

**Effects of alginate oligosaccharide mixtures on the growth and fatty acid composition of the green alga *Chlamydomonas reinhardtii***

Running title: Effects of alginate oligosaccharides on a green alga

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*Abbreviations:* Alginate oligosaccharide mixture (AOM), Acid hydrolysis (AH), Enzymatic degradation (ED)

## ABSTRACT

Alginate is a natural acidic linear polysaccharide that is produced by brown seaweeds. It is currently used in a broad range of commercial enterprises, such as the food and medical products industries. Recent evidence has demonstrated that alginate oligosaccharides may function as growth promoting agents for certain plant cells, including those of some green algae. *Chlamydomonas reinhardtii* is a green alga that is used as a model organism in fundamental molecular biology studies; it is also a producer of biohydrogen. In the present study, we examined effects of two types of alginate oligosaccharide mixtures (AOMs), which were prepared by either enzymatic degradation (ED) or acid hydrolysis (AH), on the growth of *C. reinhardtii*. Growth was significantly promoted by AOM (ED) in a concentration-dependent manner. The maximum effect was observed on day 4 of treatment. The fatty acid composition of *C. reinhardtii* was also influenced by AOM (ED); the levels of C16:0, C18:2 cis and C18:3 *n*-3 increased in treated cells. AOM (AH) and the other saccharides that we tested did not affect the growth of *C. reinhardtii*. The effects that we identified could promote efficient biomass production by reducing culture times and by changing cellular fatty acid levels.

**Keywords:** Alginate oligosaccharide; *Chlamydomonas reinhardtii*; Fatty acid composition; Green alga; Growth promotion

## INTRODUCTION

Alginate is a natural acidic linear polysaccharide that is composed of  $\alpha$ -L-guluronate and  $\beta$ -D-mannuronate (uronic acids) residues. This carbohydrate occurs in the cell walls of brown algae, such as *Laminaria japonica* and *Undaria pinnatifida*. Alginate and its derivatives are currently used in a wide range of commercial enterprises, including the food, medical, cosmetic and textile-processing industries. These industries take advantage of the unique physicochemical properties of alginate, particularly its high viscosity in aqueous solutions and its gel-forming capacity in the presence of calcium ions (1). Alginate also has pharmacological properties, such as the induction of cytokine production in human monocytes (2). The dietary administration of alginate stimulates the immune abilities of white shrimp and juvenile carp (3-4).

The properties of alginate oligosaccharides that were prepared using bacterial alginate lyase have been widely investigated (5-8). Interestingly, unsaturated alginate oligomers prepared by enzymic depolymerization induce tumor necrosis factor (TNF)- $\alpha$  secretion from RAW264.7 cells, whereas saturated alginate oligomers prepared by acid hydrolysis have only minimal effects (9). Hereafter, we refer to the alginate oligosaccharide mixture (AOM) prepared by enzymatic degradation as AOM (ED). We refer to the AOM prepared by acid hydrolysis as AOM (AH). The growth of *Nannochloropsis oculata* (Eustigmatophyceae), which is a common heterokont microalga that is used in bivalve and zooplankton aquaculture, is promoted by AOM (ED) in a concentration-dependent manner (10). Yokose et al. investigated the effects of AOM (ED) on the growth of various organisms: six mammalian cell lines (HeLa, Vero, MDCK, XC, CHO, and L929), two species of phytoplankton (*Chaetoceros gracilis* and *Skeletonema* sp.), and two strains of marine bacteria (*Edwardsiella tarda*). Among the

samples tested, this group observed a concentration-dependent growth-promoting effect only for the diatom *C. gracilis*, which suggests that AOM (ED) has species-specific growth-promoting effects (11). *C. gracilis* is a very useful dietary item in shellfish aquaculture. Although the previous evidence is inconclusive, there are sufficient reasons to postulate that AOM (ED) will have growth-promoting effects on other commercially important algae.

The green alga *Chlamydomonas reinhardtii* occurs globally in many soil and freshwater environments. This alga is physiologically flexible in that it is generally photoautotrophic but is, nevertheless, able to multiply even in total darkness through the use of alternative organic carbon sources. In addition, it is common knowledge that *C. reinhardtii* is not only an essential model species for fundamental studies in physiology, biochemistry and molecular biology, but also an important species for studies on algal hydrogen production.

Currently, marine and freshwater microalgae are the subject of intensive research and increasing commercial attention. For example, microalgal biofuel plants have distinct advantages, including high yields, near-continuous harvest streams, and the potential for siting bioreactors on non-arable land (12). Schenk et al. predicted that there will be enormous efforts in the near future to reduce the costs of cultivation and to further improve biological performance in the production processes for microalgal biofuels (12). In addition, microalgae contain large amounts of polyunsaturated fatty acids (PUFAs: fatty acids with more than one double bond) and are widely used for aquaculture and the production of functional foods and pharmacological products.

