Selection and characterization of *Euglena anabaena* var. *minor* as a new candidate *Euglena* species for industrial application

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**Euglena gracilis** is a microalgae used as a model organism. Recently, mass cultivation of this species has been achieved for industrial applications. The genus *Euglena* includes more than 200 species that share common useful features, but the potential industrial applications of other *Euglena* species have not been evaluated. Thus, we conducted a pilot screening study to identify other species that proliferate at a sufficiently rapid rate to be used for mass cultivation; we found that *Euglena anabaena* var. *minor* had a rapid growth rate. In addition, its cells accumulated more than 40% weight of carbohydrate, most of which is considered to be a euglenoid specific type of beta-1-3-glucan, paramylon. Carbohydrate is stored in *E. anabaena* var. *minor* cells during normal culture, whereas *E. gracilis* requires nitrogen limitation to facilitate paramylon accumulation. These results suggest the potential industrial application of *E. anabaena* var. *minor*.

**Key words:** *Euglena anabaena* var. *minor*; *Euglena gracilis*; paramylon

In general, microalgae exhibit more efficient photosynthesis than terrestrial plants, thereby facilitating the culture of microalgae as alternative sources of food, biomass, and materials for producing fuel. Many microalgae, such as *Chlorella*, *Spirulina*, and *Dunaliella*, are cultivated on a large scale and used in industrial applications. A photosynthetic protist species, *Euglena gracilis*, is also employed in the industrial applications of microalgae. *E. gracilis* was originally used as a model organism in basic research, especially investigations of photosynthesis, but studies showed that *E. gracilis* stores a good balance of nutrients. *E. gracilis* cells are rich in protein, which includes high rate of methionine, a characteristic of animal proteins, and all essential vitamins are synthesized or incorporated in the cells. In addition, the *E. gracilis* cells include nutritionally important fatty acids, DHA, docosahexaenoic acid, and EPA, eicosapentaenoic acid. Therefore, mass cultured cells were considered to be suitable for use as a nutritional food source. In addition, *Euglena* species are known to store a specific type of beta-1-3-glucan called paramylon. Paramylon possesses a multiple functionality represented by immunostimulatory activity (unpublished); its production has increased the suitability of *Euglena* for industrial applications.

*E. gracilis* has been used as a model organism for decades, so its culture system is well established. The mass cultivation of *E. gracilis* can be achieved using the basic information available. In contrast, little is known about the culture of other *Euglena* species. The genus *Euglena* includes more than 200 species, and many appear to have specific characteristics. For example, *E. sanguinea* produces a highly valuable carotenoid compound called astaxanthin, while *E. mutabilis* is known to tolerate very low pH and arsenic stress, and some *Euglena* species are known to propagate in salt water. Thus, each *Euglena* species has specific characteristics, but paramylon particles can be observed in the cells of each species, and some of them should also possess the capacity to produce a wax ester, which is a fascinating characteristic of *E. gracilis*.

The prerequisites for the growth of *E. gracilis* were studied comprehensively decades ago. *E. gracilis* assimilates ammonia nitrogen rather than nitrate nitrogen. It can utilize various carbon sources for growth enhancement, and it requires vitamin B1 and B12 for proliferation. Based on this information, the culture media were optimized intensively for *E. gracilis*. As a result, a basic autotrophic culture medium, i.e. Cramer–Myers (CM) medium, and a high-yield heterotrophic culture medium, i.e. Koren–Hutner (KH) medium, were formulated and they have been used widely. In contrast to the specialized culture media used for *E. gracilis*, other *Euglena* species are maintained in versatile media, which support the proliferation of a wide range of algae. To broaden the scope of industrial...
applications of *Euglena*, it would be beneficial to conduct screenings to identify the valuable species and to optimize their culture conditions. In this study, we screened some of the available *Euglena* strains to identify the species that can proliferate at a sufficiently rapid rate, and we evaluated more appropriate culture conditions for the screened strain.

