

Functional assembly of surface microbiota of *Ulva fasciata* improves nutrient absorption efficiency and growth

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Abstract

Macroalgae growth depends on biologically available nitrogen, such as ammonium and nitrate. Therefore, nitrogen is the most common growth limiting factor of macroalgae. However, it is unclear how the surface microorganisms of marine macroalgae promote nitrogen transformation and improve the utilization of nitrogen by macroalgae. In this study, 228 strains of bacteria were isolated from the surface of *U. fasciata*. The composition and structure of *U. fasciata* surface epiphytes under different nitrogen concentrations were determined by high throughput sequencing. Network analysis was used to identify highly correlated strains and predict possible functions. The bacterial strains of microbiota were identified based on the presence of nitrogen-fixing genes involved in the production of growth hormones and the appearance of dissolving P genes. The characteristics of promoting plant growth and increasing plant biomass accumulation were further determined. The ability of beneficial microbial combinations to assist in the acquisition of N/P nutrients was tested using synthetic communities (SynCom). Finally, the expression levels of several key genes were used to elucidate the effect of SynCom on the nutrient acquisition of *U. fasciata*.

Introduction

As the base of the Marine food chain, algae support a variety of benthic animals. In addition to essential carbon dioxide and water, algae also require nutrients for growth [1]. Long time evolution endows algae with the ability to live under various abiotic environmental conditions. For example, a group of large green algae *U. fasciata* grows rapidly in eutrophic waters and significantly increases biomass [2]. However, *U. fasciata* breeding in large quantities can have a great negative impact on the coastal environment, such as severely damaging the balance of coastal ecosystem, affecting air-sea exchange, and even having long-term impact on the marine biogeochemical cycle [3]. It is important to note that the growth and morphogenesis of algal species depend on their interaction with specific related bacteria [4–5].

To cope with the high fluctuation of nutrients in the ocean, algae have evolved a number of survival strategies to regulate the acquisition of nutrients [6]. At the same time, algae related microorganisms have evolved ways to regulate nutrition [7]. Although the relationship between algae and microbes remains unclear, the intimate relationship between algae and microbes can be dated back to the emergence of ecosystems. The compounds secreted by marine algae can attract bacteria to colonize and provide suitable habitat for these bacterial communities. In addition, compounds released by bacteria are beneficial to the growth and morphogenesis of their host [8]. Co-culture studies have found that polysaccharides can enhance the relationship between algae and bacteria [9]. The interaction between microorganisms and algae affects the nutrition of algae. Among them, the acquisition of nitrogen and phosphorus usually involves epiphytic microorganisms on the surface of algae [10]. The structure and composition of microorganisms on the surface of algae are closely related to the utilization of plant nutrients [11]. A large number of studies have shown that plant-related

microorganisms play an important role in host nutritional relationships [12]. However, due to the complexity of community structure, the importance of beneficial bacteria to the host is still unclear.

Under natural conditions, the interaction between microorganisms and hosts may be mutualism and/or competition. The complex interaction between them not only affects the composition of the community, but also determines the remodeling of the community structure. Changes in composition and structure of microorganisms affect the interaction between plants and specific microbial members. Therefore, we can regulate the beneficial growth of certain aspects of plants by increasing the number of functional microbiota in the community [13]. On the other hand, it can reduce the number of functional microorganisms in the community to inhibit the growth of target functions of host plants, and solve environmental problems such as green tide. Therefore, Understanding the functional bacteria that play a role in plant epiphytic microorganisms is of great significance to the ecological environment.

Therefore, in order to determine the function of the isolates, *U. fasciata* were co cultured with and without its pure strains of bacteria isolated from the surface of *U. fasciata*. Our results suggested that *U. fasciata* mold and recombine epiphytic bacteria can adapt to a wide range of nitrogen concentrations through the interaction between bacteria. In addition, we also found that the availability of nitrogen in *U. fasciata* depends on epiphytic bacteria, which they work together to absorb nitrogenous nutrients and synthesize compounds necessary for plant growth. The results of this study can fill in the knowledge gap of finding functional strains on the surface of *U. fasciata* that really play a role under nitrogen nutrient drive. It lays a foundation for revealing plant-microbiome interactions and possibly promoting plant growth.

Materials and methods

Sample Collection and *Ulva fasciata* Cultivation

Ulva fasciata is a common wild species in southern China. In November 2018, a large number of *U. fasciata* were collected and transferred to the laboratory. The algae were washed several times with filtered seawater and placed in a light incubator containing artificial seawater [14] with 50 mmol/L ($\text{NO}_3^-:\text{NH}_4^+ = 2:1$) nitrogen concentration. *U. fasciata* was acclimated in artificial seawater for one week. The environmental parameters were as follows: temperature = 20 ± 1 °C, light intensity = 800 lux (photoperiod L:D was 12h:12h), salinity = 30‰, and pH = 8.0.

Then 20 g of *U. fasciata* were cultivated in nine closed plastic incubators of 28 x 27 x 18 cm. The nitrogen concentrations of each group in the three incubators were 10 (low concentration), 50 (control), and 200 mmol/L (high concentration). During the culture period, the artificial seawater was replaced every two days. The experiment lasted for two weeks in the same light incubator as the above conditions. Two grams of *U. fasciata* were sampled to determine the contents of soluble protein, soluble sugar, phycocyanin and Chlorophyll a [15]. At the same time, seawater was taken to measure pH, salinity,

dissolved oxygen, PO_4^{2-} , NO_2^- , NH_4^+ , NO_3^- , DSi_2^+ , and NO_3^- [16]. Samples were collected at 7-time points (0 day, 2 days, 4 days, 6 days, 8 days, 10 days and 12 days) and stored at -80°C for DNA extraction.

DNA Extraction and 16S rRNA Sequencing

U. fasciata was kept in a 50-mL centrifuge tube, and then an appropriate amount of sterile seawater was added. The samples were treated with ultrasonic (1 min, 50 kHz, 5s/5s) to isolate bacteria from the surface of *U. fasciata*. After removing the *U. fasciata* with sterile tweezers, the sample was centrifuged at 4000 g for 10 min to collect cell pellet, which was then used for bacterial DNA extraction. Total genomic DNA was extracted using the CTAB method [17]. High-quality DNA was amplified by PCR performed in a PRISM® 7500 rapid real-time PCR system as described by [18]. The extracted DNA was sent to Shanghai Meiji Company (Shanghai, China) for high-throughput 16S rRNA measurement and epiphyte determination. Illumina MiSeq sequencing and universal primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') were used to amplify the V3-V4 region of bacterial 16S rRNA gene.