For all of these reasons, our objective was to determine whether an AOM affects the growth physiology of *C. reinhardtii*. We examined the effects of AOM (ED) and AOM (AH) on the growth and fatty acid composition of *C. reinhardtii*. AOM (ED)

improved the growth rate and increased the levels of the fatty acids C16:0, C18:2 cis and C18:3 *n*-3, but AOM (AH) did not.

## MATERIALS AND METHODS

**Materials** We obtained alginate polymer (IL-6M) from Kimika Co., Tokyo, Japan. Glucose, galactose, mannose, xylose, and rhamnose were purchased from Wako Pure Chemical Industries Ltd., Osaka, Japan. All other chemicals were commercial products of the highest purity available.

**Preparation of alginate oligosaccharide mixtures (AOMs)** We digested alginate polymer with bacterial alginate lyase [following Nishikawa et al. (13)] to produce AOM (ED), which has an unsaturated terminal structure with a double bond. We also used an acid hydrolysis procedure to produce AOM (AH), which lacks the unsaturated terminal structure with a double bond. To prepare AOM (AH), we incubated a 1% alginate polymer gel in 200 ml of an acidic solution (0.3M HCl, pH 4.0) at 121°C for 80 min. After cooling at room temperature, the sample solution was neutralized with 1 M NaOH and lyophilized in a vacuum freeze-dryer (FD-1, Tokyo Rikakikai Co., Ltd., Tokyo, Japan).

**Analysis of AOMs by liquid chromatography/tandem mass spectrometry (LC-MS/MS)** An LC-MS/MS analysis of the AOMs was performed as previously described (13). For the analysis, an API2000 mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with TurboIonSpray (Applied Biosystems) for the electrospray ionization was used for the detection. The MS spectrum was obtained in the Q1 scan mode. An aqueous solution of the AOM (100 µg/ml) was injected into the LC-MS/MS machine. The conditions for the Q1 scan analysis were as follows: column, Mightysil RP-18GP Aqua 2 mm × 150 mm (Kanto Chemical, Tokyo, Japan); column

temperature, 40°C; mobile phase, 5 mM ammonium acetate; flow rate, 0.2 ml/min; injection volume, 10 µl; analytical time, 10 min; source polarity, positive ion mode; capillary voltage, 5 kV; source temperature, 500°C; declustering potential, 50 V; and mass range,  $m/z$  150-1500.

**Algal species and culture conditions** *Chlamydomonas reinhardtii* (CC-3395; arginine-requiring) was maintained at 26 °C in 100-ml flasks containing 50 ml of Tris-acetate-phosphate (TAP) medium (14) supplied with filter-sterilized L-arginine at a final concentration of 100 µg/ml. The flasks were shaken (100 rpm/min) under 200 ( $\pm$ 5) µmol photons/m<sup>2</sup>/s<sup>1</sup> of cool-white fluorescent illumination, which was provided continuously. All of the cultures were handled with sterilized instruments. We used cells in the exponential growth phase for all experiments. The growth rate (divisions/d) was calculated for each well of the 48-well cultivation plates (the plates are described in the following section) at three temporally consecutive data points following the methods of Brand et al. (15). We determined the maximum growth rates over the entire incubation period.

**Growth experiments in 48-well plates** We tested effects of AOM (ED and AH) on the growth of *C. reinhardtii* raised in 48-well plates (Becton Dickinson Co., Ltd., Franklin Lakes, NJ, USA). Ten microliters of an intact cell suspension (initial cell density: 10<sup>4</sup> cells/ml) were added to 990 µl of AOM (ED) or AOM (AH) (concentrations: 0, 250, 500, and 1000 µg/ml). For controls, we replaced the AOM with an equivalent volume of TAP medium. We counted the cells daily by light microscopy using five 10-µl subsamples from each well. When necessary, the samples were diluted 100× with fresh TAP medium. Each bioassay was performed in triplicate. On the fourth

day, the growth-promoting effects of AOM (ED) were maximal; at this point, we counted the cell densities in the treated and control wells. We calculated the proliferation index as the ratio of cell numbers in AOM (ED or AH)-treated wells to the cell numbers in control wells.

*Chlamydomonas reinhardtii* is photoautotrophic but is also able to use exogenous organic carbon sources, including acetate (16). We aimed to determine whether the AOM was being used as an exogenous carbon source to support cell growth. To do this, we compared the growth rates of AOM-treated cells on the fourth culture day with the growth rates of cells treated with a range of saccharides (rhamnose, xylose, mannose, galactose and glucose) at concentrations of 1000  $\mu\text{g/ml}$ .

