Enhancing Diatom, *Cyclotella meneghiniana* Growth Using Growth-Promoting Bacteria Isolated From the Phycosphere of Chlorophytes and Chrysophytes

NUR AMIRAH IZYAN NOOR MAZLI[1], FATIMAH MD YUSOFF[2,3,*], MUHAMMAD FARHAN NAZARUDIN[1], YAM SIM KHAW[1], HUI TENG TAN[1], MURNI KARIM[1,2,3]

[1]Laboratory of Aquatic Animal Health and Therapeutics, Institute of Bioscience, Universiti Putra Malaysia, UPM Serdang, Selangor, Malaysia

[2]Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia, UPM Serdang, Selangor, Malaysia

[3]International Institute of Aquaculture and Aquatic Sciences, Universiti Putra Malaysia, Port Dickson, Negeri Sembilan, Malaysia

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Abstract

The relationship between microalgae and bacteria in a microenvironment, the phycosphere, has a significant role in enhancing the quality and quantity of microalgal production, which would in turn affect consumers' growth and nutritional quality, such as the zooplankton, which are important live feeds in aquaculture. Thus, selecting and characterising suitable microalgal growth-promoting bacteria (MGPB) for enhancing microalgal production is an important process since not all bacteria promote high growth. In this study, physcosphere bacteria associated with chlorophytes and chrysophytes were isolated, screened for their microalgae-promoting attributes (phosphorus solubilisation, indole-3-acetic acid (IAA) production, and nitrogen fixation) and re-inoculated into microalgal cultures. A total of seven bacterial isolates were recorded to have multiple growth promoting traits, with three strains (CY-2, CY-4, CY-5) showing the greatest ($P < 0.05$) link for those traits. These seven potential MGPB were molecularly characterised using 16S rRNA approach. The phylogenetic tree of the isolated bacteria demonstrated the dominant bacteria associated with the chlorophytes were in the class bacteroidetes, while the chrysophytes appeared to be associated with Firmicutes bacteria suggesting that the compositions were strictly species-specific to the microalgae host. Enhanced *Cyclotella meneghiniana* growth by the seven isolated bacterial strains was highly dependent on the growth-promoting traits; especially those demonstrated by *Pseudomonas hibiscicola* and *Ochrobactrum haematophilum*. These two bacteria showed the potential to enhance the quality of microalgae, and they could be bioencapsulated and used to improve the quality of zooplankton as one of the main live feeds in the aquaculture industry.

Keywords: aquaculture, microalgae, phosphate solubilisation, indole-3-acetic acid, nitrogen fixation, interaction

Introduction

In recent years, the perspective of bacteria has shifted greatly – from organisms seen as mere contaminants and disease-causing agents to how they can benefit biotechnological innovations, such as bioremediation and fermentation technologies. Interactions between microalgae and bacteria commonly encompass various kinds of symbiotic partnerships, including mutualism, the biological relationship in which two or more parties from different species mutually facilitate each other's growth and development (Seyedsayamdost et al., 2011; Cooper and Smith, 2015; Ramanan et al., 2016). In general, microalgae release dissolved organic matter (DOM) that bacteria use as their energy source. Bacteria, in turn, remineralise these organic nutrients into inorganic forms, for microalgae to utilise. In the case of mutualism, a bacterium provides useful compounds such as vitamin B12 to a microalgal partner in return for fixed carbon (Croft et al., 2005; Helliwell et al., 2011).

The importance of the plant growth-promoting bacteria; rhizobacterium, such as *Azospirillum*, *Mesorhizobium*, and *Rhizobium* sp. in stimulating the growth of microalgae, for example, being the source of nitrogen in oligotrophic conditions is unquestionable and has been proven by many studies (Gonzalez and Bashan, 2000; Watanabe et al., 2005;

Hernandez et al., 2009; Kim et al., 2014). Similarly, by utilising heterotrophic bacteria, it was revealed that when certain microalgae are cultured within an engineered consortium of mutualistic bacteria, they can significantly increase the consortium's supply of fixed organic carbon, thus resulting in faster microalgal growth (Cho et al., 2015). Several other researchers have revealed the significant importance of heterotrophic bacteria in microalgal development and survival (Seyedsayamdost et al., 2011; Kim et al., 2014). Hernandez et al. (2009) and Choix et al. (2012) have reported the impacts of naturally occurring microalgae-associated bacteria in promoting microalgae growth and production. There is now huge interest in exploring the bacterial diversity inside the microalgal phycosphere ever since the term was first coined by Bell and Mitchell (1972) to characterise a region that exists around a microalgal cell or colony where microalgal extracellular products promote bacterial development (Lee et al., 2013, Kim et al., 2014, Cho et al., 2015, Ramanan et al., 2015).

In aquaculture, the relationship between microalgae and bacteria inside the phycosphere, has a substantial impact on the development of nutritional quality of the consumers in the aquatic food chain such as zooplankton, which are important live feeds in aquaculture. The selection of suitable microbial consortia might significantly improve aquaculture output, efficiency, and sustainability (Natrah et al., 2013). Manipulation of the phycosphere of high-value microalgae by the inclusion of selected beneficial bacterial strains may enhance microalgal biomass, by accelerating the growth rate and reducing the cultivation time. This allows for faster turnover of production cycles, increasing the overall productivity and thus reducing the production cost. Therefore, characterising bacteria with microalgal growth-promoting (MGP) attributes could enhance microalgal biotechnological processes (Lian et al., 2018).

Phosphorus is necessary for microalgal growth, lipid and fatty acid production, and metabolic activities such energy transfer, signal transduction, and photosynthesis (Atiku et al., 2016; Ota et al., 2016; Yang et al., 2018). Using phosphorus solubilising bacteria capable of breaking down insoluble phosphorus and releasing it into the environment for microalgae growth can be a suitable technique for improving microalgal cultivation processes. Nitrogen is another key macronutrient that supports microalgal growth and development, as well as the synthesis of the amino acids required to produce chlorophyll for photosynthetic activity (Grobbelaar, 2007). Inorganic nitrogen sources utilised in microalgal cultivation include nitrate, nitrite, and ammonium salts. Nitrogen fixing bacteria might be used to fix atmospheric nitrogen in the future, notably for outdoor mass microalgal culture. On the other hand, indole-3-acetic acid (IAA) is a key significant signalling molecule in microalgae and bacteria, in addition to plants and bacteria. IAA produced by *Azospirillum brasilense*, has been reported to promote the growth of the green microalgae, *Chlorella sorokiniana* (de-Bashan et al., 2008), implying that IAA-producing bacteria should be used in microalgae culture as well.