Bioinformatic Analysis

Quantitative Insights into Microbial Ecology (QIIME, version 1.9.1) was used to de-duplicate and qualify-filter the raw FASTQ files, classify the sequences, as well as annotate and calculate beta diversity distance. MOTHUR software (version 1.30.2) was used to analyze the microbial alpha diversity. The filtered sequences were grouped into Operational Taxonomic Units (OTUs) clustered with a 97% similarity cutoff using UPARSE software (version 7.0.1001). The RDP Classifier (version 2.11) and 16S rRNA database was employed to analyze the taxonomy of each 16S rRNA gene sequence using a confidence threshold of 97%. Principal coordinate analysis (PCoA) was performed on the Weighted UniFrac distances in QIIME to study the similarity or difference in the composition of the sample community and to visualize the distance matrix. Statistical analysis was performed using Microsoft Excel. Redundancy Analysis (RDA) was used to summarize the changes of different environmental factors and epiphyte under different nitrogen concentrations. The network was analyzed with NetworkX software [19] under Spearman association coefficient and absolute value ≥ 0.5 and Spearman's rank were calculated between species to reflect correlations between them. Shannon index was calculated to describe the α diversity and the richness of the epiphyte microbiota. Different graphicss were drawn using different software (such as R language, QIIME, and MOTHUR). After CRL conversion, the test was statistic analyzed by ANCOM and used to determine whether there were significant differences in the abundance of bacterial categories. ANCOM is a method of interpreting compositional constraints to reduce false findings when detecting differentially rich taxa at the ecosystem level, while maintaining high statistical power.

Taxonomic identification and phylogenetic analysis of potentially beneficial microorganismsT

The surface of *U. fasciata* was gently scraped with a sterile knife, and the scraped materials were gradually diluted and spread on the marine agar plate 2216E, and placed in an incubator at 25°C for 3–4

days. Single colonies were selected, streaked, purified, and inoculated in marine broth 2216E. PCR amplification of the 16S rDNA gene of these strains from the surface of *U. fasciata* was performed using primers 16S-27F (3'-AGAGTTTGATCCTGGCTCAG-5') and 16S-1492R (3'-TACGGCTACCTTGTTACGACTT-5') [20]. Then the samples were sent to Bioengineering (Shanghai) for sequencing. These strains were cultured on marine broth 2216E to logarithmic growth phase. The bacterial suspension was mixed with 40% sterilized glycerol in the ratio of 1:1. A total of 228 pure strains were isolated from the surface of *U. fasciata*. The phylogenetic trees of Rhodobacteraceae and Flavobacteriaceae were constructed by Neighbor-Joining method in MEGA 7.0.

Screening potentially beneficial microbes

Specifically, the N₂ fixed marker gene (*nifH*) was confirmed by PCR assays using primer pairs: *nifH*-F (AAAGGYGGWATCGGYAARTCCACCAC) and *nifH*-R (TTGTTSGCSGCRTACATSGCCATCAT) [21]. Since IAA regulates plant growth and development, we also tested the indoleacetic acid production capacity of the strains [22]. Salkowski colorimetric method was used to screen for IAA production in strains according to the scheme proposed by Bric et al. [23]. The strains were cultured in 50 mL of 2216 E at 25°C for 3 days, and the bacterial culture medium was centrifuged at 10000 rpm at 4°C for 10 min. The cell pellet was washed twice with PBS and supplemented with 1 g/L of L-tryptophan, and then oscillated in an oscillating incubator for 7 days. After centrifugation, 1 mL of the supernatant was added to 2 mL of Salkowski reagent (1 mL of 0.5 mol/L FeCl₃ and 49 mL of 35% HClO₄). The mixture was incubated in the dark at 25°C for 30 min and the absorbance was measured at 530 nm using a spectrophotometer. The concentration of IAA was estimated using the standard IAA curve. All IAA measurements were made in triplicate. In order to determine the solubility activity of phosphorus, using Ca₃(PO₄)₂ as insoluble inorganic phosphate source, Rhodobacteraceae and Flavobacteriaceae family members that could activate and fix phosphate were screened. The strain was incubated at 25°C for 3 days, and then the phosphorus soluble ability was screened on solid NBRIP medium. In a 250-mL conical flask, 50 mL of NBRIP medium was mixed with 100 µL of strain culture at neutral initial pH, then incubated for 7 days in a shaker at 25°C, and repeated inoculation for three times. The culture was centrifuged at 5000 RPM for 20 min. The precipitation of P₂O₅ was determined by molybdenum-blue method [24].

Construction of SynCom

The changes of bacterial abundance on the surface of *U. fasciata* under different nitrogen concentrations and the important nodes in the network indicated that Rhodobacteraceae and Flavobacteriaceae may be potential bacterial strains to improve nutrient absorption efficiency and growth of *U. fasciata*. Therefore, 29 strains of Rhodobacteraceae and Flavobacteriaceae were combined into SynCom1. Based on the above functional screening, five strains with *nifH* gene, high phosphorus solubilization capacity, and high IAA yield were selected from 29 strains as candidates of SynCom2. SynCom was co-cultured with sterilized *U. fasciata* without bacteria on the surface under no nitrogen.

Co-culture Experiments without N addition

The preparation method of *U. fasciata* without surface bacteria (WSB *U. fasciata*) was as follows: *U. fasciata* was collected from the field and transported back to the laboratory in the same day. The surface of *U. fasciata* was washed three times with sterile seawater and cleaned with ultrasonic (10 min, 50 W, 5s/5s) to remove bacteria. Antibiotics, including streptomycin sulfate (final concentration of 100 ug/mL), amphotericin (0.25 ug/mL), ampicillin (100 ug/mL), and imidazole (0.1 ug/mL), were added and incubated for 4 days, and the same amount of antibiotics was added again on the third day. Next, a sterile cotton swab was dipped in multi-enzyme cleaning solution (3M) and quickly scrub the surface of *U. fasciata*. Then *U. fasciata* was rinsed with a large volume of sterile seawater to obtain a WSB *U. fasciata*. The water from the last rinse was applied to the solid medium 2216E and cultured for one week. The absence of bacteria meant that *U. fasciata* was without surface bacteria. SynCom strain was activated at 25°C. After 3 days of activation, the cell pellet was collected by centrifugation (at 4000 xg) and washed twice with sterile seawater. It was then resuspended to a concentration of 10^7 strains per mL. After 1:1 mixing, the final volume of each group of SynCom was 5 mL. The mixed bacteria were added into an airtight bottle and co-cultured with WSB *U. fasciata* at artificial seawater without nitrogen addition. The treatment of WSB *U. fasciata* without adding bacteria was treated as control. On the last day of the experiment (7 days), the co-cultured *U. fasciata* was washed with a large amount of sterile seawater, the surface strains of *U. fasciata* were obtained by ultrasonic shock, and coated on marine medium 2216E. The growth of the strain on the medium was the same as that initially added, indicating that the co-culture is unpolluted. During the experiment, each SynCom group was guaranteed to repeat at least three times.

