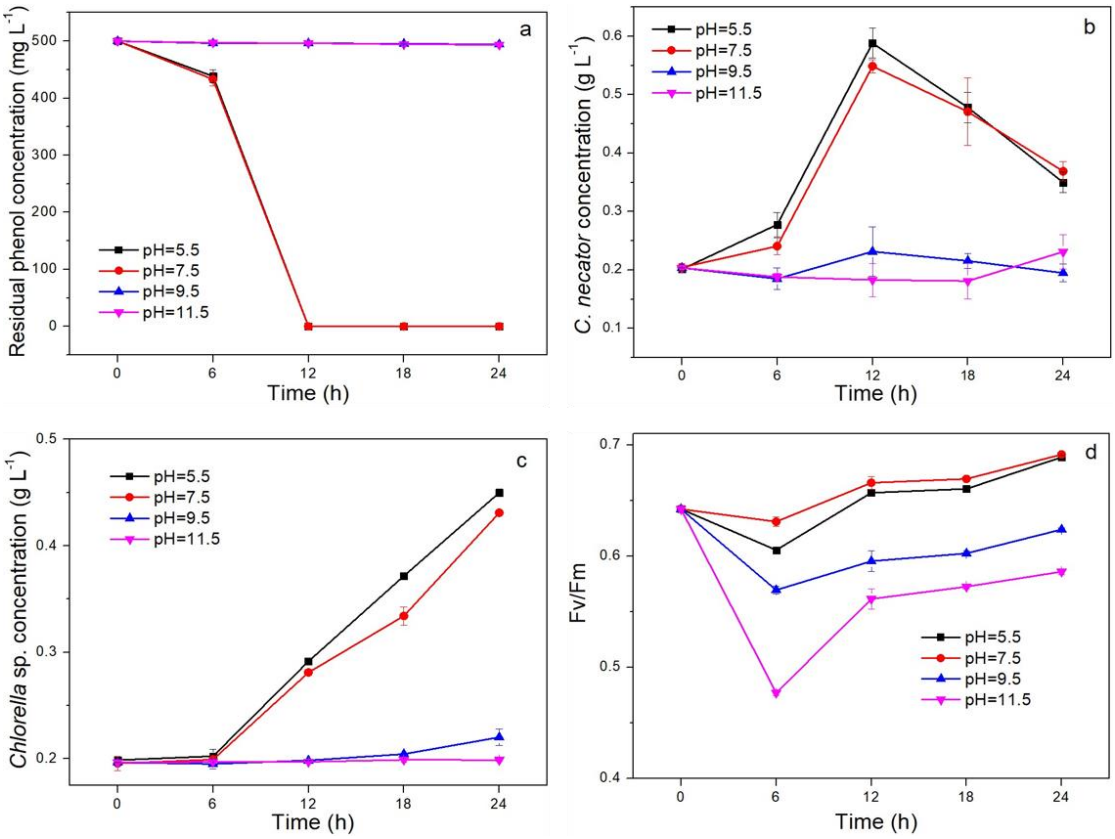


Fig. 2. Effect of *Chlorella* sp./*C. necator* ratio on the residual phenol concentration (a), *C. necator* (b) and *Chlorella* sp. (c) biomass concentration and Fv/Fm (d) of consortium under 25 ℃, 110 μmol m^{-2} s^{-1}, pH 7.5 and 500 mg L^{-1} phenol conditions.



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635 **Fig. 3.** Effect of initial pH on the residual phenol concentration (a), *C. necator* (b) and *Chlorella* sp.
636 (c) biomass concentration and Fv/Fm (d) of consortium under 0.2 g L^{-1} *Chlorella* sp., 0.2 g L^{-1} *C.*
637 *necator*, 25 ℃, 110 μmol m^{-2} s^{-1} and 500 mg L^{-1} phenol conditions.

