# Characterization and Potential Utilizations of A Marine Euglenophyte from Okinawa, Japan

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## Abstract

Among many microalgae species, certain euglenophytes stand out as excellent sources of valuable natural components with diverse biotechnological applications. This study focused on investigating the identity and potential utilizations of a green marine euglenophyte, namely EKoku01, isolated from the brackish Kokuba River in Naha City, Okinawa, Japan. Morphologically, cells of EKoku01 were 12-30μm long and 8-10 μm wide, displaying dynamic changes during euglenoid motility with a distinctively striated pellicular body. Notable features included two uneven flagella, an eyespot, and multiple green discoid chloroplasts. The 18S rDNA molecular phylogeny positioned Ekoku01 within Clade 1 of *Eutreptiella*, comprising strain CCMP389, CCMP-1594, and LIS 2000, which was distantly related to other known marine euglenophytes (*i.e.*, the genus *Eutreptia* and Clade 2 of *Eutreptiella*). Based on the morphology and molecular phylogeny, EKoku01 was identified as *Eutreptiella* sp., representing the first recorded species of this genus in Okinawa, Japan. EKoku01 showed an optimum growth performance at 20 °C (max. μ=0.17 ± 0.037 $day^{-1}$) and exhibited an unusually strong positive phototaxis response towards a light source. Its fatty acid (FAs) profile comprised predominantly saturated FAs (69.8%), polyunsaturated FAs (15.1%, with two omega-3 FAs), monounsaturated FAs (3.6%), and branched FAs (4.4%), while ~7.1% remaining unidentified. Based on its growth performance, FAs composition, and light sensitivity, we proposed several potential utilizations of this strain (*e.g.*, as a source for nutraceutical, pharmaceutical, food, and aquaculture applications), including the use of EKoku01 as a model organism for developing an innovative light-driven cell concentration system based on phototaxis.

Keywords: Euglenophyte, *Eutreptiella*, morphology, molecular, phylogeny, fatty acid

## Introduction

Microalgae, also commonly known as phytoplankton, are diverse groups of primarily unicellular eukaryotic microorganisms that inhabit various ecosystems, from terrestrial to numerous freshwater bodies and marine environments (Dolganyuk et al., 2020). They play important ecological roles in the aquatic ecosystems as primary producers that contribute to global oxygen production through photosynthesis (Juranek et al., 2020; Abdelfattah et al., 2023), while also providing food for zooplankton and other larger organisms (Ma & Hu, 2023). Some microalgae species stand out excellent sources of biomass and feedstocks that can be extracted to obtain beneficial components (*e.g.*, lipids, fatty acids, protein, carbohydrates, and pigments) and various bioactive compounds (*e.g.*, antioxidants, vitamins, and minerals) (Coêlho et al., 2020; Zhou et al., 2020; Bhatnagar et al., 2023; Castro-Cosio et al., 2023). In particular, algal lipids have the potential to be used as materials for producing valuable nutraceuticals or biofuel (Udayan et al., 2022; Khoo et al., 2023).

Among many microalgae species, some photosynthetic euglenophytes are known for their beneficial applications. For example, the freshwater *Euglena gracilis* is known as an excellent source not only for the production of a wide variety of valuable metabolites and natural products (Gissibl et al., 2019), but also as an agent for wastewater bioremediation

(Do et al., 2022; Geremia et al., 2021) as well as biorefineries for biofuel and biogas production (Khatiwada et al., 2020). In Japan, one particular company, (*i.e.*, the Euglena Co. Ltd.) has successfully developed large-scale cultures and commercialized various products derived from *E. gracilis* (Suzuki, 2017). In the marine environment, the genus *Eutreptiella* has recently been shown as a promising source of fatty acids (Kuo et al., 2021; Kang et al., 2023) and diet for clam culture (Yamasaki et al., 2019).