SynCom functional evaluation

To evaluate the effect of SynCom on the growth of *U. fasciata*, *U. fasciata* with similar growth and size were obtained from the same batch by multi-spot sampling method. The surface moisture was absorbed with sterile filter paper and weighed (wet weight). Three of the *U. fasciata* were placed in an oven at 60 °C until constant weight (dry weight), the remaining six *U. fasciata* were sterilized for co-culture experiment. After SynCom and WSB *U. fasciata* were co-cultured for one week, the wet weight and dry weight of *U. fasciata* were measured. The contents of soluble sugar, soluble protein, phycocyanin, and Chlorophyll a were determined. The concentration of N in the *U. fasciata* was determined according to Wetzal et al. [25]. The concentration of phosphorus was determined by phosphomolybdenum blue spectrophotometry with a UV-vis spectrophotometer. The control was cultured with sterile seawater.

Activity of glutamine synthetase (GS)

About 0.5 g *U. fasciata* sample (wet weight) was homogenized in liquid nitrogen with 5 mL of 0.2 M HEPES buffer, pH 7.9. The homogenate was centrifuged for 20 min, and the supernatant was used to determine GS activity by the method of Slawky & Rodier [26].

U.fasciata RNA Extraction and RT-qPCR of some Key Genes

Total RNA of co-cultured *U. fasciata* was extracted by Trizol method under low nitrogen conditions [27]. The culture of uninoculated WSB *U. fasciata* was used as a negative control. DNA enzyme was used to

remove possible DNA, and gel electrophoresis was used to analyze the selection of DNA-free RNA. RNA quantification was performed with a spectrophotometer, and RNA was used to synthesize the first strand of cDNA. The analysis was performed in 96-well plate using SYBR Green method, *U. fasciata* β -actin gene as an internal control. The effect of synthetic community (SynCom) on *U. fasciata* at nitrogen-free concentration was confirmed by quantifying the expression levels of three key genes involved in photosynthesis (*rbcL*), lipid biosynthesis (*accD*), and growth hormone precursor (*wrkY*) pathways. The primer sequences used in this study are listed in Supplementary TAB S1. RT-qPCR reaction was carried out as follows: 95°C for 30 s, followed by 40 cycles of 95°C, /10 s, 60°C, /30 s. Each reaction was carried out in triplicate, and the formula $2^{-\Delta\Delta CT}$ was used to calculate the RT-qPCR amplification data.

Results

Changes of bacteria community composition during *U. fasciata* cultivation

A total of 2,741,338 high-quality sequences were obtained from *U. fasciata* samples (n = 54). The relative abundance of bacterial communities was analyzed at the genus level and Chi-Square test statistical analysis were used. (Fig. 1). The results showed that the bacterial community changed dramatically during the culture at low nitrogen concentration. The abundance of the genera *Granulosicoccus* (0.5–5.1%, $P < 0.01$), *Maribacter* (0.4–1.4%, $P < 0.01$) and *Neptunomonas* (0.27–1.1%, $P < 0.01$), as well as members of the families Rhodobacteraceae (5.8–13.0%, $P < 0.01$), Methyloiligellaceae (0.6–7.0%, $P < 0.01$), and Flavobacteriaceae (0.8–3.9%, $P < 0.01$) were increased significantly during the growth of *U. fasciata*; while those of *Pseudomonas* (29.4–2.2%, $P < 0.01$), *Achromobacter* (24.6–2.0%, $P < 0.01$) and *Stenotrophomonas* (8.1 – 0.06%, $P < 0.01$) were decreased. When *U. fasciata* was cultured at high N concentration, the abundance of *Granulosicoccus* (2.7–8.2%, $P < 0.01$), *Maribacter* (0.9–16.8%, $P < 0.01$), *Neptunomonas* (3.1–5.2%, $P < 0.01$), and genera of Methyloiligellaceae (0.8–3.5%, $P < 0.01$) and Flavobacteriaceae (0.7–2.2%, $P < 0.01$) were increased; while those of *Pseudomonas* (25.6–1.6%, $P < 0.01$), *Achromobacter* (21.7–2.0%, $P < 0.01$), and one genera of Rhodobacteraceae (7.5–5.8%, $P < 0.01$) were decreased. In addition, the abundance of Rhodobacteraceae was increased during the culture of *U. fasciata* with the low nitrogen concentration but decreased at high N concentration. In addition, the ANCOM statistic analysis showed that not only the common nitrogen-fixing bacteria (*Methylophaga*) and the relative abundance of Flavobacteriaceae (*Cellulophaga* and *Mesonina*) and Rhodobacteraceae (*Pseudophaeobacter*) significantly different under different nitrogen concentrations (Fig. 2). Moreover, PCoA analysis based on Bray-Curtis distance showed that high concentration of nitrogen had a greater effect on the bacteria community than low concentration of nitrogen (Fig. 3). Shannon index demonstrated that the diversity of bacterial community harboring the surface of *U. fasciata* cultured at low nitrogen concentration was similar to that at high concentration (Table 1).

Figure 2 Used the ANCOM statistical analysis to calculate the relative abundance of different bacterial species at phylum and genus levels with different nitrogen concentration.

Environmental factors affect bacteria community

Environmental factor and microbiota RDA analysis were performed to determine the factors affecting the changes of the bacterial community during the growth of *U. fasciata* (Fig. 4). The results showed that the arrows representing NO_3^- were the longest compared with other influencing factors, that NO_3^- had a greater influence on the surface microbiota of *U. fasciata* cultured under different nitrogen concentrations. In this experiment, it was also found that DSi_2^+ had a greater impact on most microbiota. The results showed that nitrogen played an important role in changes of bacteria community.

The connection between various bacteria

In order to understand the relationship among bacterial communities during the growth of *U. fasciata*, the network was constructed. The nodes in the network were the top 13 species of *U. fasciata* surface bacteria with the highest total abundance (Fig. 5). The clustering coefficients of Rhodobacteraceae and Flavobacteriaceae in the network analysis were 0.76 and 0.50, respectively. The members of these families were determined as key nodes in the network analysis. Although this was not the highest in network analysis, based on the changes of bacterial abundance under different nitrogen concentrations (Fig. 1) and the species of pure bacteria were obtained [13]. Therefore, we choose Rhodobacteraceae and Flavobacteriaceae as the basis of synchronization, and their evolutionary relationship is shown in Fig. 6.

