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Microalgae-associated bacteria play a key role in the flocculation of *Chlorella vulgaris*

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HIGHLIGHTS

- Flocculation of axenic and xenic cultures of *Chlorella vulgaris* implicates bacteria.
- DGGE analysis indicates presence of five species of microalgae-associated bacteria.
- FACS treatment of xenic culture implicates three bacterial species in flocculation.
- Bacteria and its extracellular substances increase floc size.
- This study proves that bacteria play a major role in flocculation of microalgae.

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ABSTRACT

Flocculation is most preferred method for harvesting microalgae, however, the role of bacteria in microalgal flocculation process is still unknown. This study proves that bacteria play a profound role in flocculating by increasing the floc size resulting in sedimentation of microalgae. A flocculating activity of 94% was achieved with xenic *Chlorella vulgaris* culture as compared to 2% achieved with axenic culture. Denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA gene of xenic *C. vulgaris* culture revealed the presence of *Flavobacterium* sp., *Terrimonas* sp., *Sphingobacterium* sp., *Rhizobium* sp. and *Hyphomonas* sp. as microalgae-associated bacteria. However when *Flavobacterium*, *Terrimonas*, *Sphingobacterium* were eliminated by fluorescence activated cell sorter (FACS), flocculating activity reduced to 3%. Further studies with cell free extracts also suggest that bacterial extracellular substances might also have a role in enhancing flocculation. We conclude that the collective presence of certain bacteria is the determining factor in flocculation of *C. vulgaris*.

1. Introduction

Microalgae are now receiving extensive global attention as a potential source for biofuel production after being touted as an alternative fuel source some decades ago (Benemann et al., 1977; Oswald and Golueke, 1960). The production process of biodiesel using microalgal biomass includes cultivation, harvest, oil extraction, and conversion. Harvesting of microalgae is one of the most critical steps which involves separation of biomass from culture medium and contributes about 20–30% of the total biomass production cost (Gudin and Thepenier, 1986; Uduman et al., 2010). Harvesting is especially critical when the product of interest is a relatively low-value product like biodiesel (Vandamme et al., 2010). The major challenges in harvesting microalgae is because of small size of microalgal single cell (typically a few micrometer) and its low concentration in the culture medium (0.5–2 g L^{-1}) (Schlesinger et al., 2012; Vandamme et al., 2011).

Most common harvesting methods include flocculation, gravity sedimentation, centrifugation, filtration and ultrafiltration, sometimes with an additional flocculation step or with a combination of flocculation–flotation. Centrifugation is one of the preferred methods for microalgal cell recovery because of effectiveness and rapidness but it requires energy thereby increasing operating costs (Molina Grima et al., 2003). Membrane replacement is the major cost involved in filtration method and this also depends on the concentration of microalgae (Wilde et al., 1991). One of the most potential methods to reduce cost and energy usage during harvesting is flocculation (Wyatt et al., 2012). Algal cells carry negative charge on the cell surface preventing aggregation because of repulsion; however the addition of cationic metal ions such as Ca^{2+} and Fe^{3+} can neutralize this charge, leading to the aggregation of cells.

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Various flocculation methods result in higher particle sizes to enable gravity sedimentation, centrifugal recovery, and filtration (Molina Grima et al., 2003). The harvesting of microalgal cells by flocculation is more convenient process than contemporary methods such as centrifugation and filtration because it allows the treatment of large quantities of microalgal culture (Lee et al., 1998) and can be applied to a wide range of species (Pushparaj et al., 1993). Among the various flocculants, Aluminium sulphate (Alum) is most widely used for removal of algae, because of ease of use and application (Ebeling et al., 2003; Schlesinger et al., 2012). However, it cannot be applied over a wide pH range, moreover, floc size with alum when compared to ferric flocs is smaller resulting in ineffective sedimentation (Ebeling et al., 2003). Other cations such as calcium and magnesium also have a positive effect on flocculation in high pH (Vandamme et al., 2012). In addition, cationic polymers such as chitosan (Divakaran and Sivasankara Pillai, 2002), or alkalis such as NaOH have been used to achieve better flocculation (Brennan and Owende, 2010).

On the contrary, various species of algae have been reported to auto-flocculate (Spilling et al., 2011; Sukenik and Shelef, 1984). There have been reports of role of bacteria and extracellular polymeric substances in enhancing flocculating activity of algae (Grossart et al., 2006b; Kim et al., 2011; Tolhursf et al., 2002). However, the bacteria involved in flocculation and mechanism behind the process have been largely unclear. This study unveils the plausible role of algal-bacterial association in flocculation by experimenting with axenic, xenic and incomplete axenic (partially purified) cultures of *Chlorella vulgaris* under different ionic conditions.

2. Methods

2.1. Axenic and xenic culture of C. vulgaris

C. vulgaris (NCBI accession number JQ664295) used in this study was obtained from swine wastewater in Gonju, Korea, and was grown in BG11 medium (UTEX, 2009). *C. vulgaris* was inoculated into 300 ml of the BG11 medium in 1 L Erlenmeyer flask. The algal culture was stirred at 100 rpm, at 25 °C with a light intensity of 100 μmol m^{-2} s^{-1}. Axenic *C. vulgaris* colony was obtained in consequent treatment of ultrasonication, fluorescence activated cell sorter (FACS), and micropicking from swine wastewater sample (D-H.C., R.R., J.L., B-H.K., H-M.O., and H-S.K., unpublished data). Long-term laboratory xenic culture of *C. vulgaris* was maintained by routine serial subculture over 3 months. Each *C. vulgaris* culture was cultivated for 14 days in BG11 medium.