Cyclotella sp. is among the many important diatom species commonly utilised as live food source in aquaculture, and they are used in the diet of aquatic animals, due to their remarkable essential fatty acid compositions (Pahl et al., 2009; Cupo et al., 2021). Because of this, there have been attempts to boost *Cyclotella* sp. production in various ways (Jeffryes et al. 2013; Li et al. 2017; Cupo et al., 2021); and selecting and characterising bacteria that can promote its growth should be studied further. Furthermore, exploring *Cyclotella*–bacteria interactions to boost microalgal biomass production could never be considered a waste and will unquestionably have a high economic worth. Therefore, the present study focused on selecting phycosphere bacteria capable of promoting the growth of *Cyclotella meneghiniana*, to enhance its production for the aquaculture industry. Bacteria associated with chlorophytes and chrysophytes were screened for microalgal growth promoting traits and subjected to co-cultivation with *C. meneghiniana* to evaluate their effects on growth. This study found that selective phycosphere bacteria could enhance the growth and overall productivity of microalgae, and the bacteria-microalgae consortia have the potential to be bioencapsulated and used as a feed additive, to improve the quality of zooplankton as one of the main live feeds in the aquaculture industry. Highly nutritive microalgae and bacteria could improve both the growth and health of zooplankton.

Materials and Methods

Microalgal strains and cultivation condition

Xenic freshwater microalgal strain, *Cyclotella meneghiniana* (Isolate UPMC-A0070; Accession No. MK834579) was obtained from microalgae culture collection of the Laboratory of Aquatic Animal Health and Therapeutics, Institute of Bioscience, Universiti Putra Malaysia (UPM) and maintained in Bold's Basal Medium (BBM) (Nicols and Bold, 1965) at 20 °C under a light intensity of 100 µmol photons.m^{-2}.s^{-1} irradiation under a light cycle of 12 h light and 12 h dark.

Isolation of bacteria associated with microalgae

Bacteria were isolated from ten freshwater microalgae species of Chlorophyta and Chrysophyta cultured in the laboratory (Table 1). Microalgae were grown in BBM at 25 °C under a light intensity of 100 µmol photons.m^{-2}.s^{-1}. Irradiation and isolation were carried out at the late exponential phase to choose bacteria that coexist well with microalgae.

Table 1. Microalgae strains and their sources used to investigate growth-promoting bacteria.

Microalgal strain	Phylum	Classification	Isolate code	Accession number	Origin
Chlorella sp.	Chlorophyta	Trebouxiophyceae	UPMC-A0088	MT890044	Institute of Bioscience Pond, UPM
Chlamydomonas reinhardtii	Chlorophyta	Chlorophyceae	UPMC-A0054	MH166735	Sri Serdang Pond, Selangor
Scenedesmus obliquus	Chlorophyta	Chlorophyceae	UPMC-A0057	MH166738	Putrajaya Lake, Putrajaya
Scenedesmus communis	Chlorophyta	Chlorophyceae	UPMC-A0061	MK834570	Faculty of Engineering Pond, UPM
Oocystis sp.	Chlorophyta	Trebouxiophyceae	UPMC-A0084	MT039383	Sri Serdang Pond, Selangor
Poterioochromonas malhamensis	Chrysophyta	Chrysophyceae	UPMC-A0073	MK834582	Institute of Bioscience Pond, UPM
Pavlova noctivaga	Chrysophyta	Haptophyceae	UPMC-A0072	MK834581	Institute of Bioscience Pond, UPM
Navicula permitis	Chrysophyta	Bacillariophyceae	UPMC-A0071	MK834580	Taman Jaya Lake, Kuala Lumpur
Nitzschia palea	Chrysophyta	Bacillariophyceae	UPMC-A0069	MK834578	Faculty of Engineering Pond, UPM
Cyclotella meneghiniana	Chrysophyta	Bacillariophyceae	UPMC-A0070	MK834579	Lukut Lake, Seremban

UPM: Universiti Putra Malaysia.

Phycosphere bacteria were isolated following Rivas et al. (2010) to separate bacteria from microalgal colonies. The microalgae biomass was washed twice by repeated centrifugation at 4,800 ×g for 6 min, followed by resuspension with TE buffer (Tris 10 mM; Sigma Aldrich, USA); EDTA 1 Mm (pH 8.0; Sigma Aldrich, USA). The biomass was then rehydrated in 10 mL of distilled water and sonicated thrice at 60 Hz in a sonicator bath (Cole-Parmer, USA) 3 min each time, with 3-min intervals on ice to separate the bacteria adhering to microalgal colonies. The samples were then inoculated onto five different media: alkaline nutrient (DSMZ, Germany), Mueller Hinton (MH) (Merck, Germany), glucose peptone water (GPW) (DSMZ, Germany), thiosulfate citrate bile salts sucrose (TCBS) (Merck, Germany), De Man, Rogosa and Sharpe (MRS) (Merck, Germany)) agar plates to enhance the recovery of the cultivable bacteria. Morphologically distinct colonies were purified and stored at –20 °C for further study.

Preliminary in-vitro screening of microalgae growth-promoting bacteria

The purified bacterial strains were further evaluated for microalgae growth-promoting characteristics; phosphate solubilisation, nitrogen fixation, and indole-3-acetic acid (IAA) production to explore more of the role of these bacteria in the microalgae phycosphere. To select for phosphate solubilising bacteria, bacterial suspensions were inoculated into Pikovskaya broth (Pikovskaya, 1948) and incubated at 28 °C and shaken at 200 rpm for 3 days. The bacterial cultures were subjected to centrifugation (Eppendorf 5810R, Germany) at 14,000 rpm for 10 min, and the concentration of soluble phosphate in the supernatant was measured using the technique reported by Murphy and Riley (1962). The amount of soluble phosphate was obtained from the standard curve using potassium dihydrogen phosphate (Merck, Germany) which was determined spectrophotometrically (UV-1900i, Shimadzu, Japan) at 830 nm.

For the selection of nitrogen-fixing bacteria, the freshly grown bacterial cultures were inoculated in peptone water (Merck, Germany) and incubated at 28 °C for 12 days and shaken at 120 rpm. The bacterial cultures were centrifuged (Eppendorf 5810R, Germany) at 14,000 rpm for 10 min, and the ammonia production in the supernatant was determined using indophenol method of Bergersen (1980). The standard curve of ammonium chloride was used to calculate the quantity of ammonia which was measured spectrophotometrically (UV-1900i, Shimadzu, Japan) at 660 nm.