**Analysis of fatty acid composition by gas chromatography (GC)** One milliliter of *C. reinhardtii* cell suspension (initial cell density:  $10^4$  cells/ml) was added to 99 ml of test solution (AOM (ED); concentration: 1000  $\mu\text{g/ml}$ ) in 200-mL glass flasks. For the controls, *C. reinhardtii* was grown in TAP medium without AOM (ED). This experiment was performed in triplicate. After four days of incubation under 200 ( $\pm 5$ )  $\mu\text{mol photons/m}^2/\text{s}^1$  of cool-white fluorescent illumination on a 12:12 h light:dark cycle at 26 °C, the cells in each flask were counted under light microscopy. The cell densities in the treated and control cultures were  $\sim 4.3 \times 10^5$  cells/ml and  $\sim 3.2 \times 10^5$  cells/ml, respectively. We centrifuged the suspensions ( $5000 \times g$ , 5 min, 4 °C) to obtain treated and untreated cell pellets for fatty acid analysis.

We determined the fatty acid composition of *C. reinhardtii* cells following the methods of Yokose et al. (11). Figure 1 depicts the analytical procedure. Methylated fatty acids were obtained with a fatty acid methylation kit (Nacalai Tesque, Kyoto, Japan) following the manufacturer's instructions. The fatty acids were extracted with



0.5 ml of analytical grade hexane (HEX) (Wako Pure Chemical Industries, Ltd.). Impurities in the extracted fatty acids were removed by solid-phase extraction using Sep-Pak<sup>®</sup> Silica cartridges (Waters, Milford, MA, USA). Subsequently, the fatty acid composition was determined using a gas chromatograph (GC-17A, Shimadzu) equipped with an Rtx-2330 column (60 m × 0.25 mm, Restek Corp., Bellefonte, PA, USA). The conditions for GC analysis were as follows: carrier gas, helium; analysis mode, splitless; injector temperature, 250 °C; detector temperature, 280 °C; and oven temperature, 160 °C (15 min after injection, oven temperature was raised from 160 °C to 220 °C at 4 °C min<sup>-1</sup>; oven temperature was maintained at 220 °C for 10 min). This analysis was performed in duplicate.

**Statistical analyses** We tested the growth experiment data (Figs. 4 and 5) for homogeneity of variances across treatments by Levene's test. The data were analyzed by a one-way analysis of variance (ANOVA) then subjected to Dunnett's post hoc tests. These statistical analyses were performed with the Statistical Package for the Social Sciences software (SPSS<sup>®</sup> Statistics 19; SPSS, Inc., Chicago, IL, USA). Differences were considered to be significant at  $P < 0.05$ .

## RESULTS

**LC-MS/MS detection of two AOM types** We detected AOM (ED) and (AH) by LC-MS/MS under positive ionization. Figure 2A shows the Q1 mass spectrum of AOM (ED). In positive ion mode, no protonated molecules  $[M + H]^+$  were detected, but we clearly identified ammonium-adducted molecules  $[M + NH_4]^+$  at  $m/z$  ratios of 370, 546, and 722. Sodium-adducted molecules  $[M + Na]^+$ , such as those at the  $m/z$  ratios of 375, 551, and 727, were also clearly observed. The analysis demonstrated that the primary oligomers in AOM (ED) were a dimer (352 Da), a trimer (528 Da), and a tetramer (704 Da). There were no significant levels of monomers or oligomers larger than a tetramer. Accordingly, we selected the ammonium adduct ion as the precursor ion for determining the specific  $m/z$  transitions of the AOM (ED) dimer, trimer and tetramer.

Figure 2B depicts the Q1 mass spectrum of AOM (AH). In positive ion mode, we detected no protonated molecules  $[M + H]^+$ ; however, we clearly identified ammonium-adducted molecules  $[M + NH_4]^+$ , including those at  $m/z$  ratios of 388 and 564. Sodium-adducted molecules  $[M + Na]^+$ , such as those at  $m/z$  ratios of 217, 393, and 569, were also clearly observed. The analysis demonstrated that the primary oligomers in AOM (AH) were a monomer (176 Da), a dimer (352 Da), and a trimer (528 Da). There were no significant quantities of oligomers larger than a trimer. Accordingly, we selected the ammonium adduct ion as the precursor ion for determining the specific  $m/z$  transitions of the AOM (AH) monomer, dimer and trimer. In addition, several minor signals were detected at less than 500  $m/z$  (Fig. 2), but these were background signals derived from the mobile phase as determined by the comparison with the blank (data not shown). Therefore, we concluded that these signals did not come from contaminative component(s).

### **Effects of the two types of AOMs and other saccharides on the growth of *C.***

***reinhardtii*** The growth of *C. reinhardtii* was significantly promoted by the addition of 1000 µg/ml AOM (ED) (Fig. 3A). AOM (AH) did not have an equivalent effect (data not shown). The mean growth rate of *C. reinhardtii* exposed to AOM (ED) was 1.62 divisions/d, and that of the controls was 1.26 divisions/d. The growth-promoting effect of AOM (ED) was maximal on day 4 of the experiment (Fig. 3B and C). Therefore, we used the relative cell densities on day 4 of the controls to those of cells treated with AOM (ED), AOM (AH) and other saccharides as indices of the growth-promoting effects.

