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Potential of water surface-floating microalgae for biodiesel production: Floating-biomass and lipid productivities

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Microalgae have been accepted as a promising feedstock for biodiesel production owing to their capability of converting solar energy into lipids through photosynthesis. However, the high capital and operating costs, and high energy consumption, are hampering commercialization of microalgal biodiesel. In this study, the surface-floating microalga, strain AVFF007 (tentatively identified as *Botryosphaerella sudetica*), which naturally forms a biofilm on surfaces, was characterized for use in biodiesel production. The biofilm could be conveniently harvested from the surface of the water by adsorbing onto a polyethylene film. The lipid productivity of strain AVFF007 was 46.3 mg/L/day, allowing direct comparison to lipid productivities of other microalgal species. The moisture content of the surface-floating biomass was 86.0 ± 1.2%, which was much lower than that of the biomass harvested using centrifugation. These results reveal the potential of this surface-floating microalgal species as a biodiesel producer, employing a novel biomass harvesting and dewatering strategy.

[**Key words:** Microalgae; Biodiesel; Dewatering; Floating; Harvesting]

The provision of a sustainable energy supply and the need to reduce CO_2 emissions are two major challenges facing the world. Microalgal biodiesel has been recognized as a promising alternative to petroleum as a fuel, owing to its near-to-zero net CO_2 and sulfur emissions (1). As an additional benefit, microalgal biodiesel production will not compete with food production, fodder or other products derived from crops (2). However, high capital and operating costs, as well as high energy consumption, have hampered the industrial production of microalgal biodiesel significantly. The microalgal biodiesel production process can be divided briefly into five steps; mass cultivation of microalgae, harvesting and dewatering, lipid extraction, conversion into biodiesel, and management of by-products (3). Taking the entire production process into consideration, harvesting and dewatering of small microalgal cells (typically in the range of 3–40 μm diameter) from low cell density culture (typically in the range of 0.3–1 g L^{-1}) accounts for 20–30% of the total costs, and can potentially consume tremendous amounts of energy. Although harvesting and dewatering in commercial microalgae production facilities is generally performed by centrifugation, that recovery method is energy intensive.

Recently, several alternative methodologies, such as filtration (4–6), gravity-sedimentation (7,8), flocculation (9), or flotation (10,11), have been proposed in place of centrifugation for harvesting microalgae. These methodologies are generally considered more advantageous and effective than centrifugation (12). However, even using those approaches, the microalgal cells still need to be concentrated by chemical or mechanical methods. These facts suggest that processes that lead to the self-condensation of microalgae, such as self-aggregation, auto-flocculation, or biofilm formation, could provide routes to superior cost and energy savings during the production of microalgal biodiesel.

In our group, microalgae isolated from seawater and freshwater have been studied with regard to their lipid productivities (13–16). Recently, we have focused on the study of surface-floating microalgae, isolating 168 microalgal strains with a water surface-floating ability. These microalgae form floating biofilms naturally on the surface of the water during cultivation, and can contribute to efficient biomass harvesting, resulting in cost and energy savings. Following screening for microalgal oil production, strain AVFF007 was selected as one of the highest oil producers among water surface-floating microalgae. However, the possibilities and advantages afforded by surface-floating algal biomass for biodiesel production have not been studied, despite a report showing that some microalgal strains can form biofilms at the water surface.

In this study, the growth characteristics and surface-floating properties of strain AVFF007 were investigated. Furthermore, the moisture content of both surface-floating and non-floating biomass was measured. Based on these results, the potential for oil production is discussed, with consideration of efficient harvesting and dewatering systems, to evaluate the feasibility of biodiesel production using water surface-floating microalga.

