Biotechnology and Bioprocess Engineering 22: 150-160 (2017)
DOI 10.1007/s12257-016-0584-8

pISSN 1226-8372
eISSN 1976-3816

RESEARCH PAPER

Contaminated Bacterial Effects and qPCR Application to Monitor a Specific Bacterium in *Chlorella* sp. KR-1 Culture

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Received: 25 October 2016 / Revised: 27 March 2017 / Accepted: 20 April 2017

Abstract To investigate the effects of bacteria contaminated in microalgal cultivation, several bacteria were isolated from four photobioreactors for *Chlorella* sp. KR-1 culture. A total of twenty-one bacterial strains isolated from the reactors and identified by 16S rRNA gene sequencing. Six bacteria, which were found from more than two reactors of the four photobioreactors, were introduced into co-culturing experiments with *Chlorella* sp. KR-1. Then, the bacterial influences on the productivity of microalgal biomass and lipids were assessed in the photoautotrophic- and mixotrophic microalgal cultivation by comparing them with axenic culture of *Chlorella* sp. KR-1. The results showed that both biomass and lipid production were significantly enhanced under mixotrophic conditions compared to photoautotropic conditions. However, an excess ratio (more than 10%) of bacterial cells to microalgal cells at the initial stage of mixotrophic cultivation has limited the growth of *Chlorella* sp. KR-1 because of the relatively fast growth of bacteria, especially under mixotrophic conditions. Moreover, it was proven that the strong biofilm formability of *Sphingomonas* sp. MB6 is the responsible strain to cause the biomass aggregation observed during the early stage of co-culture. The high abundance of *Sphingomonas* sp. MB6 during early cultivation period shown by qPCR results was also well corresponded with the period shown a strong biofilm formation, which suggested the applicability of qPCR to monitor a specific bacterial group in a microalgal culture.

Keywords: mixotrophic condition, co-existing bacteria, biomass and lipid production, qPCR, biofilm

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1. Introduction

Microalgae are prokaryotic or eukaryotic microorganisms that grow under a wide range of environmental conditions, including aquatic and terrestrial ecosystems. These organisms are commonly photoautotrophic, converting solar energy into chemical energy. In this mechanism, they use light energy for CO_2 fixation, which contributes to global CO_2 sequestration from air. Algal biomass can be used as a feedstock for healthy food, feed additives, cosmetics, and energy production, *etc*. [1,2]. However, active applications of algae as biodiesel feedstock are still limited because of the high cost of microalgal cultivation and harvest from culture solution. Several studies have suggested a coupling of microalgal cultivation and wastewater treatment to compensate for the cost of microalgal production [3, 4], and others have suggested microalgal cultivation using wastewater as a source of essential nutrient for microalgal growth [5].

Microalgal-bacteria consortia have been investigated in many studies [5-8], but the relationships between microalgae and bacteria were not uniformly described. Nevertheless, it is known that symbiotic relationships between microalgae and bacteria can be explained by their complementary O_2/CO_2 exchange. Microalgae can grow and take up pollutants in wastewaters *via* their photosynthesis [9]. In addition, some bacteria can promote microalgal growth by releasing algal growth-promoting factors (*e.g.,* vitamins, phytohormones, siderophores, and phosphate solubilizing compounds) or

by supporting the growth through nitrogen fixation [10,11]. However, some bacteria can inhibit microalgal growth by producing algicidal metabolites and completing restricted nutrient compounds [12,13]. Microalgae also inhibit bacterial growth by producing antibacterial materials [14] and changing the pH of the culture [15]. Conversely, several studies have reported that microalgal harvesting is enhanced by self-aggregation bacteria [8,16]. Therefore, the relationships could be different depending on the purpose of their application.

Some microalgae can use both inorganic and organic carbon substrates under light and dark conditions, resulting in better growth rates and lipid contents in algal biomass than photoautotrophic conditions [17,18]. Therefore, mixotrophic culture of microalgae has received attention as a method of promoting microalgal production over recent years [18-20]. However, the environment required to achieve higher production from mixotrophic cultivation of microalgae might be vulnerable to contamination by bacteria.

Quantification of bacteria using plating methods is difficult because it requires a time to grow [21]. In addition, many bacterial habitats in natural environments cannot survive easily under limited artificial conditions. Quantitative PCR is an alternative method to compensate for the disadvantages described above. Therefore, it has been widely used to detect bacterial numbers or specific gene expression in various environmental samples [22,23]. This method is especially useful for quantification of uncultured and slow-growing bacteria. Additionally, this method has been employed for differential detection of prokaryotic- and eukaryotic microorganisms in co-culture systems [24].

This study was aimed to monitor and clarify the influences of contaminated bacteria in microalgal cultivation as described in our previous studies [25,26]. To accomplish this, several bacteria were identified after being isolated from operating photobioreactors, and the effects of co-existing bacteria on biomass and lipid production of *Chlorella* sp. KR-1 were analyzed by direct introduction of several isolates into bubble-column photobioreactors (b-PBRs). In addition, real-time qPCR was applied to monitor the population changes of *Chlorella* sp. KR-1 and *Sphingomonas* sp. MB6 in a co-culture of *Chlorella* sp. KR-1 and the isolates.

2. Materials and Methods

2.1. Microalgal strain and culture condition

The *Chlorella* sp. KR-1 used in this study was isolated from freshwater near a thermal power plant in Yeongwol, Korea by the Korea Institute of Energy Research [27,28]. This is a promising strain for biodiesel production that shows a high tolerance for actual coal-burned flue-gas and a high lipid content up to 41% (w/w) [27,28]. This strain was kept on agar plates of BG11 medium and the pH of the medium was adjusted to 7.1. Preculture in a 250 mL Erlenmeyer flask was performed at 28 ± 1°C and 140 rpm in a shaking-type incubator. The preculture was then transferred into a b-PBR (length, 35 cm; inner dia., 3.7 cm; working vol., 500 mL). All experimental results were produced from b-PBRs in which CO_2 in air (2%, v/v) was bubbled through a 0.2 μm syringe filter (BioFACT, Korea) and continuous illumination with a light intensity of 150 μmol/m^2/sec was provided at room temperature (25 ± 3°C). The pH was checked periodically and maintained at 6.8 ~ 7.2 by the CO_2 gas supply.