In order to select for IAA-producing bacteria, the bacterial strains were inoculated into nutrient broth (Merck, Germany) supplemented with 1 $g.L^{-1}$ of L-tryptophan (Merck, Germany) and incubated at 28 °C for 3 d with vigorous shaking. The IAA production was determined following Ullah et al. (2013) using a Salkowski reagent. The colour changes were read after 30 min at 530 nm using a spectrophotometer (UV-1900i, Shimadzu, Japan). Indole acetic acid (IAA) (Sigma-Aldrich, USA) was used for a standard curve.

Identification of growth-promoting bacteria

Seven bacteria (from a total of 80), isolated from the

microalgae phycosphere previously screened, had multiple microalgae growth-promoting traits (phosphate solubilisation, nitrogen fixation, and indole-3-acetic acid (IAA) production): CD-1 (isolated from *Chlamydomonas reinhardtii*), CL-5 (isolated from *Chlorella* sp.), PT-2 (isolated from *Poterioochromonas malhamensis*), PV-6 (isolated from *Pavlova noctivaga*) as well as CY-2, CY-4 and CY-5 (isolated from *Cyclotella meneghiniana*). These seven bacteria were selected for molecular identification. Bacterial DNA was extracted from overnight suspensions pelleted at 12,000 rpm (Eppendorf, Germany) using a DNeasy PowerSoil kit (QIAGEN, USA) according to the manufacturer's instructions and checked for purity. The bacterial 16S rRNA gene was amplified using universal primers of 27F (5-AGAGTTTGATCCTGGCTCAG-3) and 1492R (5-TGACTGACTGAGGYTACCTTGTTACGACTT-3) with thermal cycler (Biometra, Germany) following thermal conditions of the initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min, and a final extension at 28 °C for 10 min. Once the PCR products were resolved using agarose gel electrophoresis by staining with GelRed solution (Biotium, Canada) and visualised under UV light, they were then purified using a gel/PCR DNA fragment extraction kit (Geneaid, Taiwan) before submitting them (Apical Scientific Sdn Bhd, Malaysia) for sequencing using an ABI3770 sequencer (Applied Biosystems, USA). The resulting sequences for the MGPB isolates were submitted to NCBI and BLAST searches to find the closest match in the NCBI 16S ribosomal RNA sequences database. Table 2 showed the accession numbers for the sequences that were deposited in GenBank.

Bacterial effect on Cyclotella meneghiniana *growth*

In a screening experiment, the effects of potential growth-promoting bacteria; CD-1 (isolated from *Chlamydomonas reinhardtii*), CL-5 (isolated from *Chlorella* sp.), PT-2 (isolated from *Poterioochromonas malhamensis*), PV-6 (isolated from *Pavlova noctivaga*) as well as CY-2, CY-4 and CY-5 (isolated from *Cyclotella meneghiniana*) on the growth of *C. meneghiniana* were evaluated using 96-wells microplate cultures. In order to estimate the microalgae population within the culture, optical measurements at OD_{680} were taken. Contents of the wells were inoculated with respective microorganisms, in the above-mentioned medium, sealed with parafilm to prevent contamination, and incubated at 25 ± 1 °C for 10 days under illumination of 100 µmol photon.m^{-2}.s^{-1} on a rotary shaker at 120 rpm. Each experiment was performed in batch cultures in triplicates using the following seven treatments: xenic *C. meneghiniana* and CD-1; xenic *C. meneghiniana* and CD-5; xenic *C. meneghiniana* and PT-2; xenic *C. meneghiniana* and PV-6; xenic *C. meneghiniana* and CY-2; xenic *C. meneghiniana* and CY-4; xenic *C. meneghiniana* and CY-5; and xenic *C. meneghiniana* only (as a control). The experiment began with the centrifugation (Eppendorf, Germany) of bacterial strains that had been precultured overnight in nutrient broth at 3000 rpm for 5 min. Approximately 10 bacterial cells (2×10^6 CFU.mL^{-1}) formed associations with each microalgae cell from the xenic culture of *C. meneghiniana* (2×10^5 cells.mL^{-1}) in the co-cultures. To test bacteria for their ability to promote *C. meneghiniana* growth in dark conditions, cultures were grown in the dark i.e. without light. The growth was monitored daily at OD_{680} with a microplate spectrophotometer (Thermo Scientific™ Multiskan™ GO, USA).

Statistical analyses

The data for preliminary in vitro screening of putative microalgal promoting bacteria as well as the bacterial effect on *Cyclotella meneghiniana* growth were presented as mean and the standard error of the mean (SE) of three measurements (i.e. n = 3). To test for differences among treatments, the data were analysed using one-way ANOVA followed by Tukey's

Table 2. Identification of the bacterial isolates from microalgal phycosphere based on 16S rRNA partial sequence analysis.

Strain	Closest taxon	Phylum	Class	16S rRNA gene sequence similarity (%)	Accession number
CD-1	*Achromobacter* sp.	Proteobacteria	β-Proteobacteria	99.93	MW301667
CL-5	*Paenibacillus glucanolyticus*	Firmicutes	Bacilli	97.97	MW301673
PT-2	*Pedobacter* sp.	Bacteroidetes	Sphingobacteriia	99.63	MW301670
PV-6	*Achromobacter* sp.	Proteobacteria	β-Proteobacteria	99.93	MW301668
CY-2	*Pseudomonas hibiscicola*	Proteobacteria	γ-Proteobacteria	100.00	MW301669
CY-4	*Sphingobacterium* sp.	Bacteroidetes	Sphingobacteriia	98.85	MW301671
CY-5	*Ochrobactrum haematophilum*	Proteobacteria	α-Proteobacteria	99.92	MW301672

Note: α: Alpha, β: Beta, γ: Gamma.

multiple comparison tests to determine which treatments differed significantly. The statistical programme SPSS (IBM SPSS Statistics, version 20) was used for these analyses and *P* values <0.05 were considered significant.