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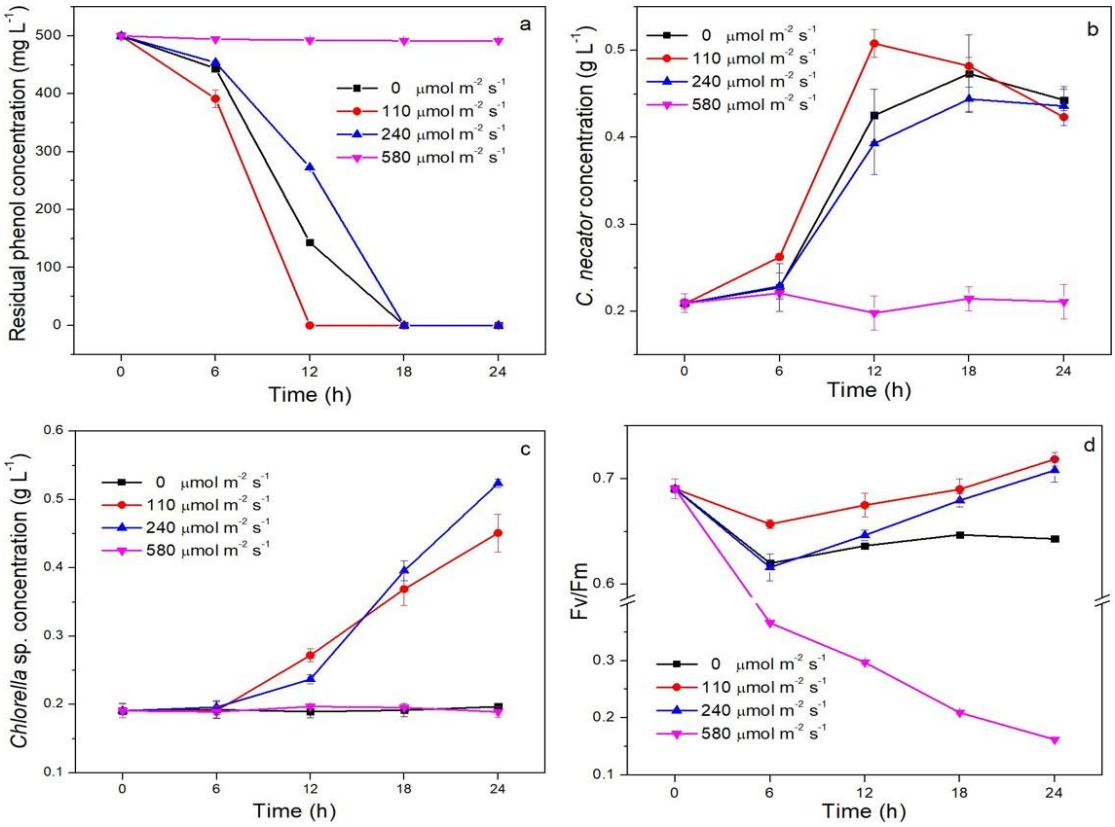


Fig. 4. Effect of light intensity on the residual phenol concentration (a), *C. necator* (b) and *Chlorella* sp. (c) biomass concentration and Fv/Fm (d) of consortium under 0.2 g L^{-1} *Chlorella* sp., 0.2 g L^{-1} *C. necator*, pH 7.5, 25 ℃ and 500 mg L^{-1} phenol conditions.