2.2. Isolation and identification of bacteria

Bacteria were isolated from four b-PBRs operating to cultivate *Chlorella* sp. KR-1 in BG-11 medium. The culture solution sampled from b-PBRs was diluted with phosphate buffer solution (pH 7.2) and spread onto R_2A agar plates (LAB M Limited, UK) and nutrient agar (Difco, Becton, Dickinson and Company, USA) plates. These plates were then incubated at 28°C for 48 h. All the culture media used for isolation of bacteria were adjusted at pH 7.2. Morphologically different bacterial colonies formed on the plates were selected and transferred on each plate. Then, the isolates were identified by partial 16S rRNA gene sequencing using the 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3') primer set.

2.3. Photoautotrophic and mixotrophic culture

The composition of a modified BG 11 medium for photoautotrophic culture was as follows: $NaNO_3$ (1.5 g/L), K_2HPO_4 0.12 (g/L), $MgSO_4 \cdot 7H_2O$ (0.075 g/L), $CaCl_2 \cdot 2H_2O$ (0.036 g/L), citric acid (0.01 g/L), ferric ammonium citrate (0.006 g/L), $Na_2 \cdot EDTA$ (0.001 g/L), Na_2CO_3 (0.02 mg/L), and trace metal solution (1 mL/L). The trace metal solution contains H_3BO_3 (61.0 mg/L), $MnSO_4 \cdot H_2O$ (169.0 mg/L), $ZnSO_4 \cdot 7H_2O$ (287 mg/L), $CuSO_4 \cdot 5H_2O$ (2.5 mg/L), and $(NH_4)_6MoO_4 \cdot 4H_2O$ (12.5 mg/L). For mixotrophic cultivation, glucose was provided as an organic carbon source in addition to the inorganic CO_2 gas. Glucose was added to diverse concentrations (0, 0.4, 0.8, 1.2, and 1.6 g/L) at the initial stage of cultivation and the inoculum concentration of *Chlorella* sp. KR-1 was adjusted to 1.5×10^6 cells/mL for all b-PBR experiments.

2.4. Co-culture of microalgae and isolates

A total of twenty-one bacteria were isolated from autotrophic b-PBR cultures and six bacterial strains, which were isolated from more than two b-PBRs among four b-PBRs, were selected for further co-culture experiments. For the

Table 1. Experimental setting for co-culture of bacteria and *Chlorella* sp. KR-1

Experiment		Microalgae inoculation	Bacteria inoculation	Glucose (g/L)
For monitoring bacterial density	A	Yes	Yes	0
	B	Yes	Yes	0.8
	C	No	Yes	0.8
For investigating effect of bacteria on microalgal growth	A0	Yes	No	0
	A1	Yes	Yes	0
	B0	Yes	No	0.8
	B1	Yes	Yes	0.8
	C0	Yes	No	1.6
	C1	Yes	Yes	1.6
For investigating effect of initial cell ratio of bacteria to microalgae	D	Yes	Yes	0
	E	Yes	Yes	0
	F	Yes	Yes	0
	G	Yes	Yes	1.6
	H	Yes	Yes	1.6
	I	Yes	Yes	1.6

co-culture experiments, these six strains were cultured aerobically in nutrient broth (Difco, Becton, Dickinson and Company, USA), harvested by centrifugation (3,000 × *g*, 10 min) and washed twice with a phosphate buffer solution (pH 7.2) to remove nutrients dissolved in broth.

Firstly, the change of bacterial cell numbers was briefly monitored by a plating method during cultivation in b-PBRs under three conditions: co-culture of the bacterial mixture with *Chlorella* sp. KR-1, and with additional glucose (0.8 g/L) or not (Table 1). The density of bacterial cells was counted directly by enumerating colonies formed on R_2A plates incubated at 28°C for 48 h. The initial cell concentrations of *Chlorella* sp. KR-1 and bacteria were adjusted to 1.5×10^6 (*Chlorella* sp. KR-1) and 9×10^3 cells per mL (bacteria mixture).

Regardless of conditions, bacterial cell numbers in the microalgal cultures were converged to 3×10^8 cells/mL, which was regarded as the maximum number of bacterial cells acceptable in microalgal culture in this study. Then, to assume a maximum permissible ratio of bacterial cells to *Chlorella* sp. KR-1 cells without deterioration of biomass production, the biomass production was compared on the following conditions; at initial ratios of bacterial cells to *Chlorella* sp. KR-1 cells were set on 1 (D and G), 0.1 (E and H) and 0.01 (F and I); under the autotrophic (D, E and F) and mixotrophic conditions (G, H and I) (Table 1).

From the above experiment, the minimum concentration of bacterial mixture was determined to affect microalgal growth, and it was used as the bacterial inoculum size at further co-culture experiments. Co-culture conditions, such as aeration, temperature, and illumination, were the same as for other experiments in this study and the co-culture conditions were summarized in Table 1.

2.5. Measurement of biomass and lipid production in b-PBSs

Microalgal growth was determined periodically by measuring dry cell weight, optical density, and chlorophyll a.

2.5.1. Dry cell weight

Most biomass of microorganisms was expressed as a dry cell weight (g/L) by measuring the total suspended solid (TSS) concentration in the solution as described in the standard methods (APHA, AWWA, WEF 2005). Briefly, 5 or 10 mL of cultivation samples from b-PBRs were filtered through 0.45 μm GF/C filters (Whatman, UK), after which the filters were dried at 105°C for at least 2 h and measured.

