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Enhancing microalgal biomass productivity by engineering a microalgal–bacterial community

Dae-Hyun Cho [a], Rishiram Ramanan [a], Jina Heo [a,b], Jimin Lee [a], Byung-Hyuk Kim [a], Hee-Mock Oh [a,b], Hee-Sik Kim [a,b,*]

[a] Sustainable Bioresource Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon 305 806, Republic of Korea
[b] Major of Green Chemistry and Environmental Biotechnology, University of Science & Technology (UST), Daejeon 305 350, Republic of Korea

HIGHLIGHTS

- Phycosphere bacterial diversity analyzed in *C. vulgaris* by DGGE and pyrosequencing.
- Growth promoting and inhibiting microorganisms from *C. vulgaris* were co-cultivated.
- Four isolated bacterial strains improved algal growth, flocculation and lipid content.
- Algae supplied DOC, bacteria in return, supplied DIC and low molecular weight DOC.
- Engineered consortium significantly enhanced algal biomass and lipid productivity.

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ABSTRACT

This study demonstrates that ecologically engineered bacterial consortium could enhance microalgal biomass and lipid productivities through carbon exchange. Phycosphere bacterial diversity analysis in xenic *Chlorella vulgaris* (XCV) confirmed the presence of growth enhancing and inhibiting microorganisms. Co-cultivation of axenic *C. vulgaris* (ACV) with four different growth enhancing bacteria revealed a symbiotic relationship with each bacterium. An artificial microalgal–bacterial consortium (AMBC) constituting these four bacteria and ACV showed that the bacterial consortium exerted a statistically significant ($P < 0.05$) growth enhancement on ACV. Moreover, AMBC had superior flocculation efficiency, lipid content and quality. Studies on carbon exchange revealed that bacteria in AMBC might utilize fixed organic carbon released by microalgae, and in return, supply inorganic and low molecular weight (LMW) organic carbon influencing algal growth and metabolism. Such exchanges, although species specific, have enormous significance in carbon cycle and can be exploitated by microalgal biotechnology industry.

1. Introduction

In large scale microalgal cultivation systems, the role of bacteria and other microorganisms cannot be ignored but are understudied (Unnithan et al., 2014). In natural ecosystems, many studies have shown the influence of these organisms over each other (Ashen and Goff, 2000; Geng and Belas, 2010). These interactions have been either mutualistic or commensalistic or parasitic and are often considered species specific (Ashen and Goff, 2000; Sapp et al., 2007). Recently, certain class of bacteria widely known as Plant Growth Promoting Bacteria (PGPB) has been acknowledged to be enhancing algal growth (Gonzalez and Bashan, 2000; Hernandez et al., 2009). Most studies on algal–bacterial interactions only address algal growth promotion and often speculate on the mode of interaction, inadequately addressing the role of algae in those interactions (Gonzalez and Bashan, 2000; Henderson et al., 2008). Moreover, algal–bacterial researchers have only dealt with effect of one species of bacteria on the growth and physiology of algae (Gonzalez and Bashan, 2000; Kim et al., 2014a). There have been no systematic studies so far that have addressed the role of several microorganisms in the mini-ecosystem surrounding algal cell walls called phycosphere (Kim et al., 2014a). Besides, use of an artificially engineered consortium to alter the dynamics of this mini-ecosystem has not been endeavored (Brenner et al., 2008).

* Corresponding author at: Sustainable Bioresource Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon 305 806, Republic of Korea. Tel.: +82 42 860 4326; fax: +82 42 860 4594.
E-mail address: hkim@kribb.re.kr (H.-S. Kim).

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Chlorella is not only a commercially exploited species but also widely studied green algae with respect to interactions with other organisms viz., bacteria and virus. *Azospirillum* sp. and *Bacillus* sp. have been implicated in growth promotion of unicellular microalgae *Chlorella vulgaris*, and has been reported to influence cell morphology, lipid, and pigment production (Gonzalez and Bashan, 2000). *Azospirillum* is a rhizosphere-dwelling, N_2-fixing bacterium that is very versatile in nitrogen fixation assimilating NH_4^+, NO_3^-, or NO_2^- under microaerobic conditions while also denitrifying under anaerobic conditions and hence can act as a general PGPB for numerous plant species and algae, including *Chlorella* (Gonzalez and Bashan, 2000; Steenhoudt and Vanderleyden, 2000). While the role of *Azospirillum* has been studied well, the roles of other PGPB and bacteria, in general, are under studied. Hence, in this study, the influence of phycosphere bacteria on microalgal growth was ascertained. *Chlorella*, a model algae used thus far for studying interactions, isolated from environmental samples was selected (Cho et al., 2013), and the associated microbial diversity as well as the effect of most isolated strains on the host were studied. Based on the results of the study, an artificial bacterial consortium was developed and their growth patterns with algae were characterized and results on mechanism of the interaction were also presented.