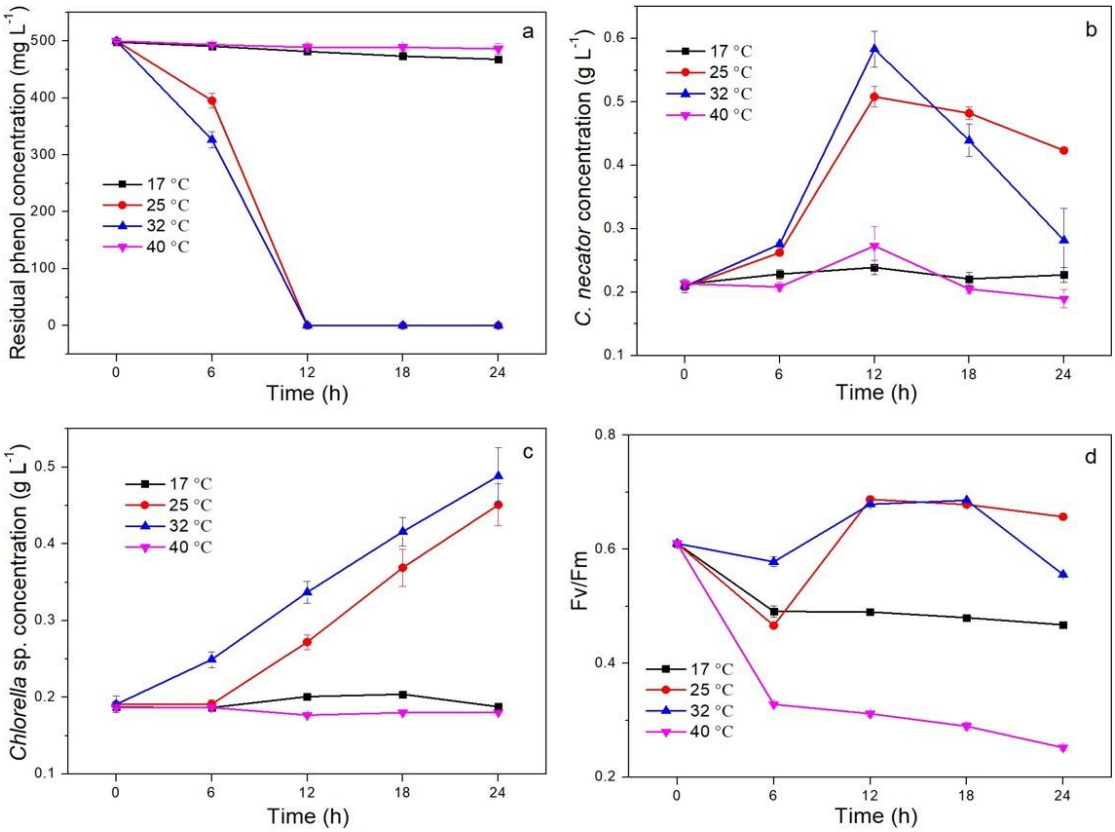


Fig. 5. Effect of temperature on the residual phenol concentration (a), *C. necator* (b) and *Chlorella*

sp. (c) biomass concentration and Fv/Fm (d) of consortium under 0.2 g L^{-1} *Chlorella* sp., 0.2 g L^{-1} *C. necator*, pH 7.5, 110 μmol m^{-2} s^{-1} and 500 mg L^{-1} phenol conditions.