The genus *Eutreptiella* da Cunha was originally described in 1914 based on the morphology of *E. marina* da Cunha. This genus is currently comprised of nine species that exclusively inhabit marine ecosystems, including brackish-water environments (Novoa, 2024). *Eutreptiella* shares a phylogenetic affinity with another photosynthetic marine euglenophyte, the genus *Eutreptia* Perty, which to some extent, can generally be differentiated from the *Eutreptiella* species by their flagella length (Walne et al., 1986). Species whose flagella are equal in length are generally assigned to *Eutreptia*, while those having distinct flagella length belong to the latter. Unlike their freshwater counterparts, information concerning the potential applications of *Eutreptiella* species remains limited. Hence, continuous efforts in sampling, isolating, identifying, and characterizing microalgal species, including marine euglenophytes, are important and an integral part of unraveling their potential.

During a routine microalgal sampling in December 2010, we successfully isolated a green marine euglenophyte, namely EKoku01, from the Kokuba River in Naha City, Okinawa, Japan. This study aimed to unravel its identity through morphological observation and molecular phylogenetic analysis, and then assess its potential applications *via* growth experiments, fatty acid (FA) analysis, and phototaxis response.

## Material and Methods

### Sampling and Single-cell Isolation

Water samples (volume: 100 mL) containing microalgal cells were collected at the surface layer (depth: 0.10-0.25 meter) using a 20 mm mesh-size plankton net from a bridge at the Kokuba River in Naha City, Okinawa Prefecture, Japan in December 2010 (Figure 1). The water temperatures were about 18-19 $^{o}$C with a salinity ranging from 14.6-15.1 psu, indicating a brackish water environment. Single-cell isolation was performed using a fine glass capillary pipette under an Olympus CKX-41 inverted microscope (Olympus, Tokyo, Japan) and the cells were transferred into culture tubes containing 8 mL of seawater-based Daigo IMK medium (Nihon, Pharmaceutical, Tokyo, Japan) at 1/4 concentration and added with soil extract (Provasoli et al., 1957). Cultures were maintained at 22 ± 1 $^{o}$C with about 50 µmol $m^{-2}s^{-1}$ white fluorescent illumination (FLR40S- 40W Lamps, Panasonic, Japan) under 14:10 light-dark cycles. Scale-ups were made to accommodate 250 mL of culture in 500 mL Erlenmeyer flasks. Initially, three isolates were established as strains (*i.e.*, EKoku01, EKoku02, and EKoku03), however, after 15 days of incubation, only

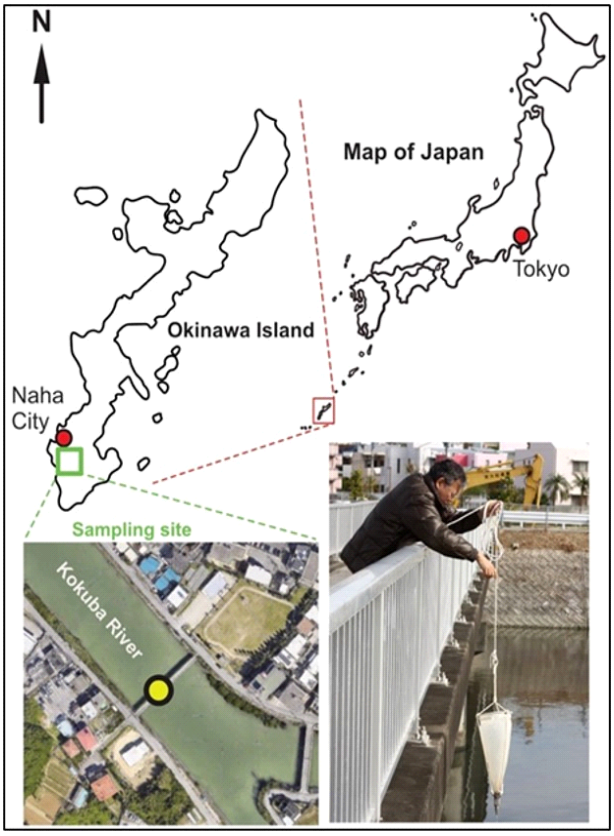


Figure 1. Map of Okinawa Island, Japan showing the sampling site (yellow dot) at the Kokuba River, Naha City in December 2010. Aerial map by Google Earth.