As shown in Fig. 4, the growth of *C. reinhardtii* was promoted by AOM (ED) in a concentration-dependent manner. None of the AOM (AH) concentrations tested had a growth-promoting effect (Fig. 4), nor did the various saccharides applied to cultures (Fig. 5,  $P < 0.05$ ).

**Effect of AOM (ED) on the fatty acid composition of *C. reinhardtii*** Only AOM (ED) promoted the growth of *C. reinhardtii*. Accordingly, we examined its influence on cellular fatty acid composition, which shifted in response to the addition of AOM (ED) at a concentration of 1000 µg/ml (Fig. 6). The levels of C16:0, C18:2 cis, and C18:3 *n*-3 increased, and the level of C18:0 decreased slightly after treatment. Moreover, several kinds of fatty acids, such as C14:1, C17:0 and C20:0, were detected only when *C. reinhardtii* was exposed to AOM (ED).

## DISCUSSION

Microalgae are an untapped natural resource. There are more than 25,000 species, of which only a few are used commercially. However, sophisticated microalgal biotechnology has been introduced only recently to develop innovative processes and products, such as functional foods and cosmetics (17). The improvement of microalgal growth physiology during cultivation will certainly advance the development of the algal industries.

In the present study, we examined effects of two types of AOMs on the growth and fatty acid composition of *C. reinhardtii*. AOM (ED) had a growth-promoting influence similar to that demonstrated in several higher plants (6, 18) and marine microalgae (10-11). The maximum effect in our study was attained at 1000 µg/ml on day 4 of cultivation (maximum yield was ~180–220% of control yield, Figures 3–5). In *N. oculata*, the maximum effect occurs on day 18 at a concentration of 20 mg/ml (maximum yield: ~500% of control yield) (10). The maximum effect on *C. gracilis* occurs at a concentration of 125 µg/ml on day 7 [maximum yield: ~140% of control yield, (11)]. Hence, *C. reinhardtii* reacted more quickly to treatment than the other two species, and the experimental effect exceeded that of trials on *C. gracilis*. However, the growth responses of *C. reinhardtii* to the addition of AOM (ED) occurred at much higher concentrations than it did in the other two species, and the experimental effect was much greater for *N. oculata*.

Sager and Granick demonstrated that most of the carbon compounds they tested, including the simple sugars glucose, galactose, and arabinose, are non-toxic to *C. reinhardtii* but do not support cell growth in the absence of photosynthesis (19). Our findings agree with this earlier work in that AOM (AH) and other saccharides did not

affect the growth of this alga (Figs. 4 and 5). It is therefore unlikely that the specific growth-promoting effect of AOM (ED) is the result of its utilization as a heterotrophic carbon substrate.

Algal oil, biofuel and biodiesel have become increasingly attractive products in the global search for alternatives to fossil fuels. Microalgal oil is particularly interesting for industrial enterprises because it is produced by diatoms and green algae, which are readily cultivated. Empirical work on the optimization of microalgal cultivation will be required to improve industrial production, but the growth physiology of microalgae at a molecular level is not well understood. Because of these circumstances, the application of various molecular biological methods to *Chlamydomonas* has increased, and a wide variety of techniques, such as genetic transformation, are now available (20). Therefore, the observation of the growth-promoting effect of AOM (ED) on *C. reinhardtii*, which is widely known as an important model species for molecular biology, is important, and the clarification of the molecular mechanism of this growth promotion on this alga will contribute to development of the algal industries through improving production efficiency.

Recently, the utilization of PUFAs, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), for functional food and pharmacological products has received increased attention. For example, Barceló-Coblijn and Murphy proposed that dietary alpha-linolenic acid (ALA) is a crucial dietary source of  $n-3$  fatty acids and that the dietary inclusion of ALA is critical for maintaining tissue long chain  $n-3$  levels (21). Furthermore, problem-solving abilities during the first year of life have been reported to be related to maternal dietary DHA intake (22). We showed that in addition to improving growth rates, the supplementation of culture medium with AOM (ED) changed the fatty acid composition of *C. reinhardtii* (Fig. 6). Specifically, the levels of

C18:3 *n*-3 (omega-3 fatty acid), C16:0, and C18:2 *cis* were notably increased by this treatment, although the level of C18:0 decreased slightly. In addition, the levels of several kinds of fatty acids, such as C14:1, C17:0 and C20:0, increased upon the addition of AOM (ED) even though they were absent in controls. Therefore, it is possible that our findings will contribute to improving the production efficiency and quality of functional foods and pharmacological products.