2.2. Optimization of pH, flocculant concentration and flocculating activity

After cultivation, pH of the cultures of axenic and xenic *C. vulgaris* was 8.1 and 8.9, respectively. The cultures were diluted with BG11 to equalize the microalgal cell concentrations to 6×10^6 cells ml^{-1}, and the pH was adjusted to 3, 5, 7, 9 and 11 with 1 N NaOH and 1 N HCl. 50 ml of *C. vulgaris* culture was mixed rapidly (300 rpm) for 30 s followed by slow mixing at 100 rpm for 1 min. Subsequently, cationic coagulant, $CaCl_2$ (10 mM) was added followed by rapid mixing at 300 rpm for 30 s and slow mixing at 100 rpm for 1 min. Then $FeCl_3$ (0.26 mM) was added to the culture, and mixed once again rapidly at 300 rpm for 30 s and slowly at 100 rpm for 1 min (Fig. 1A). The cultures were left for 2 min and 1 ml of aliquot was withdrawn and cell number was counted using C-chip hemocytometer (Digital Bio, Korea) at 200× magnification in an optical microscope (Nikon, Japan). Flocculating activity was calculated by the following equation (Kim et al., 2011; Oh et al., 2001):

$$\text{Flocculating activity } (\%) = \left(1 - \frac{A}{B}\right) \times 100 \quad (1)$$

where A is the cell number after flocculation and B is the cell number before flocculation.

The flocculating activity was also monitored at different concentrations of the flocculants used in this study, $CaCl_2$ and $FeCl_3$, respectively. At the end of flocculation experiment, cells were observed on a microscope to check for the differences in floc morphology (Microphot-FXA, Nikon, Japan). The zeta potential was measured in folded capillary cells with undiluted 1 ml samples of axenic and xenic culture (Zetasizer Nano ZS, Malvern, GK) (Henderson et al., 2008).

2.3. Flocculation of axenic C. vulgaris cells mixed with microalgal cell-free xenic culture broth

Two kinds of xenic culture broth was prepared after removing microalgal cells (bacterial cell broth) and all microbial cells (filtered broth) from the culture of xenic *C. vulgaris*. In the bacterial cell broth, only microalgal cells were removed by weak centrifugation at 3515×*g* for 10 min from xenic *C. vulgaris* culture leaving behind the bacterial cells, as the name suggests. In the filtered broth, all microbial cells were removed from xenic *C. vulgaris* culture by 0.22 μm Millipore Express PLUS membrane filter at <40 psi (Fig. 1B). The pH of each broth was adjusted to 11 with 1 N NaOH. For the flocculation analysis, 6×10^6 cells ml^{-1} of *C. vulgaris* cells were taken from the axenic culture and then mixed with the bacterial cell broth and filtered broth, respectively. Flocculating activity and pH of axenic *C. vulgaris* cells mixed with bacterial cell broth and filtered broth were measured immediately and after incubation for 24 h at room temperature.

2.4. DGGE analysis and sequencing

Four different kinds of samples were used for extraction of genomic DNA: (1) Long-term laboratory xenic culture of *C. vulgaris*, (2) incomplete axenic culture, (3) supernatant of xenic *C. vulgaris* culture medium after centrifugation and (4) filtered xenic *C. vulgaris* culture medium. For denaturing gradient gel electrophoresis (DGGE) analysis, 16S rRNA gene sequence was partially amplified. PCR was performed with two sets of primers as follows; 341F with a GC-clamp (341F, 5′-CCT ACG GGA GGC AGC AG-3′; GC-clamp 5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3′) and 786R (5′-CTA CCA GGG TAT CTA ATC-3′). DGGE was performed using the Dcode™ system (Bio-Rad Laboratories, USA). PCR products were applied directly onto 8% (w/v) polyacrylamide (37.5:1 acrylamide/bisacrylamide) gels with denaturing gradient range from 30% to 60% (100% denaturant solution contains 7 M urea and 40% v/v formamide). Electrophoresis was run in 1× TAE (40 mM Tris, 20 mM acetate, 1 mM EDTA pH 7.4) at 60 V at 60 °C for 18 h. Gels were stained with ethidium bromide (0.2 μg ml^{-1}, 1× TAE) for 15 min and destained in deionized water for 5 min and then visualized by using a KODAK Gel Logic 100 Imaging System. Each DGGE bands of interest was excised from the gels 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/). The sequences obtained in this study were deposited in the GenBank database under accession numbers from JX270632 to JX270636.

2.5. FACS treatment of xenic culture

Incomplete axenic *C. vulgaris* was a partially purified xenic culture obtained by cell sorting using a BD FACSAria cell sorter (Becton Dickinson, USA). Flow-cytometry and cell sorting experiments