**Materials and methods**

**Euglenoid strains and maintenance.** The strains used in this study were isolated from ponds and rivers in various regions of Japan, except for the *E. gracilis* strain NIES-48, which was provided by NIES, JAPAN. The isolated strains were identified as *E. anabaena var. minor*, *E. clara*, *E. deses*, *E. granulata*, *E. schmitzii*, and *E. stellata* based on careful observations using dye staining. Although the details will be reported elsewhere, *E. anabaena var. minor* was identified by the consistency with the original description, in which *E. anabaena var. minor* was described as fusiform cells under natural condition, 36–43 μm long, 9–12 μm wide, eight chloroplasts, and pyrenoids covered with saucer-shaped paramylon sheath. The cell size of our strain was 35–46 μm long and 10–14 μm wide with 5–8 chloroplasts in the log growth phase. The other variant species of *E. anabaena* could be distinguished by the cell size. Under natural condition, *E. anabaena var. anabaena* is 88–94 μm long, 20–25 μm wide, and *E. anabaena var. minima* is 26–30 μm long, 9–12 μm wide. The 16S and 18S rDNA sequences were also determined to confirm that this *E. anabaena var. minor* strain is closely related to other *E. anabaena* strains (Supplemental Figure 1).

All strains except *E. gracilis* were maintained in the AF6 medium at 23 °C with a 14:10 h light:dark cycle (50 μmol photons/m²s). *E. gracilis* was maintained in the CM medium (pH 3.5) at 26 °C with a 14:10 h light:dark cycle (50 μmol photons/m²s).

**Culture media.** The culture media used for the maintenance of the cells and in the growth rate tests were CM, KH, AF6, BG-11, C, mAC, and TAP (supplemental Table 1). The TAP medium does not include vitamin B1 and B12, which are essential for the growth of *E. gracilis*, so those vitamins were supplemented at the same concentration as that used in the AF6 medium. The CM and KH media were prepared at pH 5.5 unless stated otherwise.

**Growth tests.** We used 24-well tissue culture test plates (TPP) for small-scale culture. The plates were placed statically in an incubator set at 23 °C and equipped with lighting under a 14:10 h light:dark cycle (50 μmol photons/m²s). Larger scale cultivation tests were performed using 100 mL capacity large test tubes that contained 40 mL culture media. During culture, 150 μmol photons/m²’s of constant light illumination was provided, and the culture was aerated with aseptic air that included 5% of CO₂. The growth rate was evaluated on the basis of the optical density at a wavelength of 680 nm (OD680) using a microplate reader (SHI-1200, Corona electric) and a spectrophotometer (UVmini-1240, Shimadzu) for the 24-well plate and large test tube cultures, respectively.

**Quantification of carbohydrates.** The harvested algal cells were dried in a freeze dryer (FDV-1200, EYELA), and the deproteinized carbohydrate component was extracted as previous report. Approximately 10 mg of dried algal cells was suspended in 10 mL of acetone, and the cells were homogenized twice for 90 s, each time using a sonicator (UD-201, TOMY), and the extract was collected by centrifugation (5810R, Eppendorf, 800 g, 5 min). The extract was then boiled for 30 min in 10 mL of 1% sodium dodecyl sulfate aqueous solution and washed two times with 10 mL of 0.1% sodium dodecyl sulfate aqueous solution and water, in order. The extracted carbohydrate was quantified using the phenol–sulfuric acid method.

**Results**

**Screening for rapidly growing Euglena species**

To evaluate the growth rate of *Euglena* species other than *E. gracilis*, we selected six strains of *Euglena* species, i.e. *E. anabaena var. minor*, *E. clara*, *E. deses*, *E. granulata*, *E. schmitzii*, and *E. stellata*, and assessed their growth. The AF6 medium was used to maintain most of the *Euglena* strains in our laboratory because it can sustain the growth of a variety of algal species. However, it was not clear whether AF6 would be the best medium for the growth of each strain. To determine the growth rate of each strain, we tested several typical types of algal culture media, i.e. AF6, mAC, BG-11, TAP, and C as well as culture media optimized for *E. gracilis*, i.e. CM and KH. *E. gracilis* is tolerant to low pH and is usually cultured at pH 3.5, but the other *Euglena* species tested in this study did not exhibit growth at this pH (data not shown). Therefore, in our trials, the CM and KH media were adjusted to pH 5.5. Among the media tested, mAC and KH include glucose and various other carbon sources so they could support heterotrophic growth, whereas the other media contained no carbon sources or low levels, thereby indicating that the cells proliferate mainly in autotrophic conditions.