Some of the PUFAs that occur in nature are among the essential nutrients that determine the nutritive value of rotifers or other zooplankton for fish larvae. This has been demonstrated in correlative studies on freshwater herbivorous zooplankton, such as daphniids (23-24) and mussels (25). Fatty acids in zooplankton are derived from their microalgal diet. The fatty acid compositions of selected species of algae can be altered by temperature and nutrient limitation (26-27). However, while these environmental effects could induce industrially desirable fatty compositions in grazed phytoplankton, they could also decrease the maximum microalgal yield. This was not observed in our study. We succeeded in changing the fatty acid composition of *C. reinhardtii* by adding AOM (ED) to the culture medium while, at the same time, increasing the maximum yield.

We have not yet identified the underlying mechanisms that explain the differences in the effects of AOM (ED) and AOM (AH) on *C. reinhardtii*. Over the last few years, the *Chlamydomonas* spp. has been used for the study of lipid-based signal transduction. For example, the breakdown of phosphatidylinositol-4,5-bisphosphate (PIP<sub>2</sub>) by phospholipase C and the formation of phosphatidic acid (PA) by the activity of phospholipase D are known to be stress-stimulated reactions in plants (28), and these activities were also observed in *C. moewusii* (29). In this study, we indicated that the level of ALA (an omega-3 fatty acid) was notably increased by AOM (ED) treatment

(Fig. 6). ALA has a key role as the starting material for the synthesis of jasmonic acid (JA), which regulates plant growth and development as well as plant responses to abiotic and biotic stresses (30). Therefore, the increase in ALA levels or the fluctuation in other PUFAs in response to AOM (ED) treatment might be caused by lipid-based signal transduction as a stress response in *C. reinhardtii*. Further studies on the effect of AOM (ED) on growth, the PUFA synthesis pathway and lipid-based signal transduction are required.

Differences in molecular structures might also account for the differences in the effects of AOM (ED) and AOM (AH). For example, the unsaturated terminal structure with a double bond in AOM (ED) may be linked to its bioactivity. Iwasaki & Matsubara showed that alginate oligosaccharides had a root growth-promoting effect in lettuce seedlings and that the tri-, tetra-, penta-, and hexa-saccharides were especially active (6). More recently, Xu et al. demonstrated that penta-guluronate (G5) had the highest root growth-promoting activity among guluronate species in carrot and rice (31). Thus, the most effective structures and molecular sizes of AOM (ED) vary widely across assay systems. It is therefore possible that the differences in the effects of AOM (ED) and AOM (AH) on *C. reinhardtii* stem from differences in the molecular sizes contained in the mixtures. To gain insight into the structure-activity relationship of alginate oligomers, it is important to use highly purified samples. Hence, it will be necessary to purify various oligomers with different degrees of polymerization, and the effect of each oligomer on *C. reinhardtii* must be examined in the future.

In conclusion, we demonstrated that (i) the growth of *C. reinhardtii* is significantly promoted by an AOM prepared by enzymatic degradation (ED), and (ii) AOM treatment also increases the cellular fatty acid content. AOM (AH) and other saccharides did not affect the growth of the alga. AOMs are derived from the cell walls of seaweeds that are

used extensively in the human food industry. These mixtures are a biologically nonhazardous and environmentally friendly material. Our results may contribute to improving the production efficiency and quality of functional foods, pharmacological products, microalgal biofuel, and algal diets for aquaculture.