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MATERIALS AND METHODS

Strain and growth conditions A green microalga species (termed strain AVFF007) found floating naturally on a water surface, was isolated from a freshwater pond in Kyoto, Japan. Strain AVFF007 was maintained in CSi medium (17) under continuous illumination using cool white fluorescent lights. To obtain the floating biomass, the strain AVFF007 was statically inoculated into 40 mL volume plastic cases (size: W50 × D63 × H25 mm, AS ONE, Osaka, Japan) in a desiccator (AS ONE), at an initial cell density of 1×10^5 cells/mL. Especially, columnar cases with various heights were employed to investigate the effect of culture depth on biomass yield. The cells were grown at 25°C under continuous and cool white fluorescent lights at 200 μmol m^{-2} s^{-1}. Modified-CSi medium was used to optimize biomass yield. Modified-CSi medium: [750 mg $Ca(NO_3)_2 \cdot 4H_2O$, 500 mg KNO_3, 142 mg K_2HPO_4, 200 mg $MgSO_4 \cdot 7H_2O$, 15 mg Na_2EDTA, 111 mg KH_2PO_4, 0.5 μg vitamin B_{12}, 0.5 μg biotin, 50 μg thiamine HCl, 2.94 mg $FeCl_3 \cdot 6H_2O$, 540 μg $MnCl_2 \cdot 4H_2O$, 330 μg $ZnSO_4 \cdot 7H_2O$, 60 μg $CoCl_2 \cdot 6H_2O$, 37.5 μg $Na_2MoO_4 \cdot 2H_2O$ per liter of distilled water at pH 6.0]. Both the depth of medium (0.4, 0.8, 2.0, 3.0 and 4.5 cm) and the CO_2 concentration (0–20%) were determined to optimize biomass yield. CO_2 gas was generated in the desiccator by reacting 1.25 g sodium bicarbonate with 0.95 g citric acid. Both reagents in the vial were reacted with 0.8 mL ultrapure water. CO_2 concentrations were estimated on the basis of the desiccator volume and expected CO_2 emissions. The vial was changed every two days.

Lipid analysis BODIPY505/515 was used to stain neutral lipids in cells, as previously reported (18). Stained cells were observed under a fluorescence microscope (BX51; Olympus Corporation, Tokyo, Japan) equipped with a cooled digital camera (DP-70; Olympus), an NIBA filter set for BODIPY fluorescence and a WIB filter set for chlorophyll fluorescence.

Lipids from lyophilized algal biomass were extracted with chloroform and methanol, by slightly modifying the 'Folch method' (19). Dry algal biomass (50 mg) was ground using a mortar and pestle for 5 min, then mixed with 4 mL of chloroform and 2 mL of methanol. The mixture was then centrifuged at 1000 ×*g* for 5 min. The supernatant was collected and residual biomass was extracted once more. The supernatants were combined, and 1.25 mL of 0.1 M potassium chloride solution was added. Following centrifugation, the lower phase was transferred into a vial and then dried using argon gas. Lipid content was calculated as a percentage of the dried cell mass. Lipid productivity was calculated as: (Biomass productivity) × (Lipid content).

In order to prepare fatty acid methyl esters (FAMEs), the lyophilized cells were directly transmethylated using 1.25 M hydrochloric acid in methanol (1 h at 100°C). The FAMEs were extracted with *n*-hexane and analyzed by GC/MS (QP2010 Plus; Shimadzu, Kyoto, Japan) with FAMEWAX (Restek, Bellefonte, PA, USA) in electron impact mode. FAMEs were identified using "F.A.M.E. Mix, C4-C24 Unsaturates" analytical standards (Sigma-Aldrich, Dorset, UK).

18S rDNA sequence analysis The 18S ribosomal DNA (rDNA) was amplified by polymerase chain reaction (PCR). A 1 mL sample of cell suspension (approximately 1×10^7 cells mL^{-1}) was disrupted by boiling for 5 min. Disrupted cell lysate (1 μl) was used as the template. The following universal primers were used: Forward primer, 5′-GGT GAT CCT GCC AGT AGT CAT ATG CTT G-3′ (ss5); Reverse primer, 5′-GAT CCT TCC GCA GGT TCA CCT ACG GAA ACC-3′ (ss3) (20). PCR products were separated by electrophoresis. Sequences of 18S rDNA were determined using an ABI PRISM 3100 DNA Sequencer (Applied Biosystems, Foster City, CA, USA) using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit. The following primers were used for sequencing: Forward primers, ss5 and 5′-ATC CAA GGA AGG CAG CAG GC-3′ (18SU467F); Reverse primers, ss3 and 5′-CTC CAC CAA CTA AGA ACG GC-3′ (18SL1310R) (21). Sequence similarities were analyzed by BLAST.

Density equilibrium analysis The density equilibrium of the microalgal cells was measured as previously reported (22). Briefly, culture aliquots were centrifuged in CsCl gradients, spanning a concentration range from 35% to 105% (w/v). Concentration increments of 10% were prepared. Floating and non-floating cells of strain AVFF007 were layered on top of the CsCl gradient, and centrifuged at 20,000 ×*g* for 30 min at 4°C.