Functional screening of *U. fasciata* surface-associated beneficial microbes

As mentioned above, Rhodobacteraceae and Flavobacteriaceae were selected as potential nitrogen-fixing strains. A total of 29 strains belonging to the families Rhodobacteraceae and Flavobacteriaceae were isolated from the surface of *U. fasciata*. Among them, 12 strains have *nifH*, 15 strains produce IAA, and 10 strains acquire Pi from insoluble phosphate (Pi) (Table 2). Some strains have two or three of these potential functions. A total of five strains (including *Ruegeria atlantica* 11, *Donghicola eburneus* 5–35, *Aliiroseovarius crassostreae* Y11, *Hyunsoonleella ulvae* HU1-3, and *Croceivirga radialis* 6) with high IAA and Pi productivity and the presence of *nifH* genes were selected. And four of them (removing *H.ulvae* HU1-3) were combined into the SynCom2 and co-cultured with WSB *U. fasciata* to determine the effect of nitrogen fixation and plant growth promotion.

Evaluation of functional assemblages of microbial community

In SynComs, it is generally believed that the combined function of several microbial strains is more effective than that of a single strain [28]. Two SynComs systems were structured, the SynCom1 was based on the potential functions of members of Rhodobacteraceae and Flavobacteriaceae; whereas, the SynCom2 was established using four strains with beneficial functions. These SynComs were first co-cultured with WSB *U. fasciata* to test the physiological parameters, including soluble sugar, soluble protein, phycocyanin and chlorophyll a, wet weight, dry weight, and nitrogen and phosphorus contents. The results showed that compared with control, the changes of soluble sugar, soluble protein,

phycocyanin and Chlorophyll a, wet weight, dry weight, and nitrogen and phosphorus contents was – 8.4%-10.1%, 1.6%-16.8%, 23.9%-49.2%, -6.7%-18.3%, -6.0%-5.1%, -6.6%-10.1%, -11.7%-7.5% and – 4.4%-3.3%, respectively (Fig. 7A-H). In addition, two SynComs significantly increased glutamine synthesis activity, which was almost undetectable in the control group (Fig. 8A). The results showed that SynCom2 performed better than SynCom1 in facilitating the N and P acquisition, while SynCom2 could promote the growth of *U. fasciata* and increase the nitrogen fixation. Another thing to explain is that after the SynCom1 experiment, the surface bacteria were removed by ultrasound and coated on a flat plate. Due to the different growth of strains, the species of the strain cannot be determined. All 4 strains of SynCom2 could be found on the plate.

Figure 7 Changes of four physiological indices, N content, P content, dry weight and wet weight of sterile *U. fasciata* with two SynComs. **(A)** Phycocyanin **(B)** Chlorophyll a **(C)** Soluble protein **(D)** Soluble sugar **(E)** N content **(F)** P content **(G)** Dry weight **(H)** Wet weight.

Key genes regulation of transcription in *U. fasciata* by SynCom2

Since SynCom2 was highly correlated with the growth of *U. fasciata*, the *U. fasciata* co-cultured with SynCom2 was further analyzed by RT-qPCR to investigate the underlying mechanisms of several key genes associated with nitrogen fixation and plant growth. The expression levels of three key genes involved in photosynthesis (*rbcL*), lipid biosynthesis (*accD*), and growth hormone precursor (*wrkY*) pathway were determined by adding *U. fasciata* with SynCom2 in nitrogen-free conditions. Compared with the control group, SynCom2 gene expression increased in varying degrees. The expression of *RbcL*, *accD*, and *wrkY* was increased by 1.04-, 11.21-, and 9.54-fold, respectively.

Discussion

Nitrogen (N) is a key element of life and a limiting factor of marine productivity in many parts of the global ocean [29]. N exists in the ocean in different forms, including ammonium (NH_4^+) and nitrate (NO_3^-). In recent years, study on microbial communities in the marine N cycling has received great attention [30–31]. In this study, the surface bacteria species of *U. fasciata* under different nitrogen concentrations were determined by high throughput sequencing technology. The data indicated that the microbiota on the surface of *U. fasciata* changed with the growth time and stabilized at the late growth stage (Fig. 1). Under normal condition, nitrogen has a positive effect on Flavobacteriaceae, Thiotrichaceae, Rhodobacteraceae, Nitrospiraceae, Methylobacteriaceae, and other bacteria [32–34]. Since these bacteria are nitrogen-fixing communities, it is not surprising that the relative abundance of these bacteria increased when cultivated under low nitrogen concentration. Consistent with previous studies that the members of Rhodobacteraceae are nitrogen-fixing bacteria that involved in the physiology and health of the host [35], and the number of these bacteria decreased at the high nitrogen concentrations. Previous studies have shown that the composition of epiphytic microorganisms from algae is mainly affected by ambient environmental factors [36–37]. According to the results of PCoA analysis and RDA

analysis, our data also showed that nitrogen concentration in the environment had a great impact on the surface bacterial community of *U. fasciata* (Fig. 3 and Fig. 4).

Core microbiome is defined as a group of shared members of microbial communities from similar habitats [38]. Therefore, the discovery of core microbiome is important to understand the composition of complex microbial assemblages. In this study, the bacterial taxa with the highest abundance was defined as the core flora the core microbiota. Under the growth conditions of different nitrogen concentrations, the surface core microbiome of *U. fasciata* is consistent, including *Pseudomonas*, *Achromobacter*, a genus of Rhodobacteraceae, *Granulosicoccus*, a genus of Methylophilaceae, *Maribacter*, *Neptunomonas*, a genus of Flavobacteriaceae, *Stenotrophomonas*, *Leucothrix*. By characterizing *Ulva* related epiphytic bacteria (Chlorophyta), Lachnit et al. [39] demonstrated that each algae contains species-specific and time-adapted bacterial communities on its surface. As proposed by Marzinelli et al. [40], both biological and abiotic factors affect bacterial communities associated with macroalgae.

Network analysis was used to determine the interaction between strains [41–42]. In our results, some genera of the Rhodobacteraceae family were important nodes. Among them, the members of Rhodobacteraceae were positively correlated with other genera (red line). Due to the strong positive correlation with other genera, Rhodobacteraceae and Flavobacteriaceae family members were selected as strains with potential functions.

Recent studies have shown that plant-associated microbiota can influence the disease resistance, nutritional status, and growth rates of their host [43]. The isolation of these beneficial microorganisms is of great important to promote the growth of plants, including algae. Obviously, *nifH* gene encodes diazase, which is involved in the production and regulation of nitrogenase in the process of N_2 fixation [44]. Hence, microorganisms carrying *nifH* gene could be used as candidate bacteria for nitrogen fixation in plants. This study identified potential growth-promoting strains by obtaining microorganisms that capable of phosphorus acquisition.