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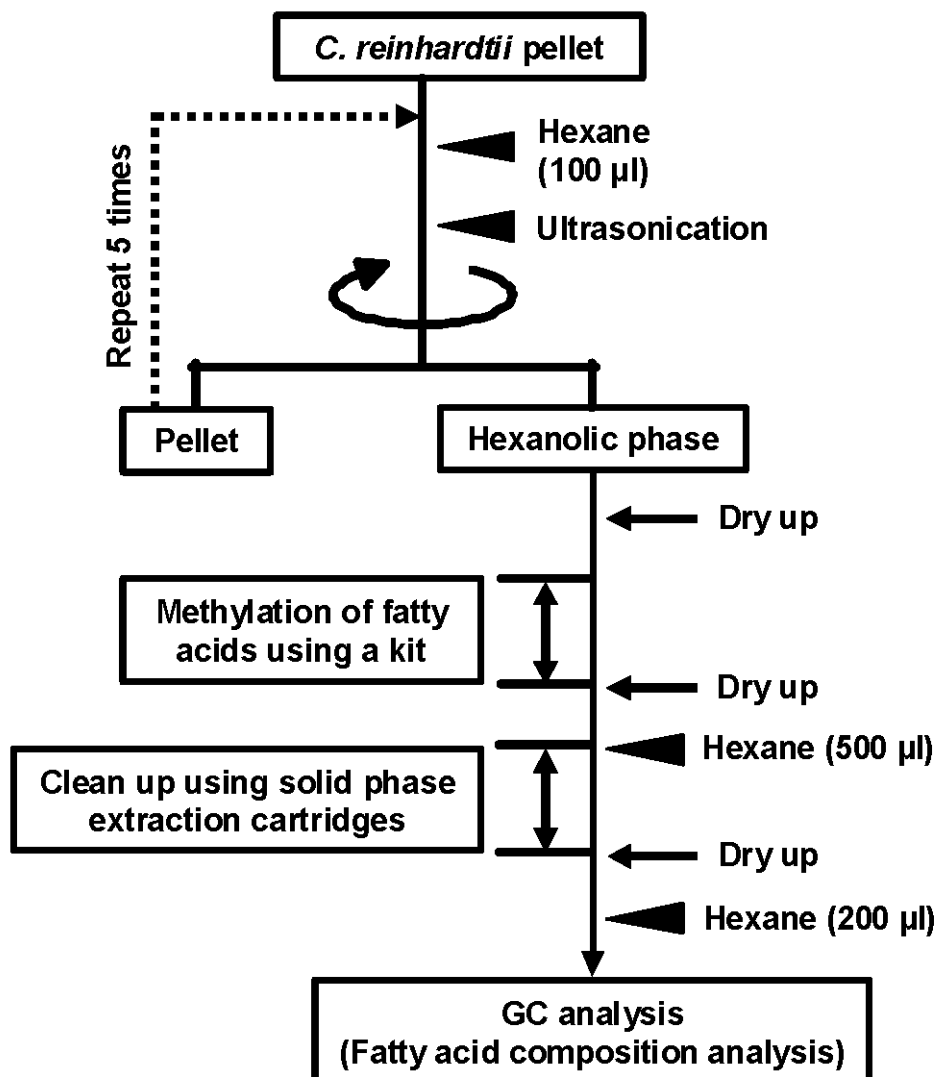
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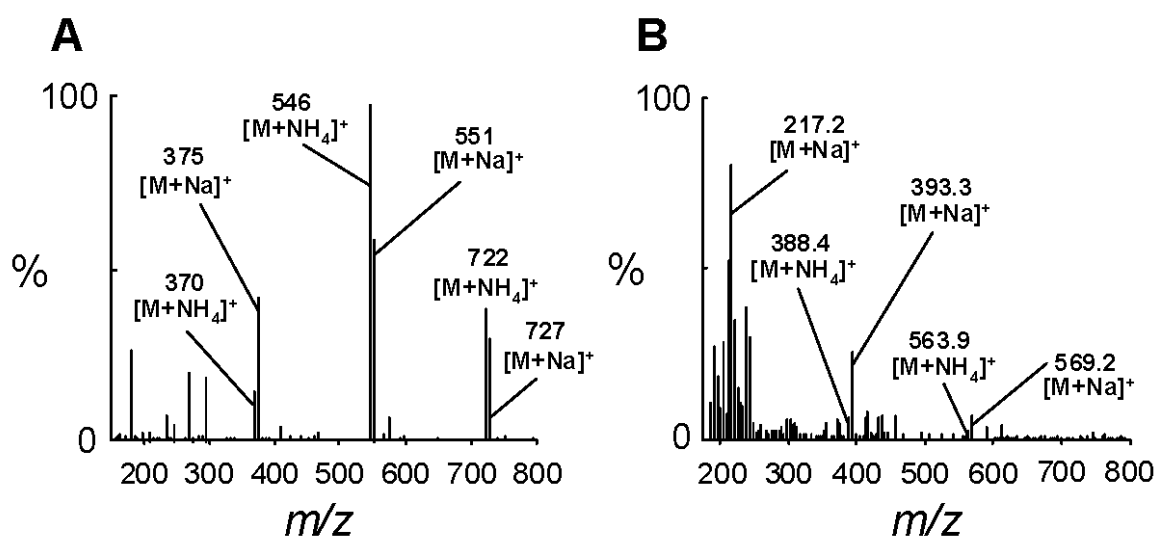
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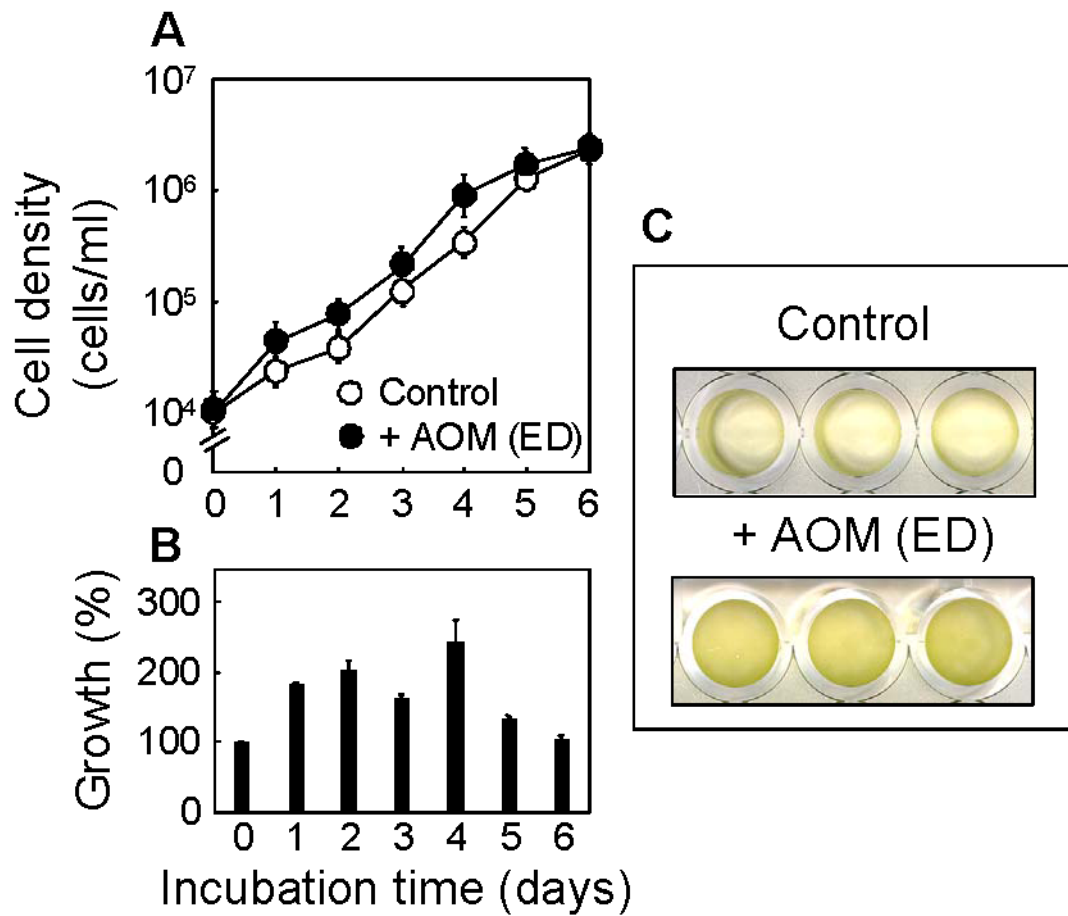
## Figure legends