Results

In vitro screening for potential microalgae growth-promoting bacteria (MGPB)

In this screening procedure, only seven of the 80 isolated bacteria were found capable of solubilising phosphate (0.08 to 2.55 mg.L^{-1} PO_4^{3-}), fixing nitrogen (0.08 to 4.70 mg.L^{-1} NH_4^+) and producing IAA growth regulator (21.00 to 452 mg.L^{-1} IAA). These seven bacteria were further analysed statistically, and Figure 1a-c summarised the results for the screening of potential microalgae growth-promoting bacteria for IAA production, phosphate solubilisation, and ammonium production, respectively. The highest (P < 0.05) phosphate solubilising activity was observed in CD-1 (2.55 mg.L^{-1} PO_4^{3-}) and CY-5 (2.36 mg.L^{-1} PO_4^{3-}) (Fig. 1a). Meanwhile, the highest ammonium production was observed in PV-6 (4.49 mg.L^{-1} NH_4^+), CY-2 (4.06 mg.L^{-1} NH_4^+) and CY-5 (4.70 mg.L^{-1} NH_4^+) (Fig. 1b). As for the IAA production, CL-5, CY-4, CY-5 yielded the highest (P < 0.05) with 343.32, 422.18, 452.06 mg.L^{-1} IAA, respectively (Fig. 1c).

Molecular identification using 16S rRNA gene analysis

Based on the 16S rRNA analysis on the PCR products (Fig. 2), the MGP bacteria strains CD-1 and PV-6 showed more than 98 % similarity to *Achromobacter* sp. Meanwhile, strain CL-5 and PT-2 showed the closest similarity to *Paenibacillus glucanolyticus* and *Pedobacter* sp., showing 97.97 % and 99.63 % similarities, respectively. The remaining three strains isolated from *C. meneghiniana*; CY-2, CY-4 and CY-6 showed more than 98 % similarity to *Pseudomonas hibiscicola*, *Sphingobacterium* sp. and *Ochrobactrum haematophilum*, respectively.

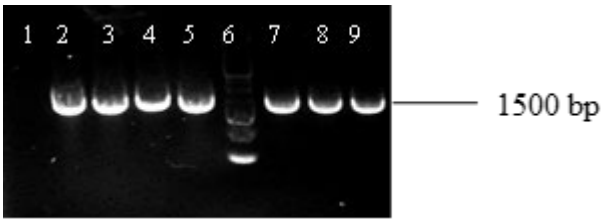

Fig. 2. Agarose gel electrophoresis analysis (1 % agarose gel) of PCR amplification of the 16S rRNA gene of potential microalgae growth promoting bacteria with an expected band size of 1500 bp. Lane 1: negative control, lane 2: CD-1, lane 3: CL-5, lane 4: PT-2, lane 5: PV-6, lane 6: 1KB DNA ladder lane 7: CY-2, lane 8: CY-4, lane 9: CY-5.

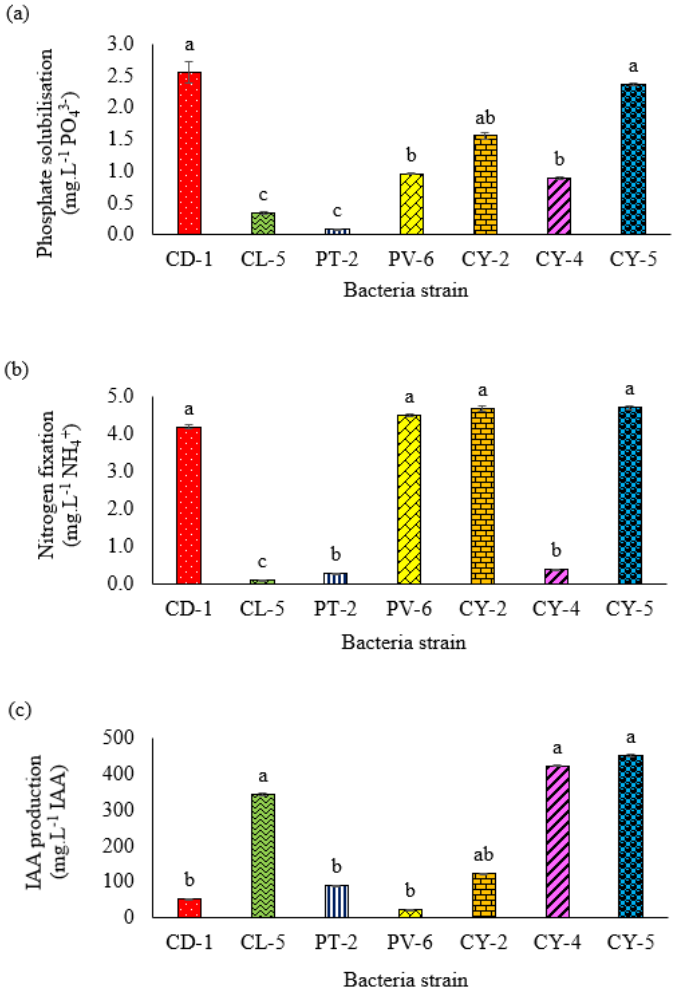

Fig. 1. Mean (± SE) values for microalgal promoting traits for seven treatments (a) Phosphate solubilisation (mg.L^{-1} PO_4^{3-}) (b) nitrogen fixation (mg.L^{-1} NH_4^+) and (c) IAA production (mg.L^{-1} IAA). The seven potential microalgae growth-promoting bacteria; CD-1 (isolated from *Chlamydomonas reinhardtii*), CL-5 (isolated from *Chlorella* sp.), PT-2 (isolated from *Poterioochromonas malhamensis*), PV-6 (isolated from *Pavlova noctivaga*) as well as CY-2, CY-4 and CY-5 (isolated from *Cyclotella meneghiniana*). Different letters in each bar represent the significant differences (P < 0.05) detected using one-way ANOVA and Tukey's multiple range test.

The potential MGP bacteria were derived from three different phyla (Table 2). At the class level, 57 % of the sequenced isolates were delegated to Proteobacteria whereby these isolates belong to α (one isolate), β (two isolates) and γ-Proteobacteria (one isolate), while the remaining 43 % of the sequenced isolates were associated with Firmicutes (one isolate), Bacteroidetes (two isolates) and Bacilli (one isolate).

Effect of bacteria on Cyclotella meneghiniana *growth*

The seven potential MGPBs strains were further investigated to examine their effect on microalgal growth. Co-cultivation experiments revealed positive effects of the individual bacteria on the growth of *C. meneghiniana* (Figs. 3, 4a). After a 10-day incubation period, the cell density of *C. meneghiniana* increased sharply with a specific growth rate of 1.7 to 4.3 times higher than the control within two days after the bacterial treatment (Fig. 3). The highest cell density was recorded in *C. meneghiniana* co-cultivated with CY-2 (*Pseudomonas hibiscicola*) and CY-5 (*Ochrobactrum haematophilum*) (Fig. 4a), were 0.33 d^{-1} and 0.48 d^{-1}, respectively where both were found to be associated with Proteobacteria class.