Through the above screening methods, 5 of the 29 strains were identified to have high IAA and Pi production, and contain the *nifH* gene. The specific effects of the SynCom2 of these four of these strains on the growth of *U. fasciata* were tested by co-culture experiment. Interestingly, *H. ulvae* HU1-3 alone can significantly promoted *U. fasciata* biomass accumulation in another study [45] but SynCom of five strains including HU1-3 did not increase the biomass. The SynCom strains of 29 strains had almost the same effect as the 28 strains after removing *H. ulvae* HU1-3 (results not shown), We selected a mixture of 29 strains (SynCom1) as positive control to demonstrate the effectiveness of the synthetic method. These four bacterias significantly increased the contents of soluble sugar, soluble protein, phycocyanin and Chlorophyll a, wet weight, dry weight, nitrogen, phosphorus, and other physiological parameters of *U. fasciata* (Fig. 7A-H). The results indicated that the four bacterial strains had health benefits to the *U. fasciata*. The experimental results also proved that high-throughput sequencing was first used to predict potential functional bacteria, and then joint functional screening was an effective method to identify

potential beneficial microorganisms. It should be noted that other potentially beneficial functions, such as Acc deaminase activity and siderophore production should also be focused on [46].

In the construction of SynComs, the function of each strain was considered. The results showed that SynCom2 based on these functions can significantly improve plant growth and nitrogen and phosphorus acquisition than SynCom1. The combination of SynComs functions adopted in this study is an effective strategy to promote plant growth and nutrient acquisition.

Then, we carried out RT-qPCR experiments using SynCom2 to observe whether the expression of some key genes was significantly affected by the colonization of the microbial community (Fig. 8B). It has been shown that *rbcL* gene is associated with chlorophyll content [47], *accD* gene regulates the activity of acetyl-CoA carboxylase and involves in the fatty acid biosynthesis pathway [48], while *wrkY* genes regulates plant development and may produce auxin precursors, which is ultimately benefit the plant growth [49]. The effect of SynCom2 on *U. fasciata* growth was further verified by quantifying the expression of key genes involved in photosynthesis (*rbcL*), lipid biosynthesis (*accD*) and plant development regulators (*wrkY*). In addition, the results of this study found that IAA genes were activated by SynCom2. These findings indicated that SynCom2 not only improve the photosynthetic capacity of *U. fasciata* leaves, but also activate plant growth promotion signals and help to increase bioaccumulation. However, how SynCom2 affects the changes of *U. fasciata* needs to be further studied. The above results indicated that there was a close genetic relationship between *U. fasciata* and its epiphytic microorganism, and the microbes in SynCom2 regulated the transcription of genes involved in the growth of the host.

Conclusion

Overall, this study determined the species and abundance of epiphytes on the surface of *U. fasciata* under different nitrogen concentrations, and then predicted the correlation between strains through network analysis. Members of the families Rhodobacteraceae and Flavobacteriaceae were selected as potential functional bacteria, and SynCom system was constructed through functional screening assays. The results showed that the functional assembly of SynCom2 could promote the growth of *U. fasciata* and increase bioaccumulation. These results were confirmed by high-throughput prediction and selection of strains based on the prediction results, which indicated that SynCom is an effective method to identify the beneficial strains to plant growth.

Declarations

Authors' contributions H W designed the study and wrote the manuscript. D L performed the experiments and data acquisition. C L and Z H contributed to the treatment of the raw data. H W and Z H contributed to the data analysis.

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Data availability Bacterial 16S rRNA gene sequencing data were uploaded to the NCBI SRA database, with accession PRJNA772012.

Ethics approval and consent to participate This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to Participate All authors agree to participate.

Consent to Publish All authors consent to publish.

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

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Tables

Tables are available in the Supplementary Files section.

Figures

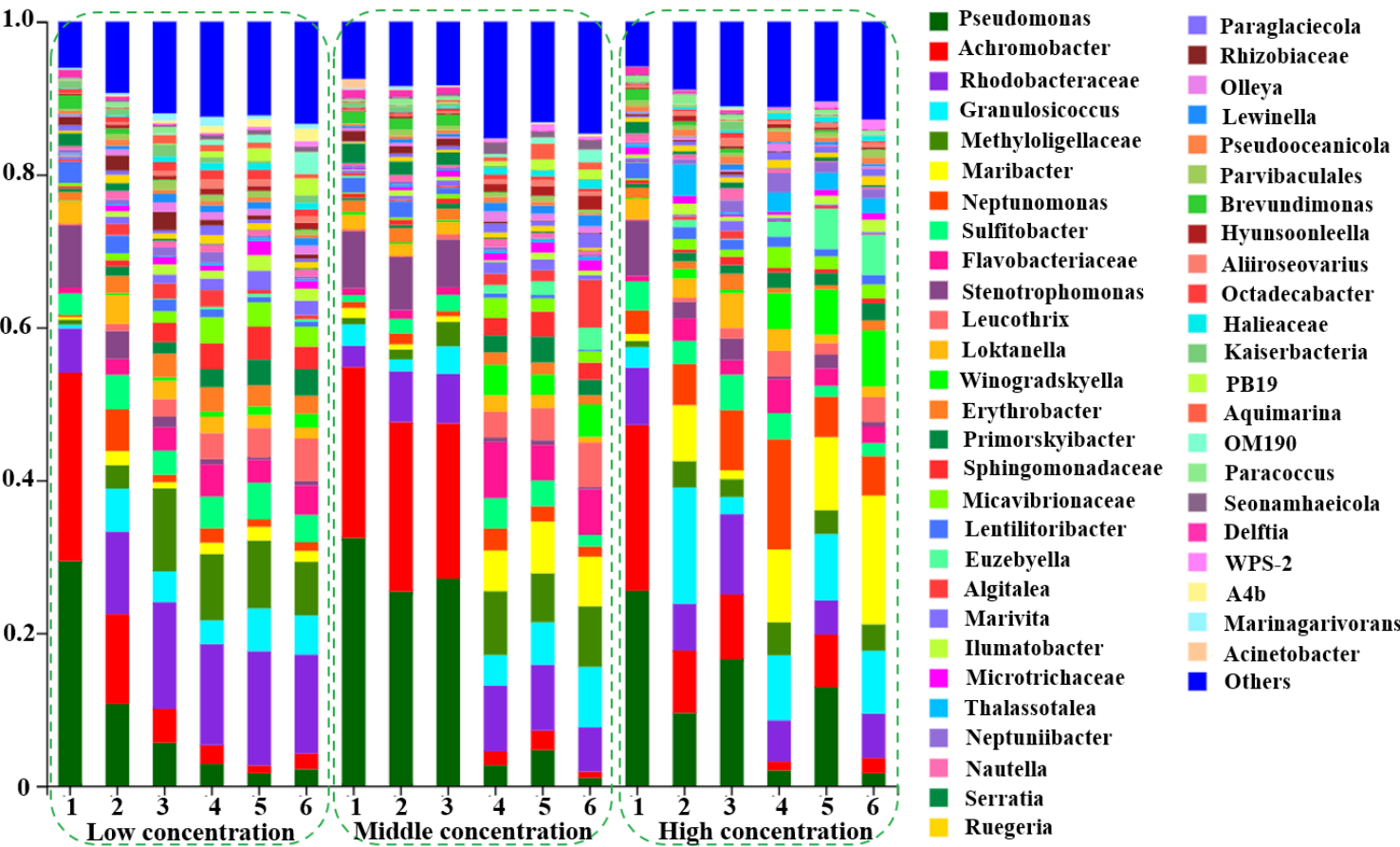


Figure 1

Changes of genus level in surface community of *Ulva* under different nitrogen concentration.