2. Methods

2.1. Samples and culture condition

C. vulgaris OW-01 (NCBI accession number JQ664295) and *Scenedesmus* sp. YC001 (NCBI accession number KC439160) used in this study, were isolated from swine wastewater in Gonju, Korea and from an open pond in Daejeon, Korea respectively. Both cultures were grown in BG11 medium (Cho et al., 2013) and xenic unialgal cultures of *C. vulgaris* (CV) and *Scenedesmus* sp. (SC) were maintained by routine serial subculture. Axenic cultures of both strains were obtained in consequent treatment of ultrasonication, fluorescence activated cell sorter (FACS), and micropicking (Cho et al., 2013) and were continuously monitored for confirmation of axenicity using the said protocols. Microalgal strains were grown in 1 L Erlenmeyer flask constituting 300 ml BG11 medium for 14 days (constant stirring at 100 rpm, 25 °C, light intensity of 100 μmol m^{-2} s^{-1}).

2.2. Biomass determination

Growth of green algae was determined by dry cell weight (DCW) and by monitoring the cell count using hemocytometer (Peters et al., 2011). In co-culture experiments, microalgae and bacteria were separated by ultrasonication and centrifuged in the presence of 40% Histodenz (Sigma, USA). For microalgal DCW determination, the cells were separated by Histodenz. The bacterial cell numbers were monitored by cell counting using epifluorescence microscopy preceded by DAPI or SYBR green staining in both bacterial fraction and microalgal fraction, as a small fraction of bacteria were still attached to microalgal cell wall even after ultrasonication and Histodenz treatment.

2.3. Lipid content and fatty acid composition

The total lipids were extracted as described previously (Lee et al., 2010). The fatty acid composition was determined using the protocol supplied by MIDI Inc. and gas chromatography (GC-2010, Shimadzu, Koyto, Japan). Each fatty acid was identified and quantified based on comparing the retention times and peak areas with FAME Mix, C8-C24 (18918-1AMP, Supelco, Sigma–Aldrich Co. LLC., St. Louis, MO, USA).

2.4. DNA extraction and PCR

The microalgal biomass was washed twice with TE buffer (Tris 10 mM, EDTA 1 mM, pH 8.0) followed by centrifugation at 4800×*g* for 5 min and mild centrifugation at 1000×*g* for 5 min to eliminate free living bacteria. The biomass was resuspended in 1.5 ml distilled water and was centrifuged at 10,000×*g* for 3 min at room temperature. DNA extractions were carried out in accordance with eukaryotic microalgal nucleic acids extraction (EMNE) method (Kim et al., 2012). The purity and quantity of DNA were examined by electrophoresis on 1% agarose gel and measured using absorbances at 260 and 280 nm with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). One microliter of extracted DNA was used to amplify 16S rRNA genes by PCR using an MJ mini Thermal cycler (Bio-Rad, Hercules, CA, USA) with primers, 27f (5′-AGA GTT TGA TCC TGG CTC GA-3′) and 518r (5′-ATTACCGCGGCTGCTGG-3′) as described elsewhere (Cho et al., 2013).

2.5. Diversity analysis

2.5.1. Denaturing gradient gel electrophoresis

Three different kinds of samples were used for extraction of genomic DNA: (1) XCV, (2) supernatant of XCV culture medium after centrifugation at 3000×*g* and (3) filtered XCV (> 1 μm) culture medium (Minisart HY syringe filter, Sartorius, Germany). DGGE was performed as mentioned in an earlier study (Lee et al., 2013). Each DGGE band of interest was excised from the gel and cut bands were amplified as template for PCR. Forward and reverse strands sequences were assembled with SeqMan software (DNA STAR, Madison, WI) and homology searches of these assembled sequences were performed with the GenBank database using the Basic Local Alignment Search Tool (BLAST) in the NCBI (http://www.ncbi.nlm.nih.gov/).

2.6. Pyrosequencing

The PCR products were analyzed using pyrosequencing with a 454 Genome Sequencer FLX Instrument (Roche 454 Life Sciences, Branford, CT, USA). The raw reads were deposited into the NCBI short-reads archive database. The sequences obtained in this study were compared using Silva rRNA database.

2.7. Isolation and identification of microalgal associated bacteria

In order to isolate microalgae associated bacteria, algae was cultured in three different liquid medium which suit bacterial growth (R2A, TSA, BG11 + glucose 100 ppm). Subsequently, culture broths were spread on the corresponding agar plate medium for picking up single colonies. After 3 days of cultivation, each single, discriminated colony was plated further and incubated at 25 ± 1 °C and cultivated for 3 days. Each isolated bacterial strain was identified by sequencing 16S rRNA gene using colony PCR (Cho et al., 2013).

2.8. Co-culture of isolated bacteria with microalgae

In the co-cultivation studies, the inoculum ratio was one of the first determining factors. The ratio of cell numbers of algae and bacteria in the exponentially growing xenic culture (6–8 days) was determined by FACS and the same population ratio was used in co-cultivation experiments throughout this study (Powell and Hill, 2013). For e.g. the total cell numbers of bacteria was kept constant at 1×10^5 cells/ml in all co-cultivation studies involving either one, two, three or four strains of bacteria. Control cultures without algae were also established in BG11 medium and BG11 medium supplemented with glucose. The cultures were stirred at