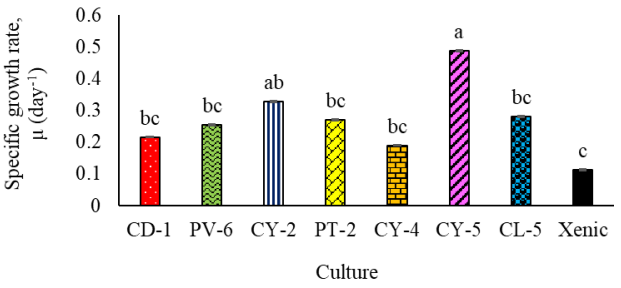

Fig. 3. Specific growth rate (μ) for *Cyclotella meneghiniana* calculated in the experiment for xenic and mixed cultures with various bacterial strains; CD-1 (isolated from *Chlamydomonas reinhardtii*), CL-5 (isolated from *Chlorella* sp.), PT-2 (isolated from *Poterioochromonas malhamensis*), PV-6 (isolated from *Pavlova noctivaga*) as well as CY-2, CY-4 and CY-5 (isolated from *Cyclotella meneghiniana*) assayed separately. For each culture, data were presented as mean and the error bars indicating the standard error (n = 3). Different letters in each bar represent the significant differences ($P < 0.05$).

Since it was known that microalgae photosynthesis is directly affected by light duration, and reducing light

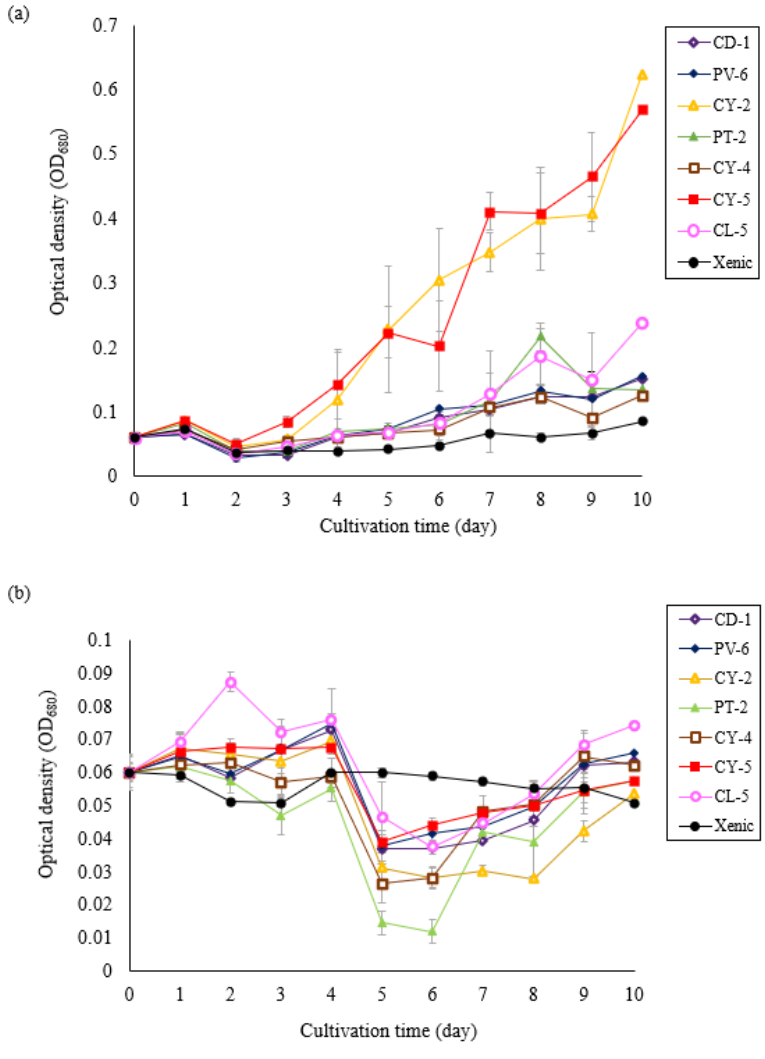

Fig. 4. Optical density (OD_{680}) for *Cyclotella meneghiniana*, measured in the experiment for xenic and mixed cultures with various bacterial strains; CD-1 (isolated from *Chlamydomonas reinhardtii*), CL-5 (isolated from *Chlorella* sp.), PT-2 (isolated from *Poterioochromonas malhamensis*), PV-6 (isolated from *Pavlova noctivaga*) as well as CY-2, CY-4 and CY-5 (isolated from *Cyclotella meneghiniana*) assayed separately, grown in (a) normal cultivation settings and in (b) the dark settings. For each culture, data were presented as mean and the error bars indicating the standard error (n = 3).

duration reduces growth rate and biomass yield, the role of these MGPBs in promoting the growth of microalgae in the absence of light were studied as well. Interestingly, although the growth rate was shown to decrease after 5 days of treatment in the dark, the growth started to increase by day starting day 6 implying that bacteria may have adapted to the condition, releasing the MGP metabolites for microalgae to survive in the absence of light (Fig. 4b).

Discussion

Bacterial collection

In the present study, bacteria were isolated from microalgae cells at the exponential growth phase in order to explore the function of phycosphere bacteria at this critical time. According to Powell and Hill (2014), *Nannochloropsis oceanica* has the greatest capacity for bacterial aggregation (up to 93.4 percent efficiency) when the microalga is in the exponential phase. On the other hand, as the cells transit into the stationary phase, the aggregation potential decreases, which suggests that the chemistry of the cell's surface changes depending on the presence of the inhabiting bacteria responsible for the aggregation process. Similarly, the colonial green microalga *Botryococcus braunii*, an oleaginous microalga, accumulates a lot of hydrocarbons, mostly in the extracellular space (Lee et al., 2015; Shen et al., 2015). The extracellular matrix (ECM) of the microalga *B. braunii*, which is mostly made of long chain polyacetal hydrocarbons that are cross-linked with hydrocarbons, mediates the association of the cells through a special colonial structure. This could explain why the *Scenedesmus* and *Oocystis* genera of the colonial-type green microalgae have a comparatively high number of bacteria isolated (Table 2) within the same phylum, since the hydrocarbons allow bacterial interaction to take place within the environment.