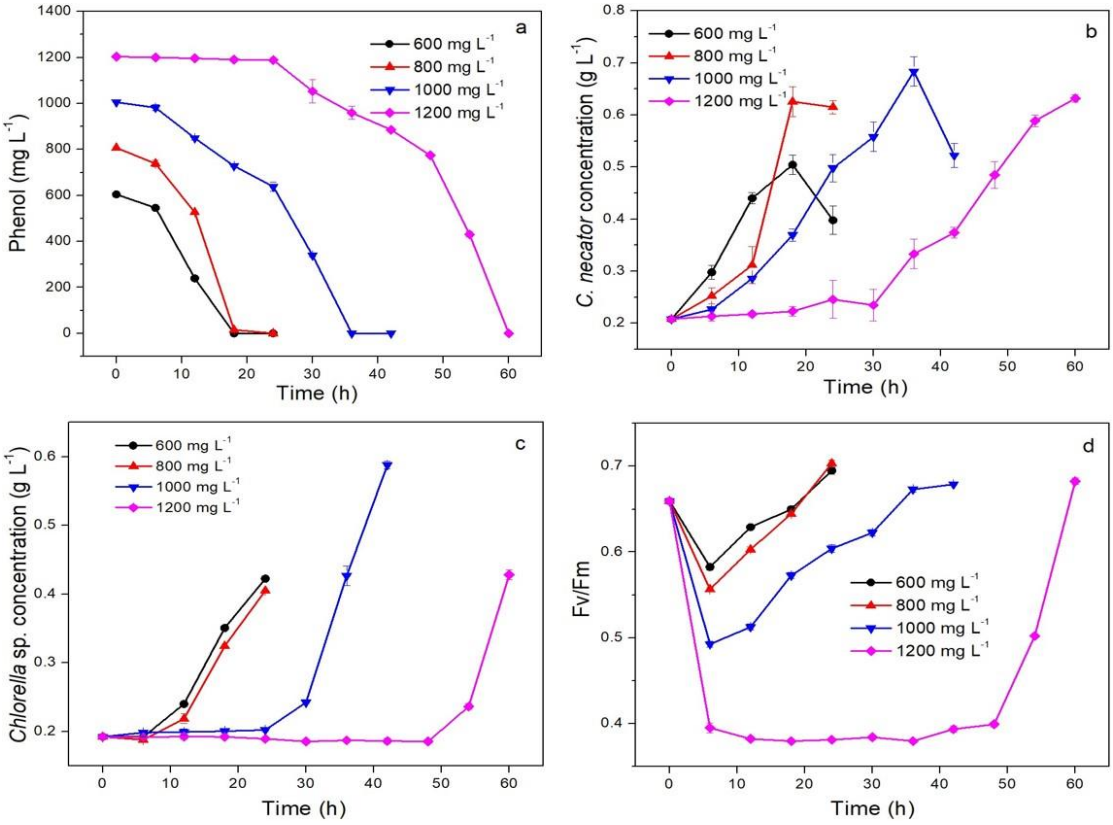


Fig. 6. Effect of phenol concentration on the residual phenol concentration (a), *C. necator* (b) and *Chlorella* sp. (c) biomass concentration and Fv/Fm (d) of consortium under 0.2 g L^{-1} *Chlorella* sp., 0.2 g L^{-1} *C. necator*, pH 7.5, 110 μmol m^{-2} s^{-1} and 25 ℃ conditions.