2.5.2. Optical density

Optical density (OD) is frequently used as a rapid and simple method of biomass measurement of unicellular microorganisms. Normally, microalgal biomass is measured at the maximum absorbance range of chlorophyll a (400 ~ 460 and 650 ~ 680 nm). However, the chlorophyll content of microalgae is affected by growth cycles and culture conditions. The absorbance at 750 nm, which exhibited minimum pigment absorbance, was much less affected by pigment content [29]; therefore, this was used to measure the growth of *Chlorella* sp. KR-1 in this study. When the value of OD at 750 nm was over 1, the culture was diluted. The biomass was derived from OD values using a standard calibration curve drawn based on corresponding OD values within a proportional range to DCW. The biomass calculated by the OD value was designated as the indirect DCW in this study.

2.5.3. Measurement of chlorophyll a

Chlorophyll a, which is the representative and common pigment of oxygenic eukaryotic phototrophs, reflects the growth of *Chlorella* sp. KR-1 in this study. Chlorophyll a was extracted by a modified methanol extraction method [30]. To accomplish this, samples were collected from b-PBRs and microalgal cells were harvested by centrifugation at 4,000 × *g* for 10 min, after which the supernatant was discarded. These harvested algal cells were frozen at -80°C for 10 min and thawed in a water bath (37°C) for 5 min. The freeze-thaw procedure was repeated three times to break the cell wall, after which algal cells were resuspended in 90% methanol solution. These samples were blocked from light and extracted at 4°C for 18 ~ 20 h. The debris of the algal cells was then separated from supernatant by centrifugation at 4,000 × *g* for 10 min. Finally, the chlorophyll a content in the supernatant was determined according to the following formula (Equation 1) after measuring the optical densities

at 650 and 665 nm [31]

$$\text{Chlorophyll a (g/L)} = 16.5 \times A_{665} - 8.3 \times A_{650} \quad (1)$$

2.5.4. Chemical analysis

The culture solution of 10 mL was collected and filtered through 0.2 μm GF/C filters (Whatman, UK) to analyze nutrient consumption, such as the organic matter (COD, Chemical Oxygen Demand) and nitrate, which were determined using an analysis kit (Humas, Korea). The pH of the samples was measured using a pH meter (B-212, Horiba, Japan).

2.5.5. Fatty acid methyl ester (FAME) analysis

Lipid contents of biomass were determined by FAME analysis [25]. Briefly, cultivation samples from a stationary phase were collected by centrifugation at 4,000 × *g* for 10 min. The biomass was then washed twice with distilled water, freeze-dried and lyophilized in glass tubes, after which it was weighed. Tubes containing each sample were amended with 2 mL of lipid extraction reagent (chloroform/methanol, 2:1 (v/v)) and vortexed well. Next, 1 mL methanol and 300 μL H_2SO_4 (pH 2-3) were added and the samples were mixed well. The reaction mixture was then incubated in a water bath (100°C, 10 min) and cooled at room temperature. After cooling, 1 mL water was added and the sample was mixed for 5 min. The mixture was then centrifuged at 4,000 × *g* for 10 min to separate the two-layers. Next, the bottom layer was extracted and filtered with a 0.2 μm syringe filter (BioFACT, Korea). The lipid phase was identified and quantified based on comparison of the retention times and peak areas using a gas chromatograph equipped with a flame ionization detector and a 0.32 mm (ID) × 30 m HP-INNOWax capillary column (Agilent Technologies, USA).

2.6. Biofilm formation assay

Some bacteria have been reported to form aggregated biofilms with microalgae, which may then be applied for easy harvest of microalgae from the culture [7,8]. To investigate the biofilm formability, the isolates were first cultured in nutrient broth medium incubated in a 37°C incubator overnight. The cultures were then diluted 1:100 into fresh tryptic soy broth (TSB) medium. For the biofilm assay [32], TSB medium was amended with glucose (0.5%), then 100 μL aliquots of the diluted cultures were inoculated in a 96-well microtiter. One isolate was replicated in four wells. The microtiter was then incubated at 28°C for 48 h and the cells in the wells were removed by turning the plates over. Next, the microtiter was gently washed twice with distilled water. These wells were filled with 125 μL of 0.1% crystal violet solution and incubated at room temperature for 10 ~ 15 min. The microtiter was cleaned up 3 or 4 times with distilled water and dried for a few hours at room temperature (25 ± 3°C). After drying, each well was filled with 125 μL of 30% acetic acid solution and incubated for 15 min. Finally, the samples were transferred into a flat bottomed 96-well cell culture plate and measured by using a microplate reader (Bio-RAD, Model 680) at 550 nm using 30% acetic acid solution as a blank [33].

2.7. Real-time PCR

Population changes in *Chlorella* sp. KR-1 and *Sphingomonas* sp. MB6 were monitored by real-time qPCR (SYBR Green real-time PCR Master Mix) during the cultivation period. Samples were collected by centrifugation at 4,000 × *g*, the total DNA from the harvest was extracted using a Fast DNA spin kit for soil (MP Biomedical, USA). Then the extracted DNA was used as the template of the following qPCR. Each PCR mixture (20 μL) was composed of 10 μL of SYBR Green PCR master mix, 1 μL of each forward and reverse primer and 1 μL of DNA template. The amount of DNA templates was measured using a Nanodrop system (Thermo Scientific, Wilmington, DE). The PCR conditions were as follows: initial denaturation at 94°C for 10 min, followed by 45 cycles of 94°C for 30 sec, 52.5°C for 30 sec and 72°C for 15 sec. PCR amplification was performed using a MJ Chromo4 (Bio-Rad, USA) and monitored with the Opticon Monitor 3.1 software. For quantification of *Sphingomonas* sp. MB6, a primer set (SM6F-SM6R) specific for the 16S rRNA was designed using the NCBI BLAST and ClustalW programs. DNA of *Chlorella* sp. KR-1 was amplified by 18S rRNA universal primers (Euk345F-Euk499R) [23,34]. The primers for real time PCR are listed in Table 2. For all qPCR assays, standard curves were generated using triplicate 10-fold dilutions of the DNA extracted from the purified *Chlorella* sp. KR-1 and *Sphingomonas* sp. MB6 cultures.