Measurement of biomass and lipid contents A polyethylene film was placed over the floating biofilms of strain AVFF007. The adhering biofilm was then scraped off using a scraper. The cells harvested from polyethylene films (0.03 mm × 950 mm, Catlog No. 107-12401, Kokugo Co., Ltd. Tokyo, Japan) were defined as water surface-floating cells (or floating cells) for the purposes of this study. The cells that remained in the liquid culture medium, non-floating cells, were then filtered through a glass fiber filter (GA-55; pore size 0.6 μm; Advantec, Tokyo, Japan). The harvested floating and non-floating cells were then lyophilized and weighed.

Measurement of moisture content For moisture content measurements, the floating cells were harvested as described above, while the non-floating cells were harvested by centrifugation at 8500 ×*g* for 10 min. The harvested biomass was weighed before and after lyophilization. The moisture content of harvested biomass was calculated as follows:

$$\text{Moisture content (\%)} = [(\text{Mass of wet biomass}) - (\text{Mass of dry biomass})]/(\text{Mass of wet biomass}) \times 100 \quad (1)$$

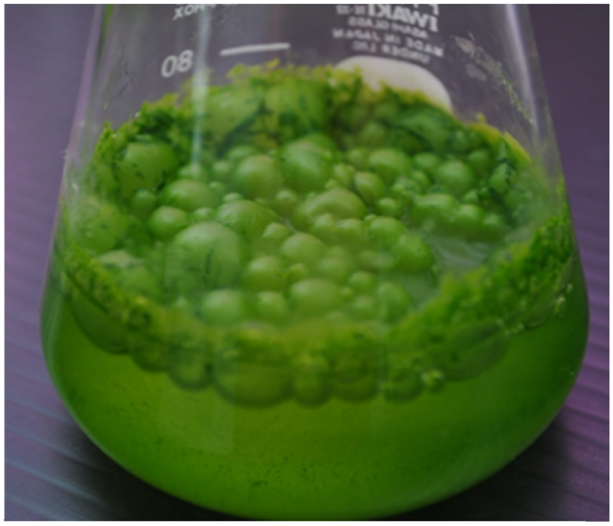

FIG. 1. Photograph of strain AVFF007 culture in CSi medium.

RESULTS

Characterization of strain AVFF007 Fig. 1 shows a typical static culture of strain AVFF007 in a glass flask. Most of the cells floated naturally upon the surface of the medium during static cultivation, while a portion of cells settled to the bottom of the medium. The floating cells formed biofilm, the surface of which was completely dry. Static cultivation was essential for the flotation ability of the cells to result in biofilm formation, since biofilms were not observed under well-mixed culture conditions with continuous agitation and aeration. The cells started to form a biofilm on the surface of the medium after 4 days of cultivation (Fig. 2A). After another 3 days of cultivation (7 days in total), most of the water surface was covered with a biofilm, along with gas bubbles that may have comprised oxygen generated by photosynthesis. The generation of gas may contribute, in part, to ability of strain AVFF007 to float. After 10 days, older biofilms exhibited chlorosis. The biofilm was too stiff to permit re-suspension in aqueous solution, however that stiffness facilitated harvesting from the culture surface. Fortuitously, the biofilm adhered to a polyethylene film, which allowed the biomass on the surface to be completely recovered (Fig. 2B). It should be noted that the biofilm attached onto a polyethylene film can be readily removed from the surface using a scraper.

Both floating and non-floating cells had a spherical morphology (Fig. 3A and B), with cell diameters ranging from 4 to 28 μm (Fig. 3C). The average diameter of the floating cells was 22 ± 3 μm, which was more than two times that of non-floating cells (average cell diameter: 8 ± 2 μm). This difference could be due to increased light availability for surface-floating cells than for non-floating cells. The floating cells had a sandy yellow appearance under the microscope, as shown in Fig. 2A, suggesting chloroplast degradation and subsequent accumulation of oil. As we expected, BODIPY staining revealed that more oil bodies had formed in the floating cells than in the non-floating cells (Fig. 3A and B). To further confirm the difference in oil contents, BODIPY/chlorophyll a ratio was determined to evaluate the oil accumulation. There is a negative correlation between oil and chlorophyll contents, so it can be utilized as an indicator of oil accumulation in microalgae. The fluorescence ratio of BODIPY/chlorophyll *a* was 0.93 ± 0.12 for floating cells and 0.52 ± 0.17 for non-floating cells, indicating that the neutral lipid content in floating cells was higher in floating cells. The difference may also be due, in part, to the flotation ability of this