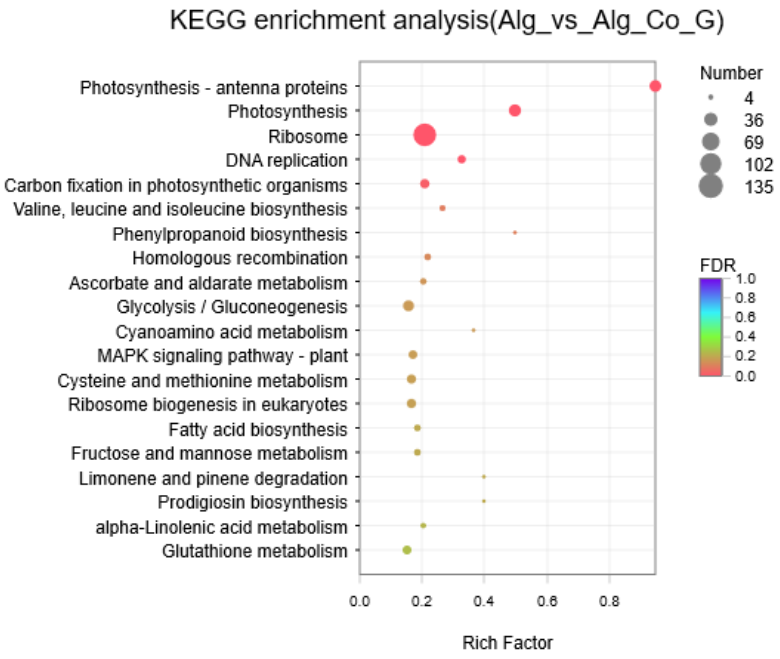
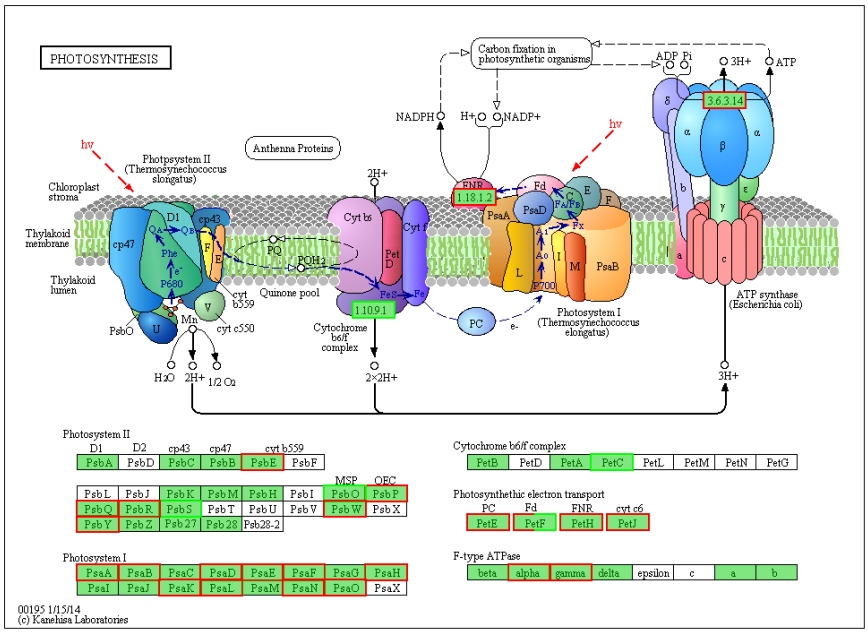


Fig. 7. KEGG pathway enrichment analyses of DEGs. The size of the point indicates the number of DEGs enriched in the pathway, and the redder the color, the more significant the enrichment result is. The different colors represent different FDR, correction of which is performed on the *P*-value.



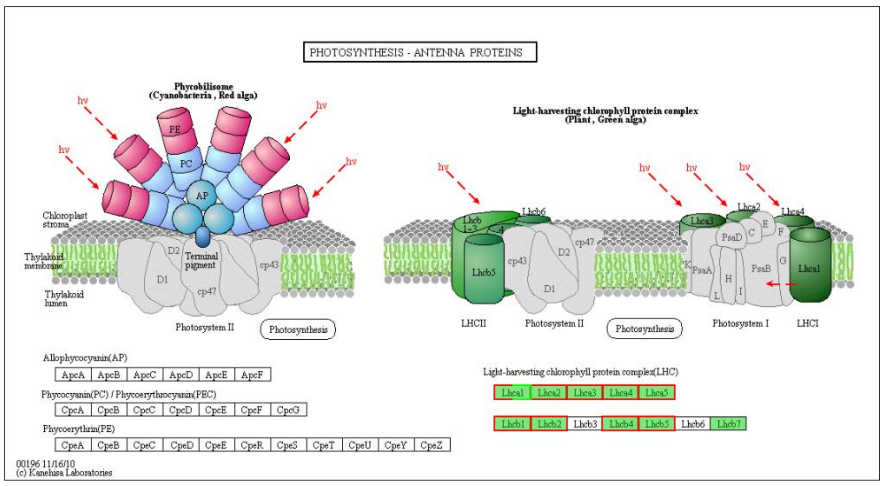


Fig. 8. Comparative transcriptomic analysis for the related genes of photosynthesis (a) and photosynthesis antenna protein (b) of *Chlorella* sp. monoculture (Alg) and *Chlorella* sp. -*C. necator* co-culture (Alg_Co). The red frame suggests up-regulated genes and the blue frame suggests down-regulated genes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