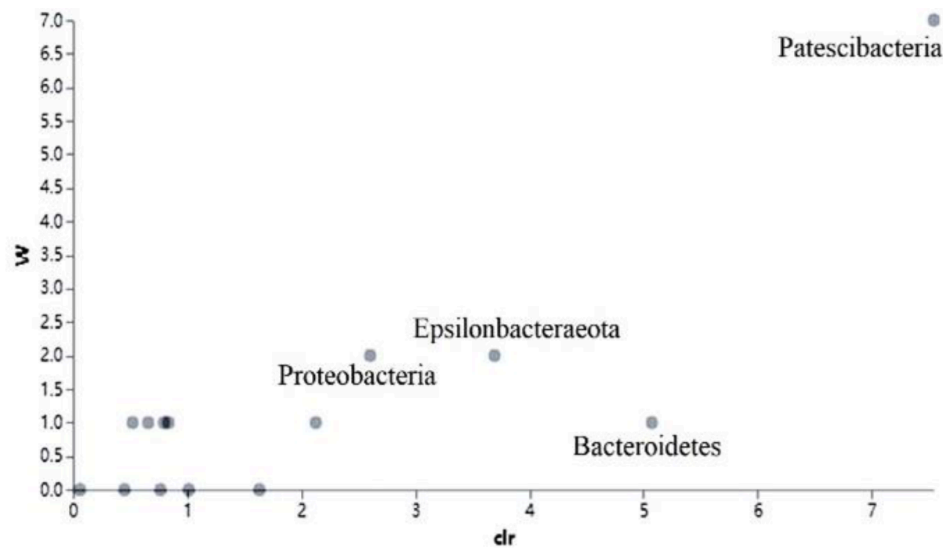


Fig.2(A)

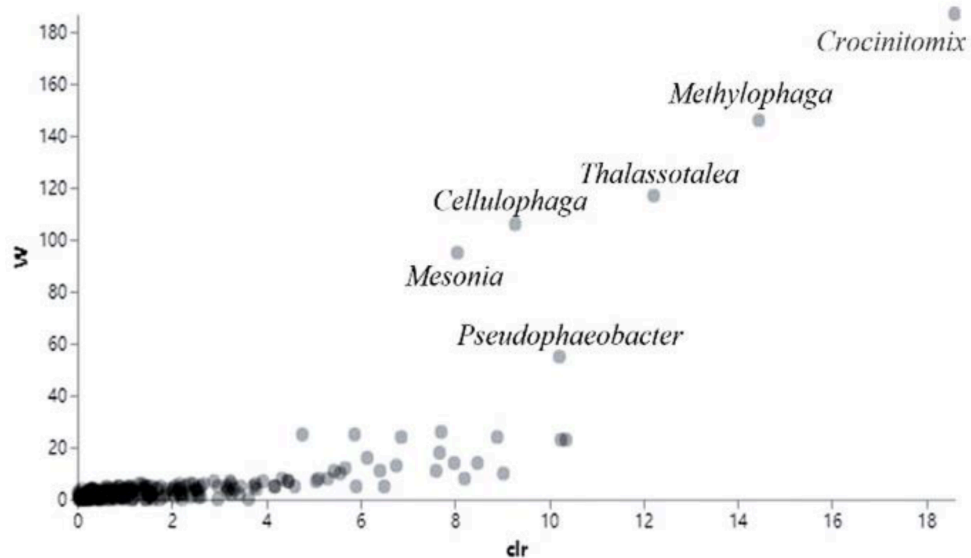


Fig.2(B)

Fig.2 Used the ANCOM statistical analysis to calculate the relative abundance of different bacterial species at phylum and genus levels with different nitrogen concentration.

Figure 2

Used the ANCOM statistical analysis to calculate the relative abundance of different bacterial species at phylum and genus levels with different nitrogen concentration.