As opposed to that, transparent exopolymer particles (TEP), are the extracellular organic macromolecules released by chrysophytes, or more precisely diatoms, either actively or as a byproduct of the cell lysis (Passow, 2002; Fukao et al., 2010). These primarily particulate acidic polysaccharides are ubiquitously found in the ocean and frequently have bacteria living on them (Passow, 2002). Similar to how plants produce flavonoids, active diatoms may employ TEP to entice particular bacterial species. The bacteria would start to adhere to TEP once they detected the diatom's presence, which may also serve as a source of nutrients for the bacteria. According to a study by Gärdes et al. (2011), when bacteria were introduced to axenic *Thalassiosira weissflogii* diatom cells, the bacterial strain has been observed to influence TEP synthesis. As the bacteria adhere to the diatom, cell aggregation was observed. This also explains why chrysophytes in this study could still harbour a high number of cultivable bacteria (Table 2) despite being subjected to multiple pre-treatments beforehand.

From the five different culture media used to extract all the cultivable microorganisms, oligotrophic bacteria were isolated in the alkaline nutrient medium, whereas fastidious bacteria were isolated from the glucose peptone water medium. For a non-selective, non-differential medium that can support the growth of a variety of non-fastidious organisms, Mueller Hinton (MH) medium was employed. Probiotic lactic acid bacteria were selectively isolated using De Man, Rogosa and Sharpe (MRS) medium whereas *Vibrio* spp. were isolated using thiosulfate citrate bile salts sucrose (TCBS) medium. None of the colonies were formed after streaking on the TCBS agar, indicating that none of the isolates belonged to pathogenic *Vibrio* species. This procedure was carried out in order to get rid of the potentially pathogenic *Vibrio* strains, which have conferred serious threats to animals reared in aquaculture for decades (Austin and Austin, 1993; Hjeltnes and Roberts, 1993; Lightner 1993; Austin and Austin, 1999; Bergh et al., 2001).

In vitro screening for potential microalgae growth-promoting bacteria

This study revealed a co-occurrence of potential microalgae growth-promoting bacteria in a natural phycosphere setting when the culturable bacteria community was assessed. Although the identified bacteria isolates do not entirely represent the whole bacterial community within the phycosphere of the microalgae, which might be due to the limited capacity of the isolation procedure, this study discovered a number of characteristics related to microalgal growth-promotion. These characteristics include the production of auxins, as well as phosphorus and nitrogen metabolism by the symbiotic MGPB, which were examined by adopting the screening procedures for plant growth promoting bacteria (PGPB) in terrestrial plant studies. Plant beneficial microorganisms (PBM), specifically the plant growth-promoting bacteria (PGPB), have demonstrated symbiotic relationships with plants by reducing heavy metal toxicity, promoting a diverse mode of plant tolerance to metals and climatic stresses, and influencing macronutrient and micronutrient bioavailability in soils (Ma et al., 2016). In addition, they also provide plants with multimodal complex tolerance to a variety of climatic stresses. These PGPB strains have also been found to protect plants against phytopathogens by producing antibiotics and inducing systemic resistance (Grover et al., 2021).

As non-diazotrophic organisms, microalgae generally rely on macronutrients such as phosphorus and nitrogen from the surroundings for their growth in which long-term deprivation of these macronutrients can induce severe stress, stagnation, and ultimately death (Ramanan et al., 2013, Schmollinger et al., 2014). This is where MGPB comes into play, when they

directly or indirectly promote growth of microalgae by offering inorganic components (nitrogen, carbon and phosphate) and other nutrients and metabolites (vitamins, growth hormones and iron) in exchange for carbon (Ramanan et al., 2016). Among the well-known MPGBs are, *Bacillus pumilus* that supports the development of *Chlorella vulgaris* by nitrogen fixation (Hernandez et al., 2009) and *Azospirillum brasilense* that benefits *Chlorella* strains through the secretion of phytohormones such as IAA (Palacios et al., 2016).

Phosphorus is required for microalgae development, the production of lipids and fatty acids, and metabolic processes such as energy transfer, signal transduction, and photosynthesis (Atiku et al., 2016; Ota et al., 2016; Yang et al., 2018). Therefore, for microalgae to develop, phosphorus is considered a crucial nutrient, albeit the amount needed varies depending on the species. Microalgae usually take up phosphorus in the form of polyphosphate or orthophosphate. Additionally, phosphorus is necessary for microalgal growth at a concentration of about 0.03–0.06 % in the medium (Hannon et al., 2010; Procházková et al., 2014; Ota et al., 2016). Meanwhile, the ideal phosphorus content for microalgae is between 0.001 $g.L^{-1}$ to 0.179 $g.L^{-1}$, according to Roopnarain et al. (2014). Therefore, it is critical for microalgae to effectively capture, store and manage phosphorus, particularly when it is scarce. Phosphorus solubilising bacteria are capable of breaking down insoluble phosphorus and releasing it into the environment (Shenbagarathai, 1993; Rashid et al., 2004; Zaidi et al., 2009; Liu et al., 2015) where it is utilised by microalgae. In the present study, the highest phosphate solubilising activity was observed in CD-1 (2.55 $mg.L^{-1}$ PO_4^{3-}) and CY-5 (2.36 $mg.L^{-1}$ PO_4^{3-}), which falls within the range of ideal phosphorus content for microalgae, as suggested by Roopnarain et al. (2014). These strains are therefore good candidates for MGPB.

Another important macronutrient is nitrogen, which promotes the growth and development of microalgae, as well as the synthesis of the amino acids needed to produce chlorophyll for photosynthetic activity (Grobbelaar, 2007). Nitrate, nitrite, and ammonium in the form of salts are generally the inorganic nitrogen sources used in microalgae cultivations. Shi et al. (2000) discovered that when ammonium was used instead of nitrate, *Chlorella protothecoides* absorbed more ammonium and generated greater biomass. Several studies have also shown that ammonium is a superior nitrogen source for a range of freshwater and marine algae (Dortch, 1990; Raven et al., 1992). On the other hand, Feng et al. (2020) discovered that *Chlorella* sp. preferred nitrate, where the growth rate increased by 11-fold compared to ammonium. Thus, various algal species require different types and concentrations of nitrogen sources (Raven et al., 1992; Ramli et al., 2017; Zhuang et al., 2018; Feng et al., 2020).