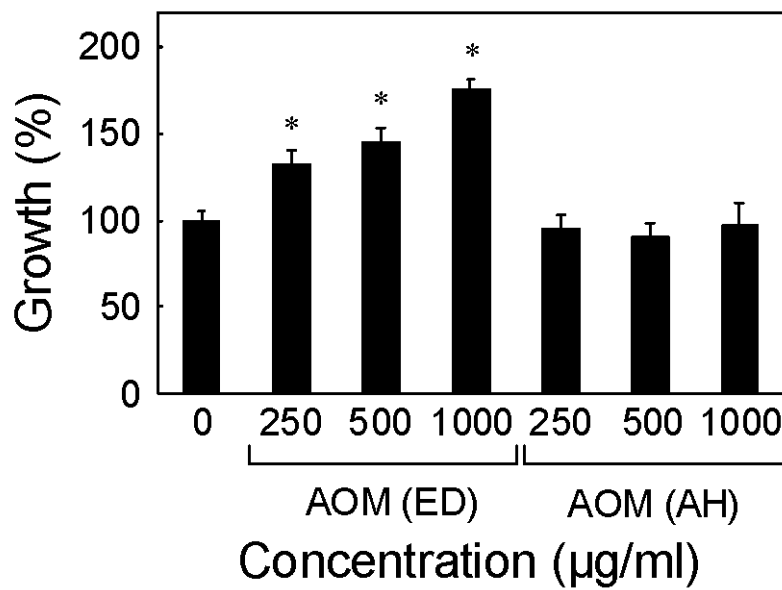
**FIG. 1.** Schematic of the analysis of fatty acid composition. See the “MATERIALS AND METHODS” for full details.



**FIG. 2.** MS spectrum of (A) AOM (ED) and (B) AOM (AH). Aqueous solutions of AOM (ED) and (AH) (100  $\mu\text{g/ml}$ ) were injected into the LC-MS/MS machine. The conditions for the Q1 scan analysis are detailed in the “MATERIALS AND METHODS”. *m/z*: mass to charge ratio.