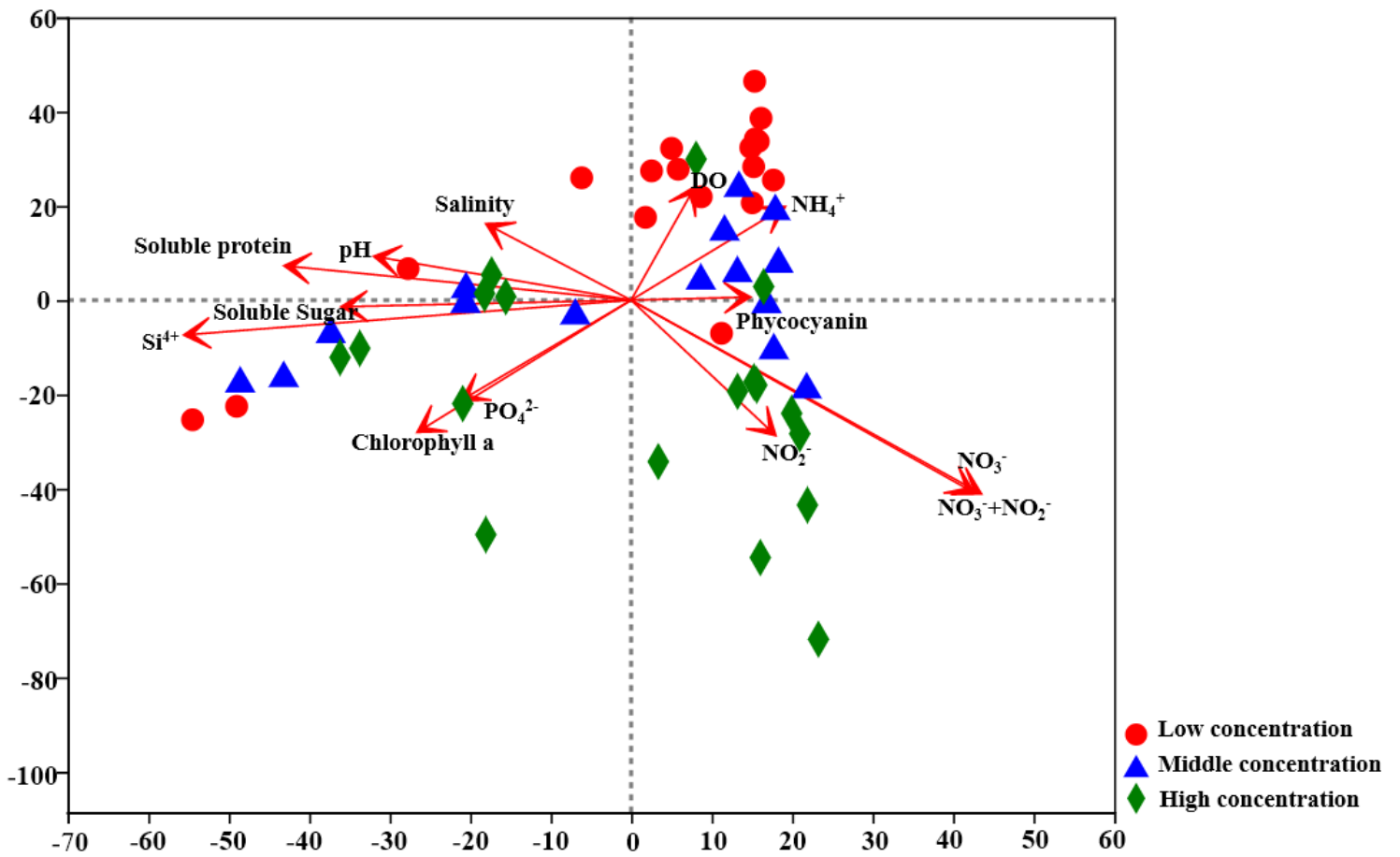


Figure 4

RDA analysis of environmental factors and bacterial genera under different nitrogen concentration.

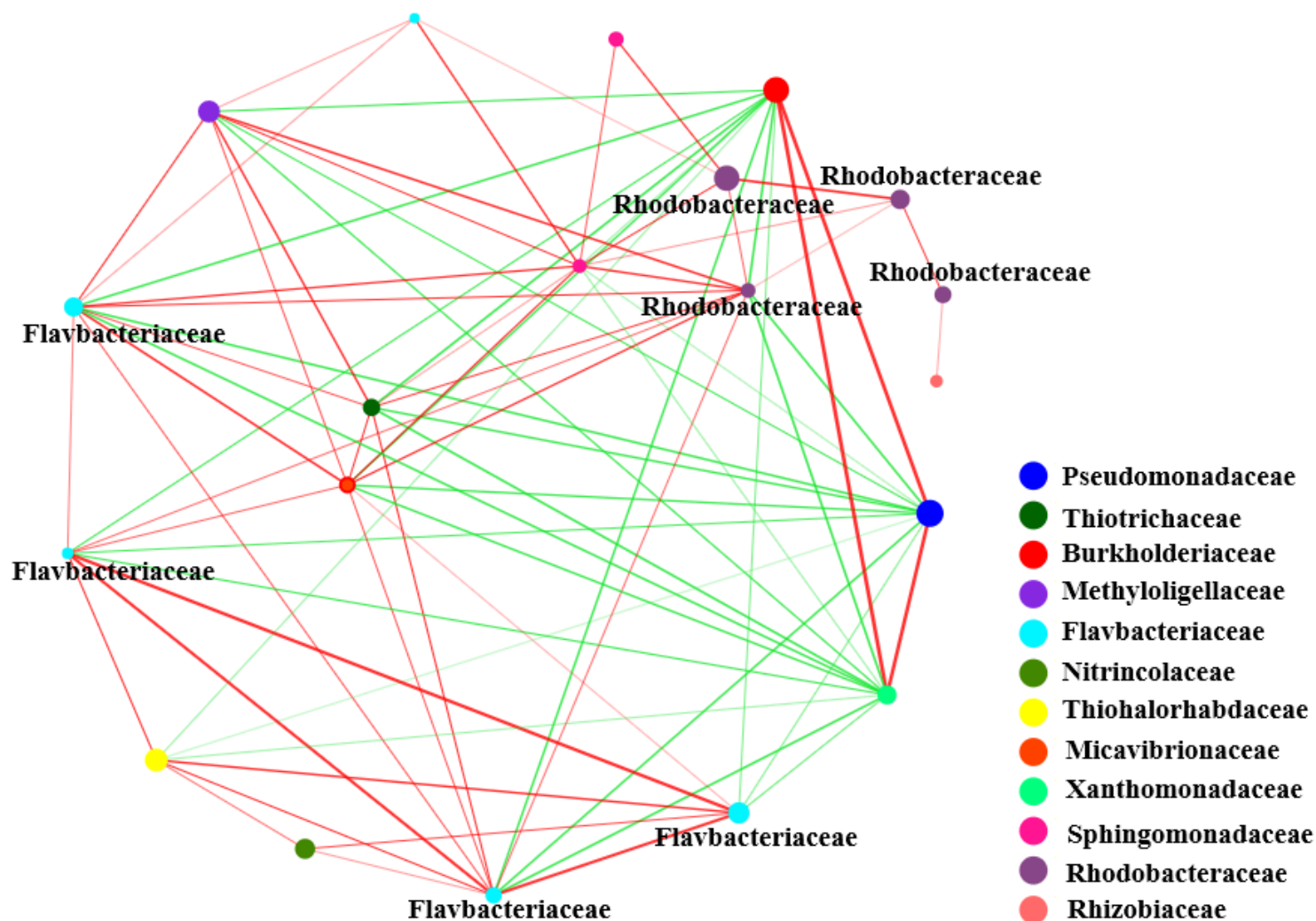


Figure 5

Correlation network diagram of bacterial genus levels in samples treated with different nitrogen concentration.