EKoku01 remained viable for further observation and analyses.

### Morphological and Molecular Phylogenetic Identification

Morphological identification of both living and 2.5%-glutaraldehyde fixed-cells was conducted using a light microscope (LM) (Nikon Eclipse 80i, Nikon Corp., Tokyo, Japan) equipped with a SPOT-Idea digital camera (SPOT Imaging, Michigan, USA), whereas preparation for scanning electron microscopy (SEM) using JEOL JSM 6060 LV (JEOL, Tokyo, Japan) followed a method described in Prabowo et al. (2016). Molecular identification was performed by amplifying the DNA extract of EKoku01 targeting the 18S rDNA

gene using a set of dedicated primers (Nakayama et al.,1996; Riewluang and Wakeman, 2023) and sequenced through Macrogen Japan. Forward and reverse sequences assembly and multiple sequence alignment (MSA) involving 39 DNA sequences of euglenophytes and one species outgroup (*i.e.*, *Chlamydomonas reinhardtii*) were conducted in MEGA 11 (Tamura et al., 2021) using ClustalW (Thompson et al., 2003). Phylogenetic analyses and tree reconstructions were performed implementing maximum likelihood analysis (ML; 1000 replications) in raxmlGUI version 2.0.10 (Edler et al., 2021) and Bayesian inference (BI; 1 million generation) using mrBayes 3.2.7a (Ronquist et al., 2012). Specific settings used in BI were based on Prabowo et al. (2016). The best-fit model was determined using ModelTest-NG in raxmlGUI, and the GTR+*I*+*G* was selected based on the Bayesian inference criterion (BIC) and Akaike inference criterion (AIC). The assembled DNA sequence of EKoku01 was submitted to GenBank and are accessible through the GenBank Accession Number PP140744.

## Growth Experiments

A logarithmic-growing stock culture of EKoku01 maintained at 22 ± 1 °C was used as a source of inoculation for the growth experiments. Five temperature treatments (*i.e.*, 15, 20, 25, 30, and 22 ± 1 °C as control) were implemented in an EYELA MTI-201 Multi Thermo Incubator (Tokyo Rikakikai Co. Ltd., Japan). A salinity of 28 psu was used in the experiment. For each temperature condition, three Erlenmeyer flasks containing 50 mL IMK medium and stock inoculate of 10 mL with initial cell density 1.0-1.2 x $10^5$ cells.mL$^{-1}$ were prepared and added into each flask (final cell density 0.17-0.2 x $10^5$ cell.mL$^{-1}$). The cell density of each treatment was measured every three days for a total of 31 days using a modified protocol of Rodrigues et al. (2011) and Hotos (2023), *i.e.*, by estimating the microalgal cell density of each treatment (n=3 replicates) as a function of light absorbance based on spectrophotometry using a Shimadzu UV-1800 (Shimadzu, Tokyo, Japan) screened between 600-700 nm. The specific growth rate (μ) of EKoku01 in the exponential or logarithmic phase of growth is estimated at an optimum detection wavelength of 680 nm using the following formula:

$$\mu = (\ln N2 - \ln N1) / (t2 - t1)$$

where N1 and N2 are cell densities of the culture at a moment of time t1 and t2 (Peng et al., 2020).