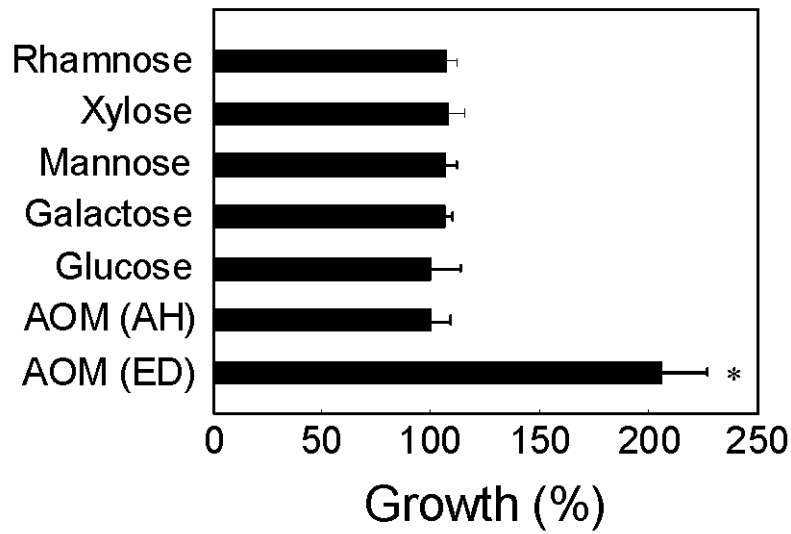


**FIG. 3.** Growth-promoting effect of AOM (ED) on *Chlamydomonas reinhardtii*. (A) Growth of *C. reinhardtii* when cultured alone (open circles) or with AOM (ED) (closed circles). (B) Daily fluctuations in the growth rate of AOM (ED)-treated cells. (C) *C. reinhardtii* (initial cell density:  $10^4$  cells/ml) was incubated in 48-well plates with 1000  $\mu\text{g/ml}$  of AOM (ED) at 26 °C. The cells were counted under light microscopy every day. (C) Impact of AOM (ED) on the growth of cells. A photograph was taken on day 4 of the experiment. The data are the means  $\pm$  (or +) SD (cells/ml) of triplicate measurements.

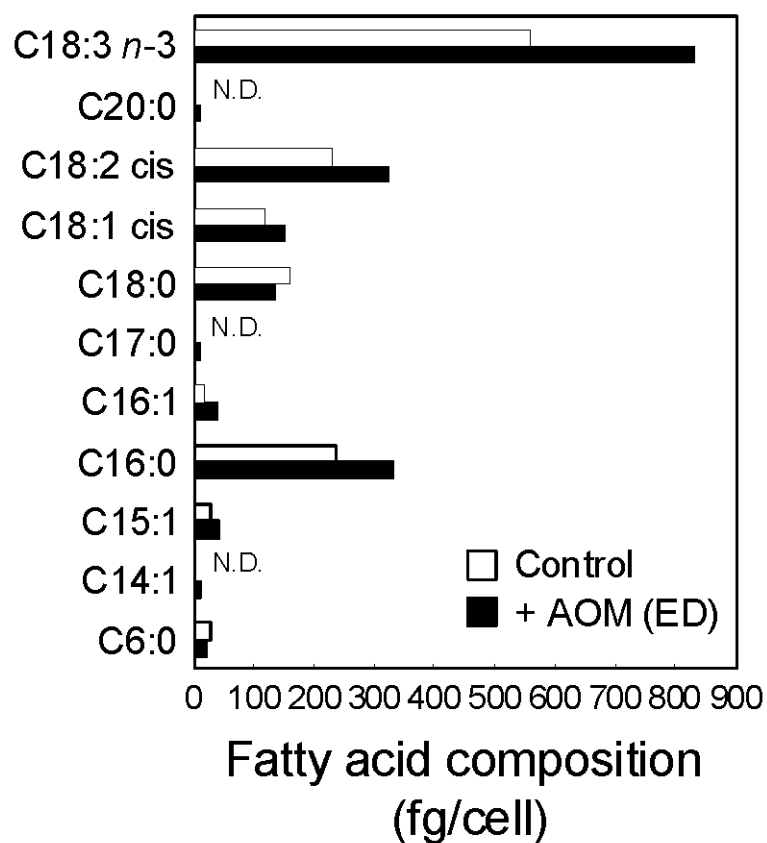


**FIG. 4.** Effects of AOM (ED) and AOM (AH) concentrations on the growth of *Chlamydomonas reinhardtii*. Algae (initial cell density:  $10^4$  cells/ml) were incubated in 48-well plates for 4 days at 26 °C at the AOM concentrations shown. Subsequently, the cells were counted under light microscopy. The data are the means + SD (%) of triplicate measurements, and asterisks indicate significant differences ( $P < 0.05$ ) between the treatment and control conditions.





**FIG. 5.** Effects of AOM (ED), AOM (AH) and other saccharides on the growth of *Chlamydomonas reinhardtii*. Algae (initial cell density:  $10^4$  cells/ml) were incubated in 48-well plates for 4 days at 26 °C at the AOM concentrations shown. Subsequently, the cells were counted under light microscopy. The data are the means  $\pm$  SD (%) of triplicate measurements, and asterisks indicate significant differences ( $P < 0.05$ ) between each treatment and control.



**FIG. 6.** Effect of AOM (ED) on the fatty acid composition of *Chlamydomonas reinhardtii*. Cultures at an initial cell density of  $10^4$  cells/ml in 200-ml glass flasks were incubated with 1000  $\mu\text{g/ml}$  of AOM (ED) for 4 days at 26 °C. The protocol for fatty acid analysis is detailed in the “MATERIALS AND METHODS”. The values are means of duplicates ( $n = 2$ ).