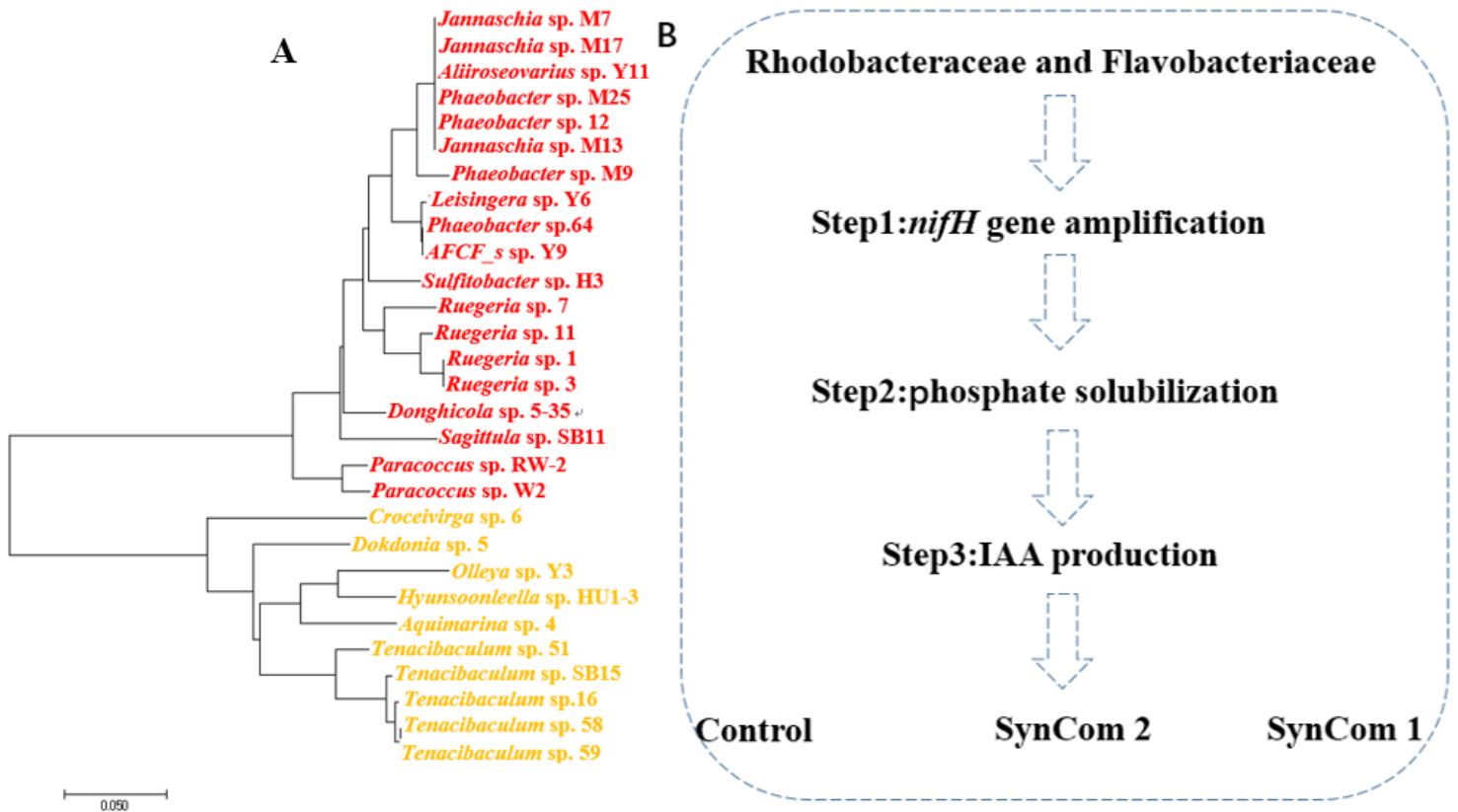


Figure 6

Rhodobacteraceae and Flavobacteriaceae stains **(A)**phylogenetic analysis and **(B)**functional screening of strains.

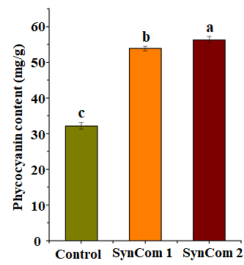


Fig.7(A)

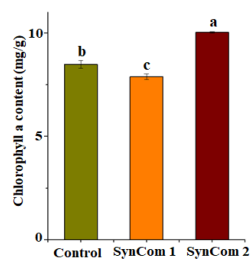


Fig.7(B)

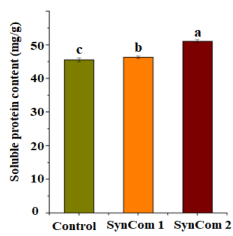


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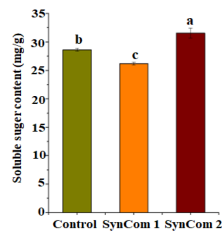


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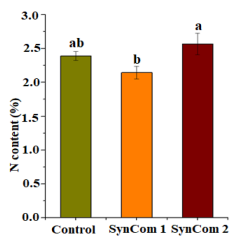


Fig.7(E)

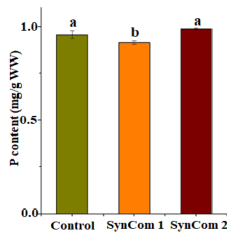


Fig.7(F)

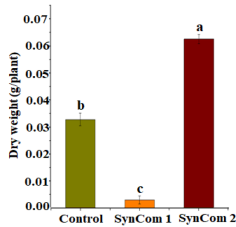


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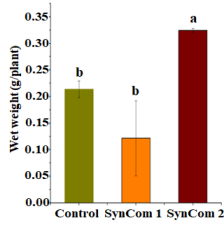


Fig.7(H)

Figure 7

Changes of four physiological indices, N content, P content, dry weight and wet weight of sterile *U. fasciata* with two SynComs. **(A)** Phycocyanin **(B)** Chlorophyll a **(C)** Soluble protein **(D)** Soluble sugar **(E)** N content **(F)** P content **(G)** Dry weight **(H)** Wet weight.

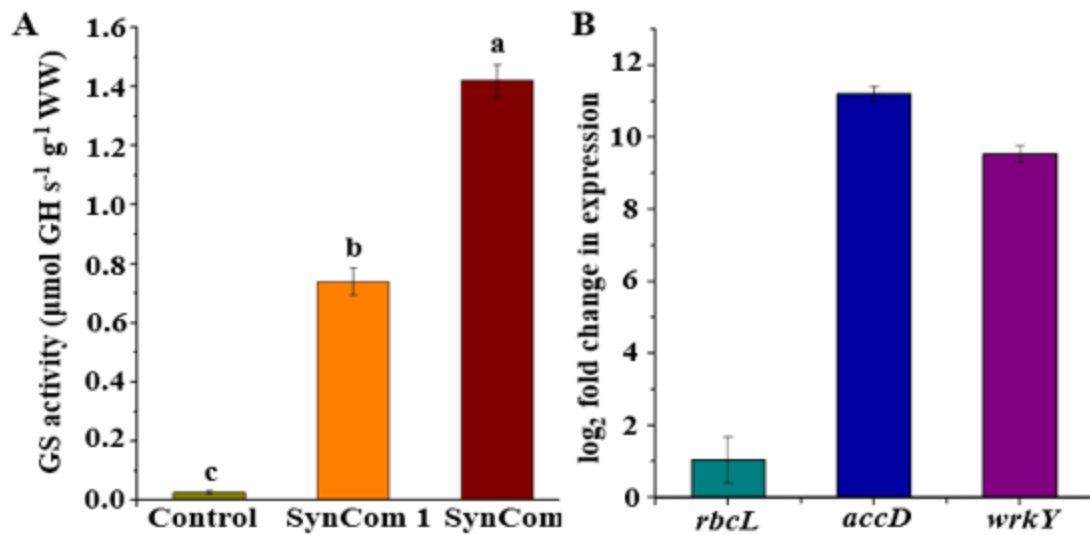


Figure 8

(A) Activity of glutamine synthetase with two SynComs and (B) *rbcL*, *accD* and *wrkY* gene expression multiples in SynCom2 compared with control.

Supplementary Files

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