Table 2. List of real-time PCR primers used in this study

Target	Primer	Sequence (5' - 3')	Amplicon length (bp)	References
MB6 16S rRNA	SM6F	GATGATAATGACAGTACCTGG	195	This study
	SM6R	CTCTCCAAGATTCCAGTCAC		
Eukaryotic 18S rRNA	EUK345F	AAGGAAGGCAGCAGGCG	154	[45]
	EUK499R	CACCAGACTTGCCCTCYAAT		

The standard curves for assuming the gene copies of *Chlorella* sp. KR-1 and *Sphingomonas* sp. MB6 were constructed from a series of 10-fold dilutions (corresponded from 10^4 to 10^9 copies) of plamid DNA carrying the PCR products of SM6F-SM6R (MB 6-specific) and Euk345F-Euk499R (*Chlorella* sp. KR-1 specific in this study) (Fig. 5A). There were linear relationships between threshold cycle (C_t) and the log amount of DNA concentration ($R^2 > 0.99$) for real-time qPCR. The slopes of amplification efficiencies were -3.16 (MB 6 specific) and -3.36 (Chlorella sp. KR-1 specific), indicating that they are appropriate for qPCR.

3. Results and Discussion

3.1. Identification of bacteria isolated from *Chlorella* sp. KR-1 culture

Occasionally, bacterial contamination was found with a pale-green colored biomass in photobioreactors for *Chlorella* sp. KR-1. A total of twenty-one bacteria differently shown on agar plates were isolated on three different times from four PBRs in which bacterial contamination was doubtful. The partial 16S rRNA genes derived from the isolates were sequenced and utilized for BLAST searches on the NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/) database. The sequences were deposited in the GenBank database under accession numbers LC036616–LC036636 and the BLAST results are summarized in Table 3. The microorganisms were classified into four different phyla, including nine *Proteobacteria*, four *Firmicutes*, three *Bacteroidetes*, and five *Actinobacteria*. Several isolates were present in more than two b-PBRs; namely, two *Pseudomonas* spp. (MB2 and HM2), two *Sphingomonas* spp. (MB6 and MB18), *Chryseobacterium* sp., *Leifsoiasp* sp., *Flectobacillus* sp. and *Herbaspirillum* sp. The six strains, MB2, MB6, MB14, HM3, HM5, and HM8, indicated in Table 3 were selected as representative bacteria for co-culture experiments to investigate the effects of contaminated bacteria in further experiments.

3.2. Photoautotrophic and mixotrophic culture of *Chlorella* sp. KR-1

Many *Chlorella* spp. can utilize organic carbon under mixotrophic conditions [35,36]. Moreover, it has been reported that *Chlorella vulgaris* has an inducible hexose transport system that allows it to utilize glucose [37]. This would also provide an advantage to remove organic compounds dissolved in wastewater when the microalga is applied for microalgal biomass production or wastewater treatment. Actually, high biomass productivity under autotrophic condition is limited since mutual shading of cells can block penetration of light. Therefore, it might be recommended that microalgae are cultivated to improve microalgal biomass production with organic carbon compounds. The promoted growth of *Chlorella* sp. KR-1 under mixotrophic conditions was reported in our previous

Table 3. Identification of bacteria isolated from *Chlorella* sp. KR-1 culture of this study

Strain	Most closest species	Accession no. in Genbank	Similarity (%)	Selected strain
MB2	*Pseudomonas putida*	KT030908	99	Yes
MB6	*Sphingomonas* sp.	AB696775	99	Yes
MB7	*Cupriavidus* sp.	KR189892	99	No
MB8	*Bacillus thuringiensis*	FJ174628	100	No
MB9	*Bacillus luciferensis*	JQ579634	96	No
MB10	*Bacillus acidiceler*	KF914412	99	No
MB12	*Bacillus methylotrophicus*	KM659215	100	No
MB13	*Arthrobacter* sp.	AB987933	100	No
MB14	*Chryseobacterium daecheongense*	KJ147083	100	Yes
MB15	*Brevundimonas vesicularis*	KT751295	100	No
MB16	*Microbacterium oxidans*	KT580637	100	No
MB18	*Sphingomonas* sp.	KP866806	99	No
MB20	*Stenotrophomonas rhizophila*	JN700143	100	No
HM2	*Pseudomonas fluorescens*	KP126778	100	No
HM3	*Leifsonia* sp.	KT462730	99	Yes
HM4	*Pseudacidovorax* sp.	FJ581042	99	No
HM5	*Flectobacillus roseus*	KM507292	99	Yes
HM6	*Microbacterium trichothecenolyticum*	KP980598	100	No
HM7	*Candidatus Chryseobacterium massiliae*	AF531766	100	No
HM8	*Herbaspirillum huttiense*	KR856354	100	Yes
HM9	*Micrococcus leteus*	KT805418	100	No