## Phototaxis Observation

During the experiment, cells EKoku01 often densely accumulated at the surface of the medium (*i.e.*, showing dark green coloration) where the distance between the culture and the light source was the nearest. This suggested that EKoku01 exhibited a positive phototaxis response. To observe this phototaxis phenomenon, a simple cell accumulation device (*i.e.*, LED cell accumulator) was used and comprised of a 20 cm long acrylic tube with a diameter of 1.8 cm (Figure 2). One of its ends was sealed with a removable rubber cap, while the other end was attached to an LED light source (CB-01BK, Ohm Electric, Tokyo, Japan) emitting ~41.67 μmol m$^{-2}$ s$^{-1}$ within a 10 cm radius. A small hole was drilled on top of the acrylic tube located near the LED end which allowed subsampling of the culture using a 1.0 mL syringe. The device was then filled with EKoku01 culture, illuminated at dark, and

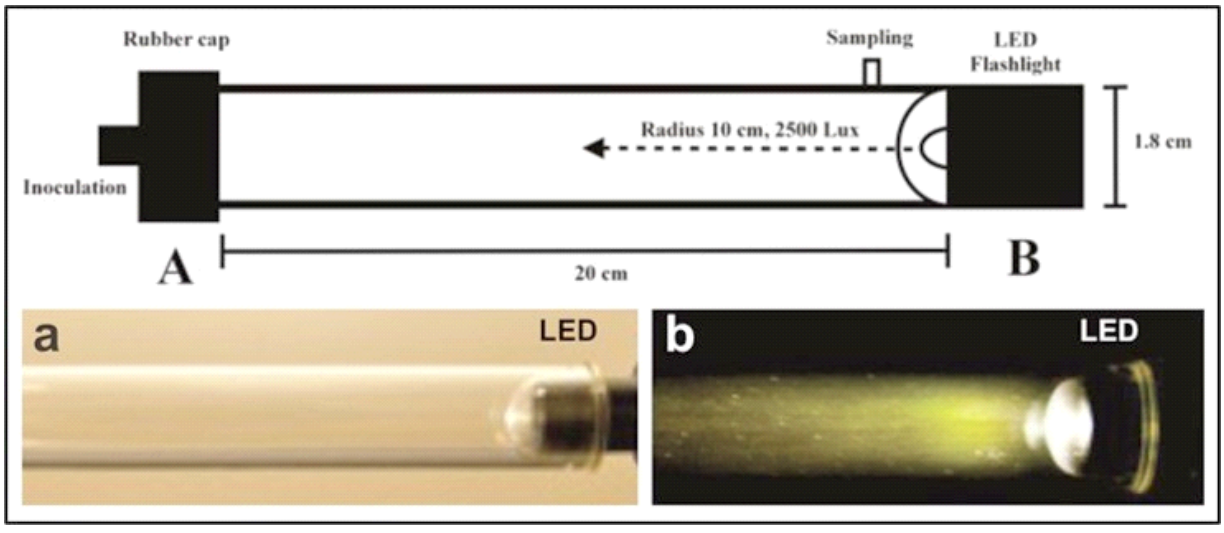


Figure 2. Design of an LED cell accumulator for phototaxis observation of EKoku01 showing the empty device (a) and during the experiment (inoculum illuminated with LED at dark) (b).

the cell density was estimated after 5, 30, and 60 min using a hemocytometer.