Each strain was cultured in triplicates at 23 °C in 1 mL of medium on 24-well plates and the growth rates of cells were evaluated by determining the OD₆₈₀ of each well (Fig. 1). In contrast to *E. gracilis*, which grew well in the CM and KH media (Fig. 1(A)), the other strains did not grow well in these media (Fig. 1(B)–(G)). The sole exception to this was *E. schmitzii*, which grew well in the KH medium (Fig. 1(F)). Although *E. granulata* exhibited a low level of growth in the KH medium, all descendents died after prolonged culture (Fig. 1(E)). *E. anabaena var. minor*, *E. deses*, and *E. stellata* exhibited the most rapid growth in the TAP medium (Fig. 1(B), (D), and (G)). *E. granulata* exhibited the most rapid growth in the C medium (Fig. 1(E)), and *E. clara* grew the best in the mAC medium (Fig. 1(C)). Some of the strains expressed medium-specific phenotypes in TAP and C media. *E. anabaena var. minor* and *E. deses* turned fat...
and dark in color in the TAP medium, while their movement was accelerated in the C medium. To compare the growth rates of *E. gracilis* and the other species, we determined the growth curves for *E. gracilis* in the CM medium (pH 5.5) and for the other species in the C medium (Fig. 2). The growth of *E. gracilis* in the CM medium was the most rapid. Among the species other than *E. gracilis*, *E. anabaena* var. *minor* exhibited by far the fastest growth. In the C medium, the growth rate of *E. anabaena* var. *minor* was one-half to one-third of that of *E. gracilis* in the CM medium.

Optimization of the culture conditions for *E. anabaena* var. *minor*

*E. gracilis* has been reported to exhibit rapid growth at 29 °C. Because *E. clara*, *E. granulata*, and *E. schmitzii* were relatively sensitive to high temperatures,
such as 29 °C, we tested the culture media (Fig. 1) in an incubator set at 23 °C. To further improve the growth of E. anabaena var. minor, its optimum growth temperature was evaluated. Using 40 mL of the AF6 medium in 100 mL test tubes, the cells were cultured at 20, 23, 26, 29, and 32 °C, and the OD_{680} was recorded each day (Fig. 3). Among the temperatures tested, the cells cultured at 29 °C exhibited the fastest proliferation, whereas those tested at 32 °C exhibited poor growth due to heat stress. Therefore, the successive experiments were performed at 29 °C.

In the culture media trials (Fig. 1), E. anabaena var. minor exhibited the fastest growth in the TAP medium, although the cultured cells were large and darkly colored in appearance. These results suggest that the TAP medium may have specific effects on E. anabaena var. minor cells. To further understand the effects of the TAP medium, we compared the growth of E. anabaena var. minor in the C, TAP, and equivalent mixtures of C and TAP media (Fig. 4). These experiments were performed in 100 mL test tubes that contained 40 mL of each medium, and the OD_{680} was recorded every day. The equivalent mixtures of C and TAP media supported similar rapid growth rates to the TAP medium alone. Furthermore, the appearance of the cells cultured in the mixture was the same as that in the TAP medium, i.e. large and dark in color, thereby suggesting that the cell phenotype in the TAP medium was related to a specific ingredient in the medium rather than the lack of some elements. We also compared the growth of E. anabaena var. minor and E. gracilis in the CM medium (pH 3.5) using the same culture conditions except for the culture media (Fig. 4). E. anabaena var. minor could exhibit a growth rate about half of E. gracilis in the 40 mL scale culture.

Carbohydrate content of E. anabaena var. minor
In the culture trials performed in large test tubes, most E. anabaena var. minor cells settled after a specific period of time without aeration (Fig. 5(A)). In contrast, the E. gracilis cells did not sink within 60 min. The rapid sinking of E. anabaena var. minor cells may be attributable to their high carbohydrate content. Especially, paramylon is the most plausible polysaccharide reserve in euglenoids. The particle density of paramylon and other carbohydrates are approximately 1.5,^{24}
so the specific gravity of the cells would be large with high carbohydrate content. The *E. anabaena* var. *minor* cells contained numerous paramylon-like particles, especially the cells grown in the TAP medium (Fig. 5(B)–(E)). To examine the carbohydrate content of *E. anabaena* var. *minor*, carbohydrate was extracted from the cells and then quantified after a week of culture in the C, TAP, and the mixture of C and TAP media (Fig. 5(F)). As expected, *E. anabaena* var. *minor* contained a high level of carbohydrate. After culture in the TAP medium, the carbohydrate content of *E. anabaena* var. *minor* comprised more than 40% of its weight. However, *E. gracilis* stored carbohydrate only after nitrogen restriction, as reported previously. In contrast, *E. anabaena* var. *minor* stored carbohydrate even in an environment that was permissive to growth.