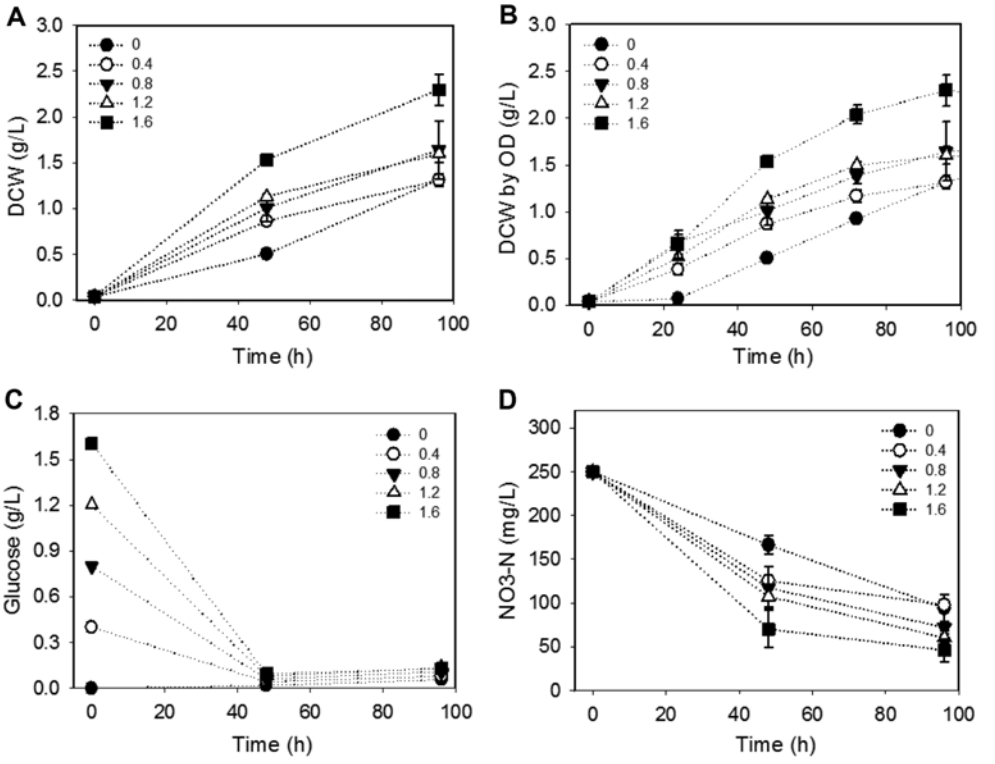

Fig. 1. Growth profiles of *Chlorella* sp. KR-1 under photoautotrophic and mixotrophic conditions (A) DCW, (B) indirect DCW (DCW by OD_{750}), (C) glucose consumption, and (D) nutrient (nitrate) removal rate. The number in the legend indicates the concentration of glucose at the unit of g/L.

study [19]. However, the bacterial influences on microalgal growth in mixotrophic condition should be appropriately taken into account because this condition is vulnerable to be contaminated by bacteria.

Initially, the axenic growth of *Chlorella* sp. KR-1 was compared after cultured under photoautotrophic and mixotrophic conditions (Fig. 1). Additional glucose was confirmed to promote the growth of *Chlorella* sp. KR-1 because biomass production increased on the increase of glucose in the range of 0, 0.4, 0.8, 1.2 and 1.6 g/L. The cell growth rates were 0.23, 0.41, 0.48, 0.55 and 0.75/day in response to 0, 0.4, 0.8, 1.2 and 1.6 g/L conditions at 48 h, respectively. However, the increase of cell growth rates slowly decreased after 48 h as glucose was depleted (Fig. 1). The highest efficiency showed 2.2-fold higher biomass production than autotrophic conditions at 48 h when there was an additional 1.6 g/L in the medium. The nitrate removal rate under mixotrophic conditions was also faster than under autotrophic conditions owing to the promoted cell growth (Fig. 1D). Therefore, it was clear that the additional glucose resulted in a significant increase of biomass productivity ($P < 0.05$, *t*-test). Finally, the cultivation for 96 h led to 1.3, 1.3, 1.6, 1.6 and 2.3 g DCW/L with 0, 0.4, 0.8, 1.2 and 1.6 g/L, respectively. Axenic conditions were confirmed every two days by a plating method and no bacterial colonies were found on plates.

3.3. Determination of bacterial cell density for co-culture experiments

The growth of the bacterial mixture was poor in the modified BG 11 medium, in which less organic carbon exists, without any microalgae. These findings demonstrated that the six isolates could not grow well with inorganic carbon sources. However, when the bacteria mixture (9×10^3 cells per mL) were cultured with microalgae (1.5×10^6 cells per mL), they grew rapidly within 24 h of cultivation to bacterial cell concentrations of 8.0×10^7 after 24 h without any glucose (A condition), 1.9×10^9 with additional glucose of 0.8 g/L (B condition) while the culture having only the bacterial cells showed 1.3×10^{10} cells per mL with the additional glucose (Fig. 2). Then, the bacterial cell concentrations decreased slightly after 24 h under all conditions, especially B and C (having additional glucose), owing to glucose depletion. Finally, the bacterial cell concentration in all PBRs converged to a constant concentration of approximately 10^8 cell/mL, which was used as the inoculum size for further co-culture experiments in this study.

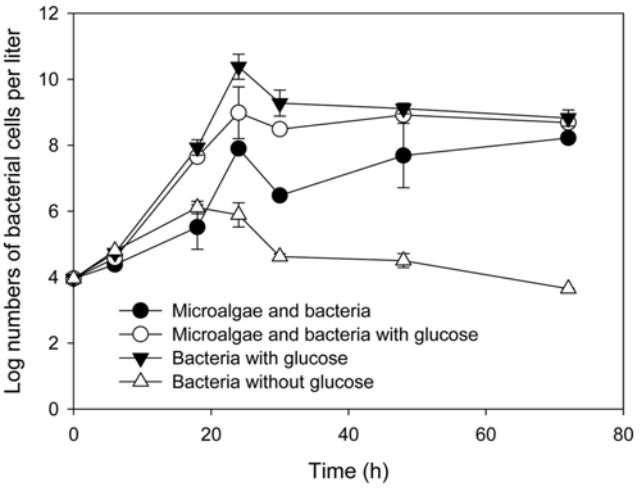

Fig. 2. Bacterial growth was monitored in different conditions by a plating method.