In the current study, the capacity of each cultivable phycosphere bacteria for nitrogen fixation was elucidated by determining the ammonium production in the culture medium. The highest ammonium production was observed in CY-5 (4.70 ± 0.06 $mg.L^{-1}$ NH_4^+) followed by PV-6 (4.49 ± 0.03 $mg.L^{-1}$ NH_4^+), CD-1 (4.18 ± 0.05 $mg.L^{-1}$ NH_4^+), and CY-2 (4.06 ± 0.08 $mg.L^{-1}$ NH_4^+). These bacterial isolates may be useful in fixing atmospheric nitrogen, particularly during outdoor mass cultivation by fixing and providing ammonium as the sole nitrogen source. This would enable microalgal cultivation to be accomplished at a cheaper cost, which would greatly assist the aquaculture industry.

Indole-3-acetic acid (IAA) is one of the key significant signalling molecules in microalgae and bacteria. Studies have revealed successful microalgal growth promotion benefiting from IAA secretion by symbiotic bacteria (Mazur et al. 2001). Microalgae on the other hand, simultaneously produced additional signalling molecules that induce bacteria to release IAA. Within the terrestrial environment, this phytohormone generally initiates the production of roots and causes them to grow longer in plants. Similarly, it has been demonstrated that exogenous IAA applied to a microalgal culture encourages the proliferation of single-celled microalgae (Vance, 1987). *Azospirillum brasilense* is a well-studied example of a plant growth-promoting bacterium (PGPB) that has also been discovered to promote microalgal growth and development (Bashan et al., 2010). IAA produced by *A. brasilense*, promotes the growth of the green microalgae, *Chlorella sorokiniana* (de-Bashan et al., 2008). Similarly, although brown microalgae are genetically distinct from both green microalgae and plants, IAA and other auxin hormones are also found to have elongation effects on their cells, much like they do in plants (Le Bail et al., 2010). Studies have shown that tryptophan biosynthesis acts as a stimulant to IAA synthesis, whereby the secretion of tryptophan by microalgae may be the cause of the increase in IAA production by bacteria.

In the current study, bacterial cultures were given 1 $g.L^{-1}$ tryptophan and the IAA production was evaluated. Three bacterial strains (CY-5, CL-5 and CL-4) secreted significantly higher IAA concentrations compared to other bacterial species with CY-5 (452.06 ± 3.72 $mg.L^{-1}$ IAA) yielding the greatest IAA concentration, followed by CL-5 (343.32 ± 2.42 $mg.L^{-1}$ IAA), and CL-4 (422.18 ± 1.21 $mg.L^{-1}$ IAA). The ranges of IAA concentrations from this study are considerably higher than the concentrations considered suitable for *C. sorokiniana* culture (Palacios et al., 2016; Peng et al., 2020), suggesting that these three bacterial strains could be effectively used as MGPB.

Molecular identification using 16S rRNA gene analysis

Previous research has linked specific bacterial populations to green algae via the metabolites released by the naturally occurring bacteria.

Proteobacteria and Bacteroidetes have been demonstrated to be affiliated more with green algae than other bacterial phylotypes (Ramanan et al., 2015; Ramanan et al., 2016). This group of bacteria linked with green algae can exhibit a comparable or identical effect to that of known terrestrial plant growth promoting microorganisms, such as *Rhizobium* when interacting with algae (Kim et al., 2014), and performs activities similar to plant-bacteria interactions.

In the current study, the bacterial strains isolated from green algae were associated with Proteobacteria and Firmicutes. Members of the Alpha-, Beta-, and Gamma-Proteobacteria and Bacteroidetes were repeatedly found to be associated with chrysophytes such as *Roseobacter* spp., *Sulfitobacter* spp. and *Flavobacterium* spp. (Amin et al., 2012). The results from the present study are consistent with the previous report whereby the chrysophytes were represented by *Achromobacter*, *Pseudomonas*, *Pedobacter*, *Sphingobacterium* and *Ochrobactum* genera, from the same phyla (Piampiano et al., 2019).

Recent investigations have categorised the known phosphate-solubilising bacterial isolates into 17 genera and three phyla (Actinobacteria, Firmicutes, and Proteobacteria) (Alori et al., 2017). According to this study, Bacterioidetes, another putative phylum, also possessed the same traits. The findings from the current study are also consistent with previous studies that found nitrogen-fixing activities were substantially related to the presence of the phyla Actinobacteria, Bacteroidetes, Cyanobacteria, Chlorobi, Chloroflexi, Firmicutes, and Proteobacteria (Raymond et al., 2004; Dos Santos et al., 2012; Boyd and Peters, 2013). As for the IAA biosynthesis, Acidobacteria, Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria were the five phyla that have been identified to be capable of synthesising IAA (Zhang et al., 2019), which is also consistent with our findings.

The potential MGP bacteria identified in the current study were derived from three different phyla. At the class level, 57 % of the sequenced isolates were delegated to Proteobacteria, whereby these isolates belong to α (one isolate), β (two isolates) and γ-Proteobacteria (one isolate), while the remaining 43 % of the sequenced isolates were associated with Firmicutes (one isolate), Bacteroidetes (two isolates) and Bacilli (one isolate). Apart from Proteobacteria, bacteria associated with Chlorophyta were from the Bacteroidetes class, while bacteria associated with Chrysophyta were from the Firmicutes class, suggesting that bacterial composition was strictly species-specific to the host microalgae.

The importance of the plant growth-promoting bacteria, rhizobacterium, such as *Azospirillum*, *Mesorhizobium*, and *Rhizobium* sp. in stimulating the growth of microalgae (Gonzalez and Bashan, 2000; Watanabe et al., 2005; Hernandez et al., 2009; Kim et al., 2014), is well known. Cho et al. (2015) revealed that when certain microalgae were cultured within an engineered consortium of mutualistic bacteria, they significantly increased the consortium's supply of fixed organic carbon thus, resulting in faster growth. Several studies have revealed the significant importance of heterotrophic bacteria in microalgal development and survival (Seyedsayamdost et al., 2011; Kim et al., 2014). Hernandez et al. (2009), and Choix et al. (2012) reported the impacts of naturally occurring microalgae-associated bacteria in promoting microalgae growth and production.

In the current study, the best three bacteria strains that exhibited multiple traits of these MGP, were *Ochrobactrum haematophilum* strain CY-5, *Pseudomonas hibiscicola* strain CY-2, and *Sphingobacterium* sp. strain CY-4. According to Oehmen et al. (2007) and Carvalheira et al. (2014), *Pseudomonas* spp. are capable of fixing nitrogen, thus it is expected that they are able to fix atmospheric nitrogen (Chan et al., 1994; Hatayama et al., 2005; Yan et al., 2008; Toyofuku et al., 2012). Previous research demonstrated that the development of *Chaetoceros calcitrans* and *Nannochloropsis oculata* may be greatly accelerated by the presence of the bacteria *Pseudomonas* and *Bacillus* (Hatha et al., 2014). Similarly, *Pseudomonas* may promote plant growth in the terrestrial environment by secreting IAA and boosting the soil's availability of phosphorus (Patten and Glick, 2002; Ali et al., 2009; Babalola and Glick, 2012; Glick, 2012).