### Total Lipid Extraction, Fatty Acid Analysis, and Artemia Bioassay

Actively growing culture of EKoku01 (250 mL, n=3 flasks) maintained under the optimum temperature condition was harvested by centrifugation at 17,800 xg (~14,000 rpm) under 4 °C for 10 min using a Tomy MX-201 micro-centrifuge (Tomy SEIKO, Tokyo, Japan). The supernatant was carefully removed and the resulting pellets (n=3) were combined for total lipid and fatty acids analyses. In summary, upon freeze-drying the pellets for 24 hours, the total lipid was extracted following a modified method of Bligh and Dyer (1959), whereas fatty acid (FA) analyses were performed following Abdulkadir and Tsuchiya (2008). Total lipids were extracted from the pellets ultrasonically using an US-1 Ultra Sonic Automatic Washer (Iuchi, Co. Japan) for 20 min using a mixture of distilled water-methanol-chloroform (1:2:1; v/v/v). The extracts were subsequently centrifuged at 3,000 rpm for 5 min using a Tomy-201 centrifuge and a rotor rack AR510-4 (Tomy SEIKO, Tokyo, Japan) followed by solvent evaporation under nitrogen aeration. Purifications of fatty acid methyl esters (FAMEs) were performed after saponification and transmethylation using a high-performance thin-layer chromatography technique (HPTLC) (Meziane et al., 2002). FAMEs were then analyzed by gas chromatography using a GC 14-B Shimadzu equipped with flame ionization (Shimadzu, Tokyo, Japan). FAMEs were separated with an FFAP-polar capillary column with hydrogen as a carrier gas. The oven temperature was raised to 150 °C at a rate of 40 °C $min^{-1}$, after injection at 60 °C, then raised to 230 °C at a rate of 3 °C $min^{-1}$, and finally held constant for 30 minutes. The ionization flame was held at 240 °C. An authentic standard (C19:0) was used to manually compare the printed retention times of the FAMEs peaks followed by the identification and estimation of the concentration of each component following the methods of Meziane et al. (2002) and Abdulkadir and Tsuchiya (2008).

Finally, to preliminary assess the harmful potential of EKoku01 (*i.e.*, whether the strain is a toxin producer), a simple artemia bioassay was performed following a modified method of Pavaux et al. (2020). EKoku01 biomass was extracted from a stock culture using acetone and tested against *Artemia salina*. The mortality rate of the *A. salin*a was observed for a total of 48 hours at 12-hour intervals.

## Results and Discussion

### Identity of The EKoku01 Strain

Correct taxonomic identification of microalga species is essential for any subsequent studies and bioprospecting applications. This is mainly due to the fact that some beneficial microalgae, especially rare or undescribed species, might share indistinguishable morphology with harmful or toxic microalgae species. Therefore, ensuring the correct identity of microalgae is crucial and should be an integral part of biodiversity and biotechnological studies. Regarding the EKoku01 strain, its morphology based on LM and SEM is presented in Figure 3. Overall, the cells were 12-30 μm long and 8-10 μm wide, depending on the irregular changes (*i.e.*, expanding and contracting) of the cell body during its euglenoid motility. Longitudinally, the cell outline was spindle-shaped, often showing a widened cell body at the middle, but tapering towards the anterior and posterior ends, with the latter being more narrowly pronounced, forming a colorless tail-like extension towards the end of the posterior part (Figure 3a). A distinct eyespot (stigma) was presented in the anterior part of the cell, just above the reservoir system, while numerous discoid green chloroplasts were observed (Figure 3a). No pyrenoid was detected using LM. At the anterior, two flagella of unequal length (*i.e.*, F1 and F2) emerged, with the shorter one being about half the size of the longer one, and both showed irregular hyperdynamic scourging when observed under LM (Figure 3b). The nucleus was likely located at the center of the cell, surrounded by chloroplasts. When observed under SEM (Figure c,d), multiple pellicular striations were observed (8-14 striae per 10 μm), arranged in grooves that resembled accordion bellows, allowing the cell to flexibly expand and contract (Figure 3e-k). Normally, the cells swam freely in the culture medium by retaining their spindle shape, but will occasionally change to euglenoid movements. These morphological features are unique to the euglenophytes (Walne et al., 1986; Marin et al., 2023; Kang et al., 2023). Thus, considering the presence of two flagella with unequal length, the stigma, the green discoid chloroplasts, and particularly its brackish habitat, EKoku01 was identified as a species of the genus *Eutreptiella*.