3.4. Effect of co-existing bacteria on *Chlorella* sp. KR-1 growth and FAME production

The above experiments revealed that additional organic sources can influence both the growths of *Chlorella* sp. KR-1 as well as co-existing bacteria. The total biomass production under axenic and artificially xenic conditions were then compared with each other under different conditions; namely, axenic conditions without (A0) and with glucose 0.8 (B0) and 1.6 g/L (C0); xenic conditions without (A1) and with glucose 0.8 (B1) and 1.6 g/L (C1) (Table 1).

Under axenic conditions, the growth rate and biomass production of *Chlorella* sp. KR-1 were higher than those under xenic conditions (Fig. 3). This trend was clearly observed under mixotrophic conditions with additional glucose. Specifically, the DCWs under axenic conditions were 1.5, 1.84 and 1.94 g DCW/L for A0, B0, and C0 condition at 96 h, while they were 1.15, 1.43 and 1.59 g DCW/L for xenic A1, B1 and C1 conditions. The indirect DCWs determined by OD showed larger differences in the values between axenic conditions of A0, B0, C0 (1.5, 1.84 and 1.98 g/L) and xenic conditions of A1, B1, C1 (1.0, 1.16 and 1.46 g/L).

However, dry cell weights showed less difference because bacterial cell biomass also contributes into total dry cell weight, especially under mixotrophic conditions. Therefore, the differences among the conditions were also monitored by the chlorophyll a contents (Fig. 3C). This is why it is necessary to develop an effective monitoring technique to estimate each potential of microalgae and bacteria in a mixed culture or contaminated culture. It is also required to

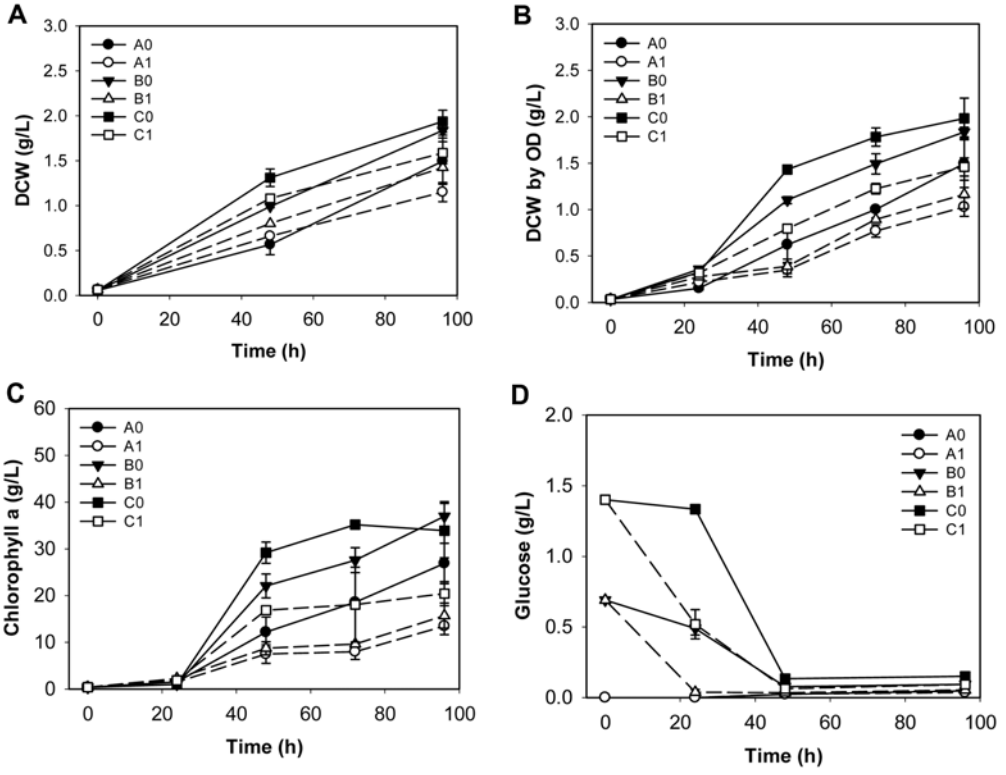

Fig. 3. Time course of *Chlorella* sp. KR-1 growth at axenic and artificial xenic conditions; axenic conditions without (A0) and with glucose 0.8 (B0) and 1.6 g/L (C0); xenic conditions without (A1) and with glucose 0.8 (B1) and 1.6 g/L (C1). The biomass production was measured by (A) DCW, (B) indirect DCW by OD, (C) chlorophyll a content, and (D) nutrient removal rate (glucose).

monitor the practical population dynamics of microalgae in a co-culture system such as microalgae involved wastewater treatment systems.

The glucose consumption with time is shown in Fig. 3D. The glucose concentrations were dramatically decreased with a faster rate in xenic culture than axenic culture. Glucose reduction efficiencies at 24 h in the cultures with 0.8 and 1.6 g/L were only 29% (B0) and 5% (C0), respectively, under axenic condition, while they were 93% (B1) and 63% (C1) under xenic conditions. At 48 h, the glucose reduction efficiencies were 89% (B0) and 90% (C0) under axenic conditions and 95% (B1) and 95% (C1) under xenic conditions. These results demonstrated that organic matter would be utilized more rapidly by co-existing bacteria than *Chlorella* sp. KR-1, while *Chlorella* sp. KR-1 under axenic culture could utilize organic compounds by mixotrophic metabolism with a retarded time.

Production of FAMEs under axenic (glucose concentration of A0 and C0 is 0 and 1.6 g/L) and xenic conditions (glucose concentration of A1 and C1 is 0 and 1.6 g/L) are shown in Table 4. The maximum production of FAMEs occurred in the order of C0 > A0 > A1 > C1. Mixotrophic conditions enhanced the FAMEs production in biomass under condition C0 (axenic with 1.6 g/L of glucose), but bacterial growth inhibits this effect under xenic conditions of C1 (xenic with 1.6 g/L of glucose). The enhancement of FAMEs production under mixotrophic conditions was observed in our previous study [25], but this effect might disappear at the cases of bacterial contamination. Considering the lower FAME contents in the xenic biomass than in the axenic biomass of the co-culture, the FAME productivity would be aggravated largely with decreased biomass productivity in xenic condition.