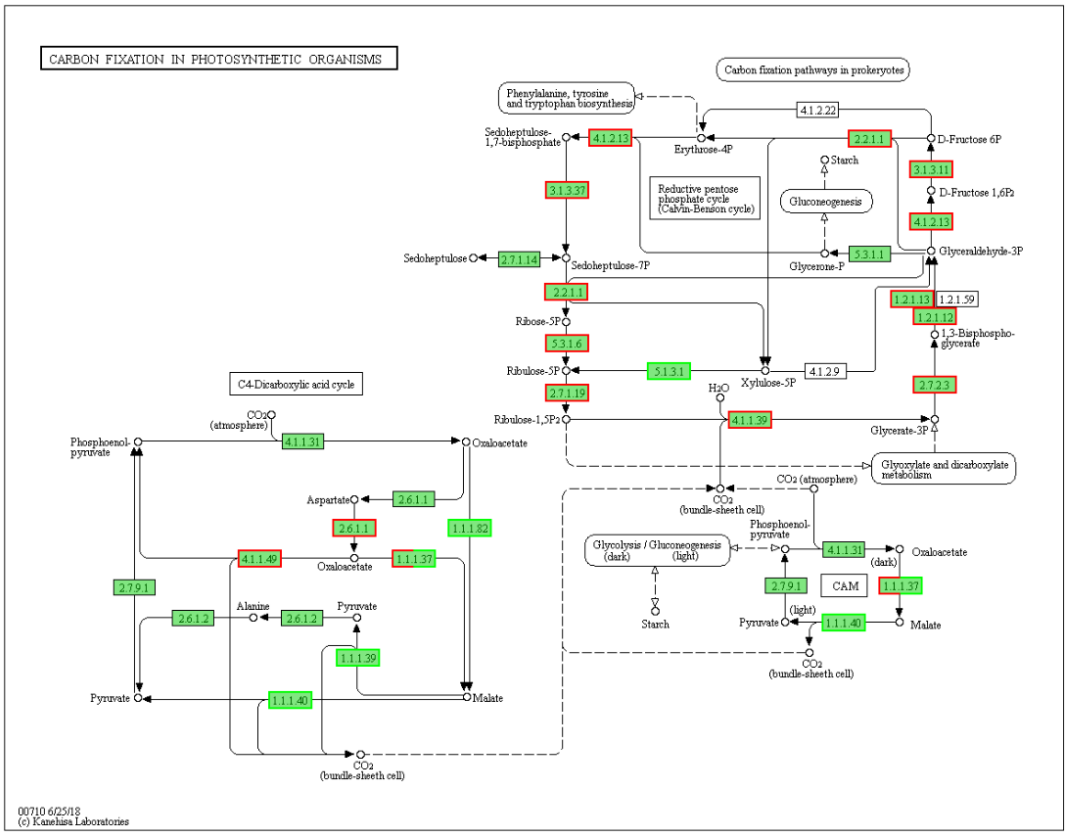


Fig. 9. Comparative transcriptomic analysis for the related genes of carbon fixation of *Chlorella* sp. monoculture (Alg) and *Chlorella* sp. -*C. necator* co-culture (Alg_Co). The red frame suggests up-regulated genes and the blue frame suggests down-regulated genes.