Studies have shown that *Sphingobacterium* spp. has the capacity to synthesise vitamin B12 in addition to the various MGP properties evaluated (Krohn-Molt et al., 2013). Additionally, this genus is crucial for the flocculation of microalgae, which may be of great benefit to the harvesting of microalgae (Lee et al., 2013).

Effect of bacteria on Cyclotella meneghiniana *growth*

Microalgae are of the important trophic source in the marine food chain that has been contributing to sustainable zooplankton and fish nutrition due to their substantial omega fatty acids content. Much previous research has concentrated on the production of valuable products of these microalgae by manipulating the growth condition of the cultures. In this study, *C. meneghiniana* growth was manipulated by co-cultivating the cultures with the identified symbiotic MGPB analysed from the phycosphere of *C. meneghiniana* and other microalgae (*Chlamydomonas*, *Chlorella*, *Pavlova* and *Poterioochromonas* genera). The majority of prior studies on interactions between microalgae and bacteria were undertaken in Erlenmeyer or bigger flasks. These culture volumes, however, are insufficient for screening a wide range of species at one time. Therefore, the present study adopted the microplate-based assessment method developed by Le Chevanton et al. (2013), and a similar

approach used previously (Skjelbred et al., 2012), to assess microalgal growth.

Furthermore, the study's comparison of microplate and flask experiments revealed good correlation for both growth parameters, confirming the method's reliability (Le Chevanton et al., 2013). Microalgal growth-promoting bacteria (MGPB) were used to cultivate microalgae in laboratory conditions and the results generated from these unnatural settings are useful for guiding large-scale microalgal culture. The highest growth rate (0.48 d^{-1}) was demonstrated in *Cyclotella meneghiniana* co-cultivated with *Ochrobactrum haematophilum*. Out of three bacterial strains isolated from the phycosphere of *C. meneghiniana*, only *O. haematophilum* had a significantly higher specific growth rate ($P < 0.05$), when re-introduced to the host microalgae cultures with a much higher concentration (5×10^6 CFU.mL^{-1}) than before, as opposed to *Pseudomonas hibiscicola* and *Spingobacterium* sp. The growth rate of *C. meneghiniana - O. haematophilum* consortium was enhanced, to almost a 3-fold increase, compared to those reported in other studies (Higgins and VanderGheynsta, 2014; Toyama et al., 2019; Krug et al., 2020).

Thus, bacteria play an important role in promoting microalgal growth because as the bacterial concentration increased, higher concentrations of IAA (452.06 mg.L^{-1} IAA), soluble phosphate (2.36 mg.L^{-1} PO_4^{3-}) and ammonium (4.70 mg.L^{-1} NH_4^+) were available for *C. meneghiniana* to stimulate the growth and reproduction of the host microalgae compared to the initial concentrations from the natural setting. This is because the concentration of bacteria must be maintained below a maximum threshold, in order to promote microalgae growth and at the same time to prevent competition for nutrients between microalgae and bacteria (Guo et al., 2014). However, the interactions between bacteria and microalgae within the phycosphere are complex and entail specific cellular processes such as quorum sensing (Fouilland et al., 2018), and thus require further study.

Bacteria are well recognised for their ability to affect microalgal growth by influencing either growth rate or biomass accumulation. In the present study, the experiment resulted in promotion effects by the MGPB shown by the growth curves under both light and dark conditions that displayed a similar trend (Fig. 4). This is similar to studies reported by Cupo et al. (2021), when the diatom *Cyclotella crytica* was cultivated under autotrophic (14:10 h light/dark photoperiod) and heterotrophic (totally in the dark and with glucose supplemented as an organic carbon source) conditions. Under an adequate nutritional regime, the growth curves under autotrophic and heterotrophic conditions showed a similar trend, but the cellular density was significantly lower in the dark ($P < 0.05$). After a three to four day of adaption period to heterotrophic condition, the growth rate showed a fast rise in cell division (Cupo et al., 2021).

In this present study, microalgae and bacteria may have acclimated to the dark environment, and once the adaptation phase was completed, the symbiotic bacteria may have secreted growth promoters to impact microalgae development (Amin et al. 2012). In addition to direct food exchange, signal transduction is another mechanism for synergistic mutualism between microalgae and bacteria (Kouzuma et al., 2015). Previous studies demonstrated that tiny molecular exchange between microalgae and bacteria may occur through other mechanisms within the growth environment and is not restricted solely to direct contact to allow for the exchange to happen (Amavizca et al., 2017).

Interactions between bacteria and microalgae are remarkably complex mechanisms that can produce a variety of patterns depending on settings and the protagonists involved. The present study demonstrated that the interactions are highly species specific. Since culture conditions can alter the composition and rates of microalgal exudation, they can also have a significant impact on the results of interactions in mixed cultures. As a result, depending on whether the culture is batch or continuous, the outcome of mixed cultures under nutrient-limited conditions is likely to differ.

Furthermore, physical factors such as temperature, can influence the rates of microalgal or organic macronutrient remineralisation (Kamatani, 1969; Garber, 1984). Finally, as reported in the current study, it is suspected that the initial microalgae-to-bacteria ratio is another critical concern in mixed culture and may turn symbiosis into competition for the macronutrient resource; with high ratios favouring competition for the macronutrient resource and low ratios resulting in higher macronutrient availability for microalgae.

Conclusion

The phylogenetic analysis of the isolated bacteria demonstrated the dominant bacteria associated with Chlorophyta were of Proteobacteria and Firmicutes phyla, while Proteobacteria and Bacteroidetes phyla were associated with Chrysophyta (diatoms). This study demonstrated that the bacterial composition was strictly species-specific to the host, microalgae. Enhanced growth-promoting activity by the isolated bacterial strains was shown to be highly dependent on IAA production, phosphorus solubilizing, and nitrogen-fixing activity. This study also illustrated that the bacteria *Ochrobactrum haematophilum* and *Pseudomonas hibiscicola* could enhance the growth and overall productivity of the microalgae *Cyclotella meneghiniana*. Thus, suitable microalgae-bacterial consortia can be established to improve the growth and nutritional quality of zooplankton which can be

used as effective live feed for commercially important fish/invertebrate larval production.

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