Table 4. FAMEs production of *Chlorella* sp. KR-1 under different culture conditions

	Fatty acid contents (mg/g Cell)						
	Total	C14:0	C16:0	C16:1n	C18:1n	C18:2n	C18:3n
A0	101	2.2	25	1.3	3.9	23	5.7
A1	98	2.4	23	1.5	5	23	3.8
C0	183	1.6	40	1	21	53	15.7
C1	94	2.3	25	1.5	7.7	21	3.3

*A0; axenic and 0 g-glucose/L, A1; xenic and 0 g-glucose/L, C0; axenic and 1.6 g-glucose/L, and C1; xenic and 1.6 g-glucose/L condition.

The biomass production at several different ratios of bacterial cells to *Chlorella* sp. KR-1 cells of 1 (D and G), 0.1 (E and H) and 0.01 (F and I) was investigated under the autotrophic (D, E and F) and mixotrophic conditions (G, H and I). The obtained results showed that the initial ratios of bacterial cells to microalgal cells could affect total biomass production (Table 5). Then, the *Chlorella* sp. KR-1 growth rate was not significantly influenced by the ratio of less than 1 in the case of autotrophic condition while it was only less than 0.1 under the mixotrophic condition. Therefore, the reuse of wastewater including organic compounds and recycling wastewater in microalgal cultivation systems would be limited by this effect due to bacterial contamination.

These results are different from those of several previous studies that showed co-existing bacteria could promote the growth of microalgae by releasing phytohormones and vitamin B_{12} [1,11,38]. Those studies did not consider the bacterial cell concentration, and general bacteria did not have positive effects, especially under artificial microalgal cultivation under mixotrophic conditions. Therefore, it is necessary to further investigate methods of controlling the numbers of contaminating bacteria when utilizing organic wastewater as the source of nutrients in an industrial microalgal cultivation system.

3.5. Biofilm formability of isolated bacteria

During co-culture of the bacterial mixture and *Chlorella* sp. KR-1, the aggregation of biomass was regularly observed, especially during the early cultivation period within 48 h. Therefore, each of the six isolates was co-cultured with *Chlorella* sp. KR-1, respectively, and their biofilm formability of the six isolates was examined by performing biofilm assays on microtiter plates. The control for biofilm assay was *Pseudomonas aeruginosa* ATCC 15692 (PAO1 strain) (positive control), which is well known as an excellent strain to produce biofilm aggregates, and nutrient broth medium without any bacterium was used for negative control [39]. The biofilm formation is facilitated by self-

Table 5. Growth rates measured by biomass in b-PBRs with various ratios of bacteria cell numbers to a fixed cell number of *Chlorella* sp. KR-1

Symbol	Autotrophic culture			Mixotrophic culture		
	D	E	F	G	H	I
The ratios of bacteria to *Chlorella* sp. KR-1*	1	0.1	0.01	1	0.1	0.01
Growth rate (/day) (standard deviation)	0.17 (0.007)	0.22 (0.007)	0.21 (0.002)	0.28 (0.007)	0.29 (0.004)	0.36 (0.011)

*The ratios indicates the cell numbers of the mixed bacteria to a fixed cell number of *Chlorella* sp. KR-1 (1.5×10^6 cells/mL) inoculated into b-PBRs. The number of bacteria cells was counted by colony count on plates and the cell number of *Chlorella* sp. KR-1 was counted by direct observation using a microscopy.

excreted extracellular polymeric substances (EPS) which are usually described as slime. The microorganisms which can produce EPS with high conglomeration ability are defined as a microorganism having high biofilm-forming ability. During the growth, these microorganisms usually generate the growth medium with a high optical density. Therefore, the biofilm-forming ability was determined through optical density of growth medium after a specific period. The results indicated that *Sphingomonas* sp. MB6 showed the strongest biofilm-forming ability among the six isolates (Fig. 4).

In general, the biofilm productivity is directly associated with the EPS production which is controlled by growth factors including pH, temperature, nutrient, oxygen (for aerobic bacteria). In the co-culture of *Sphingomonas* sp. MB6 and *Chlorella* sp. KR-1, the pH was maintained from 6.8 to 7.2, the temperature was controlled at 25 ± 3°C, the nutrient was supplied with glucose and trace element in the medium and the oxygen was available in the mixing gas that was purging continuously. Thus, the current co-culture mixotrophic conditions were sufficient and favorable for the growth and biofilm formation of *Sphingomonas* sp. MB6. These results explained why only co-culture of *Chlorella* sp. KR-1 and *Sphingomonas* sp. MB6 showed granular flocculation in the culture, especially under mixotrophic condition, unlike other co-cultures. The biofilm formation of *Sphingomonas* spp. in microalgal cultures was also mentioned in a previous study [8] and *Sphingomonas* spp. have frequently been detected in other microalgae studies [38,40-43].

Unlike other Gram-negative bacteria, *Sphingomonas* spp. are known to contain glycosphingolipids (GDLs) instead of lipopolysaccharides in their cell envelopes. Accordingly, they are more hydrophobic than other Gram-negative bacteria [44]. The hydrophobicity might result in aggregation with eukaryotic *Chlorella* sp. KR-1 [8]. In addition, its yellow pigment might protect low concentrations of *Chlorella* sp. KR-1 from the damage caused by strong light intensity, especially at an early cultivation time.