**Discussion**

**Potential of *E. anabaena* var. *minor* for industrial use**

The nutrient requirements and preferred culture conditions were relatively different according to our comparisons based on the culture of *E. gracilis* and other
Euglena species. Most of the Euglena species tested could proliferate in the C medium, which contains nitrate nitrogen, but not ammonium nitrogen, whereas E. gracilis has been reported to exhibit negligible growth with nitrate.\(^{12}\) In addition, E. clara, E. granulata, and E. schmitzii were relatively sensitive to a higher temperature, i.e. 29 °C, and all the tested species were sensitive to low pH (pH 3.5), except for E. gracilis.

Among the Euglena strains available, we found that E. anabaena var. minor exhibited good proliferation. Although this strain was not as tolerant to low pH as E. gracilis, its tolerance to high temperature was comparable to that of E. gracilis. Furthermore, we found that E. anabaena var. minor has three highly beneficial features. First, the cell size of E. anabaena var. minor, i.e. approximately 20 μm, is smaller than that of E. gracilis, i.e. approximately 50 μm (Fig. 5(B)-(E)). The difficulty of stirring large-scale cultures increases in proportion to cell size because Euglena is sensitive to shear forces. Therefore, the small cell size of E. anabaena var. minor suggests that it would be easier to stir cultures of this species. Second, the cells settled well without stirring, thereby indicating that the harvest process could be simplified. Finally, the cells stored a high level of carbohydrate in normal culture conditions (Fig. 5(E)). In E. gracilis, nitrogen source deprivation is required to stimulate the storage of high levels of carbohydrate. Therefore, the production of high carbohydrate content even in an environment permissive to growth would also be an advantage during the industrial application of this species.

**Nutrient requirements for E. anabaena var. minor growth**

E. anabaena var. minor proliferated well in the TAP medium, although it was larger and darker colored than when grown in the other media. These differences may be attributable to ingredients that are present only in the TAP medium because the mixture with the C medium also obtained the same effect. Although no ingredient was specific to the TAP medium, acetate is a component that characterizes the TAP medium. Other than the TAP medium, the mAC medium also includes acetate but much less than the TAP medium\(^ {20}\) (Supplemental Table 2). However, the addition of acetic acid to the C medium was detrimental, and the addition of a lower concentration appeared to have little effect (data not shown). Thus, acetic acid may be the main factor responsible for specific cell appearance in the TAP medium, but the combination of ingredients could also be important for the expression of the specific phenotype. Acetic acid was the sole carbon source in the TAP medium; therefore, E. anabaena var. minor may possess an efficient pathway for assimilating acetic acid. However, the acetic acid content of the TAP medium was only 0.1%, and most of the acetic acid probably evaporated during sterilization in an autoclave. Even if acetic acid can be used as a carbon source, the main carbon source is the fixation of carbon dioxide by photosynthesis.

In contrast to the culture of E. gracilis, the cultures of other Euglena species have not been studied and optimized well in previous investigations. Thus, in the present study, we tested several established culture media to determine their effects on the growth of E. anabaena var. minor, which suggested that there is considerable capacity for improving the culture of this species. Given further culture improvements and basic research, E. anabaena var. minor may have the potential for mass culture. Although our results indicate that E. anabaena var. minor grew faster than the other species, this does not necessarily mean that the other species should be excluded from industrial use because the growth rates of these species may improve dramatically after further screening in more suitable culture conditions. In addition, screening a wider range of Euglena species may identify species that are more suited to mass culture.

**Author contribution**

K.S., S.M., O.I., and K.Y. designed experiments. K.S., S.M., and K.Y. performed experiments. S.K. provided the strains. K.S., O.I., and K.Y. analyzed data. K.S., O.I., T.I., S.K., and K.Y. wrote the article. T.I., S.K. and K.Y. supervised the project.

**Supplementary material**

The supplementary material for this paper is available online at http://dx.doi.10.1080/09168451.2015.1045828.

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**Disclosure statement**

No potential conflict of interest was reported by the authors.

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