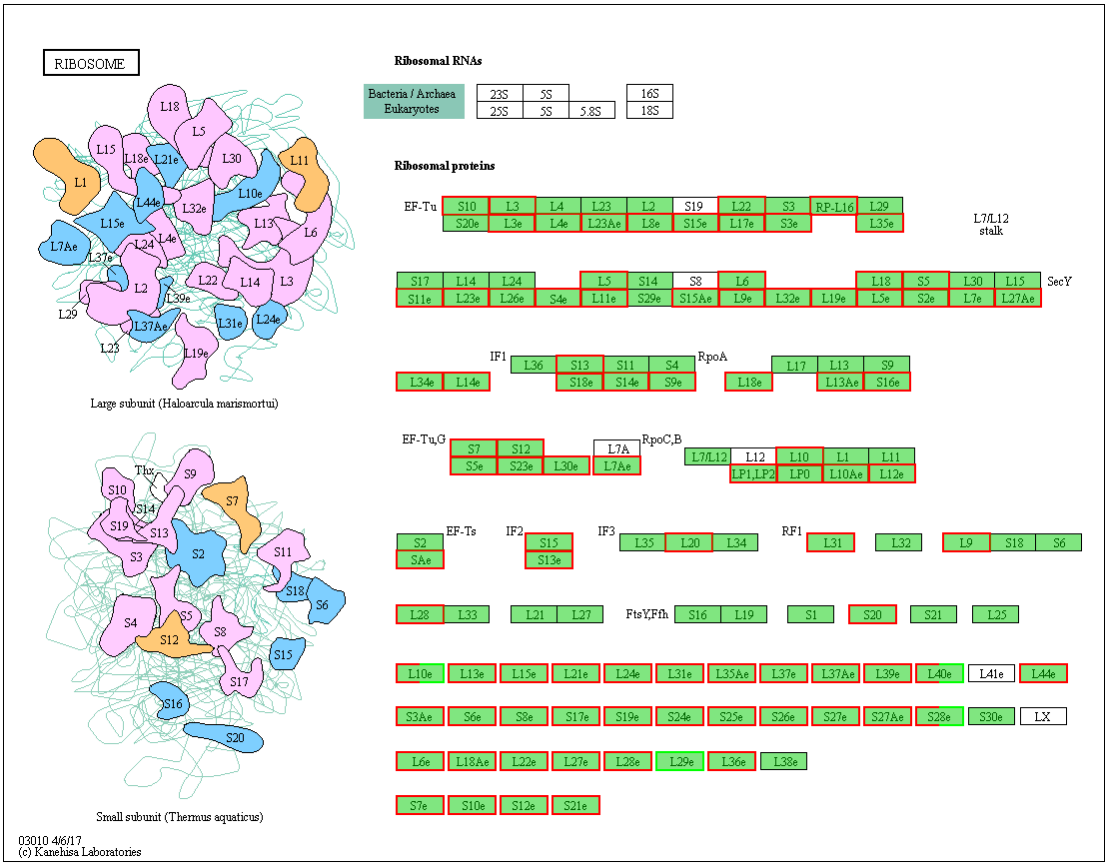


Fig. 10. Comparative transcriptomic analysis for the related genes of ribosome biosynthesis of *Chlorella* sp. monoculture (Alg) and *Chlorella* sp. -*C. necator* co-culture (Alg_Co). The red frame suggests up-regulated genes and the blue frame suggests down-regulated genes.

Table 1. The culture parameters of different operating conditions*

Operating conditions	C_{phenol} (mg L^{-1})	$C_{Chlorella\ sp.}$ (g L^{-1})	$C_{C.\ necator}$ (g L^{-1})	pH	Light intensity (μmol m^{-2} s^{-1})	Temperature (℃)
Chlorella sp./*C. necator* ratio	500	0.2	0.05, 0.1, 0.2, 0.3	7.5	110	25
pH	500	0.2	0.2	5.5, 7.5, 9.5, 11.5	110	25
Light intensity	500	0.2	0.2	7.5	0, 110, 240, 580	25
Temperature	500	0.2	0.2	7.5	110	17, 25, 32, 40

*C_{phenol}, $C_{Chlorella\ sp.}$, and $C_{C.\ necator}$ are the initial concentrations of phenol, *Chlorella* sp. and *C. necator*, respectively.