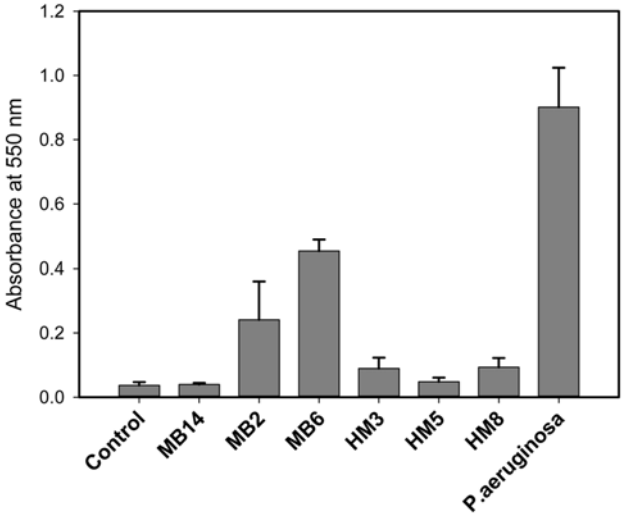

Fig. 4. Biofilm formability of isolated bacteria was determined on a microtiter plate. Control (fresh medium) and *P. aeruginosa* ATCC 15692 (PAO1 strain) was used as negative control (neg.) and positive control (pos.) for quantifying ability of biofilm production.

3.6. Population of *Chlorella* sp. KR-1 and *Sphingomonas* sp. MB6 during co-culture

The changes in abundance of *Sphingomonas* sp. MB6 under co-culture condition (having 0.8 g/L) with *Chlorella* sp. KR-1 during 144 h is depicted in Fig. 5. The population of *Sphingomonas* sp. MB6 and *Chlorella* sp. KR-1 in a same amount of DNA changed with opposite patterns during cultivation. The population of *Sphingomonas* sp.

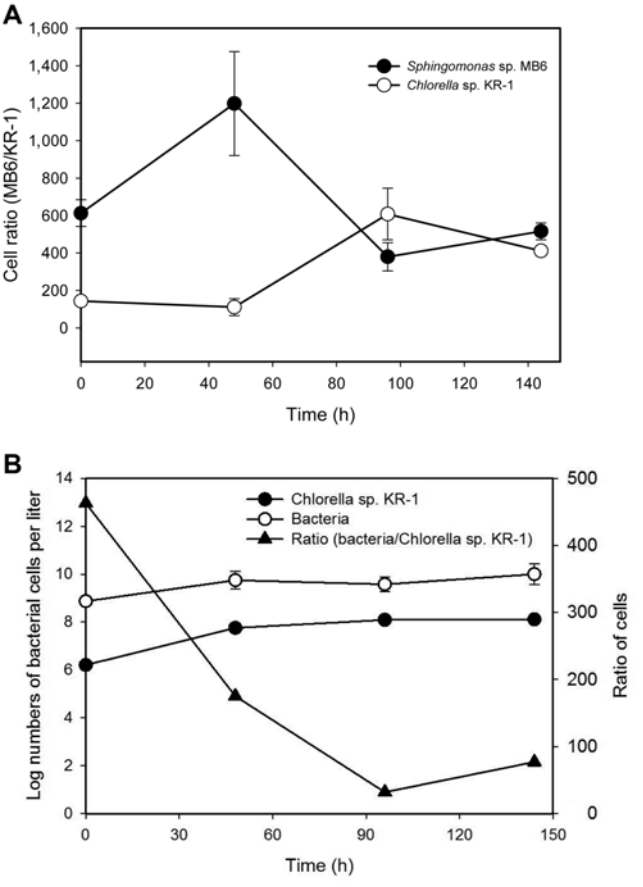

Fig. 5. Monitoring the growth of *Sphingomonas* sp. MB6 and *Chlorella* sp. KR-1 co-culture by the established qPCR. (A) The standard curves were generated from serial 10-fold 10^4 copies to 10^9 copies of plasmid DNA carrying on 16S rRNA gene of *Sphingomonas* sp. MB6 specific and 18S rRNA specific gene which was considered as *Chlorella* sp. KR-1 specific in this study. (B) Population change of *Sphingomonas* sp. MB6 during co-culture of *Chlorella* sp. KR-1 and bacterial mixture in glucose starvation condition (no additional glucose).

MB6 increased during the first 48 h, and then decreased after 48 h, whereas the population of *Chlorella* sp. KR-1 decreased slightly during the first 48 h and increased significantly thereafter. This difference indicated that bacterial growth or at least the growth of *Sphingomonas* sp. MB6 is faster than *Chlorella* sp, KR-1 during the first 48 h.

It is important to retain high microalgal productivity without excess bacterial growth because the extent of bacterial contamination during the early stage of microalgal cultivation has a significant effect on microalgal growth (Fig. 3). Therefore, this experiment showed the possibility that quantitative PCR can apply to monitor a major specific bacterial contamination in microalgae culture successfully.

4. Conclusion

Chlorella sp. KR-1 can produce more biomass and lipids under mixotrophic conditions. However, mixotrophic growth using organic wastewater and recycled wastewater in industrial cultivation can be easily contaminated with bacteria, which would inhibit the lipid and biomass production of *Chlorella* sp. KR-1. One commonly co-existing bacteria isolated from the photobioreactors of *Chlorella* sp. KR-1, *Sphingomonas* sp. MB6, showed strong biofilm formation, especially during the early stages of the cultivation, suggesting that this strain interacts with *Chlorella* sp. KR-1 more actively. In addition, this study showed that real time qPCR assay using specific primers is a promising technique to monitor a specific bacterium and microalgae in co-culture systems. The qPCR data presented here corresponded well with those based on conventional plating methods that showed co-existing bacteria can grow faster than *Chlorella* sp. KR-1 under mixotrophic conditions. This result has obvious practical implications for industrial microalgal cultivation for biofuel production and will be useful for forthcoming studies in this research field.

Acknowledgements

This study was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and Future Planning (NRF2013011775).

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