The inclusion of EKoku01 within the genus *Eutreptiella* based on morphology was strongly supported by the 18S rDNA molecular phylogeny (Figure 4), although its specific identity was not well resolved. EKoku01 was placed within the highly

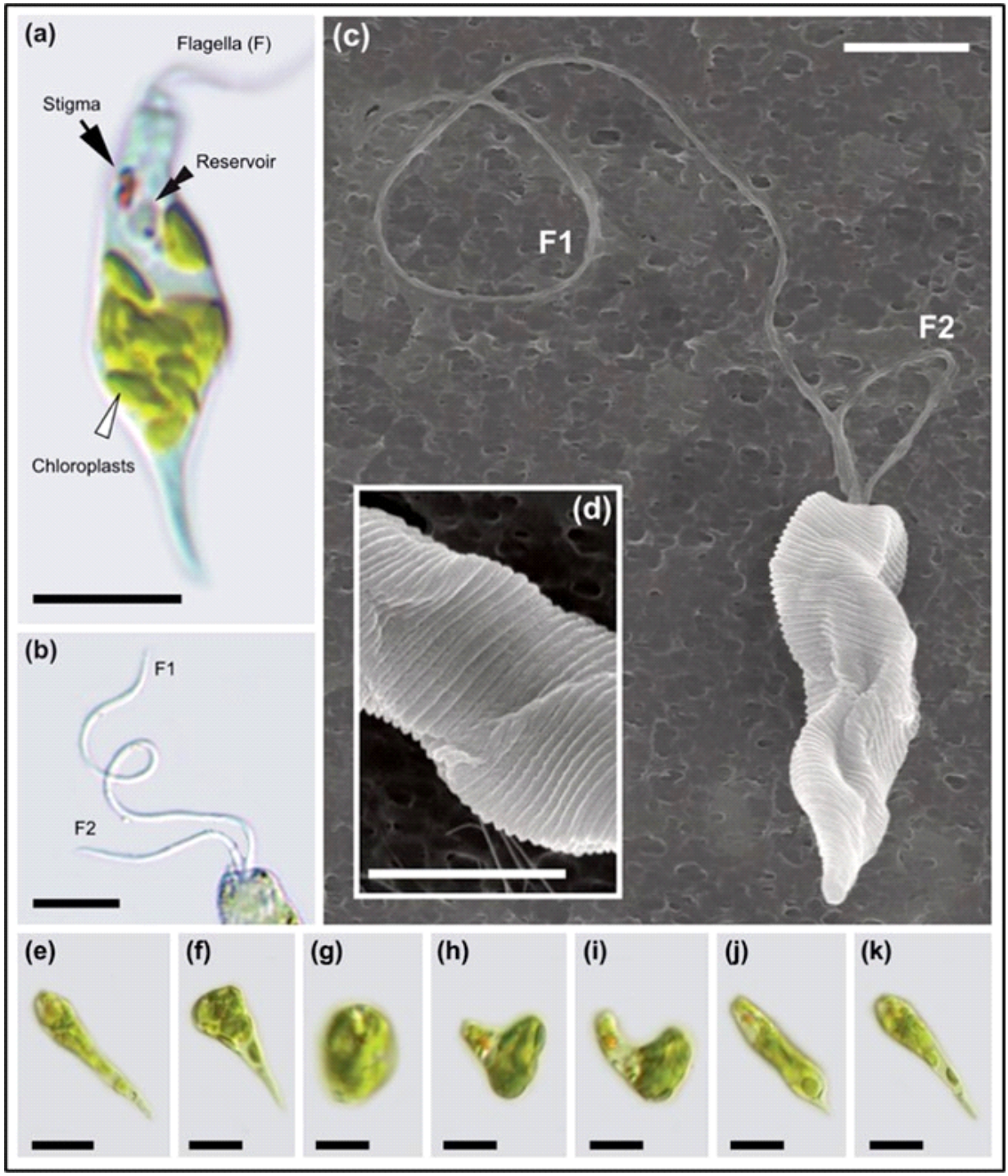


Figure 3. Light and scanning electron microphotographs (SEM) of *Eutreptiella* sp. strain EKoku01 showing the general cell morphology of the species (*i.e.*, cell outline, a stigma, chloroplasts, the unequal flagella, the pellicular striae, etc.) (a-d) and the time-series images of the cell during the euglenoid motility (e-k). Scale bars=10 mm.

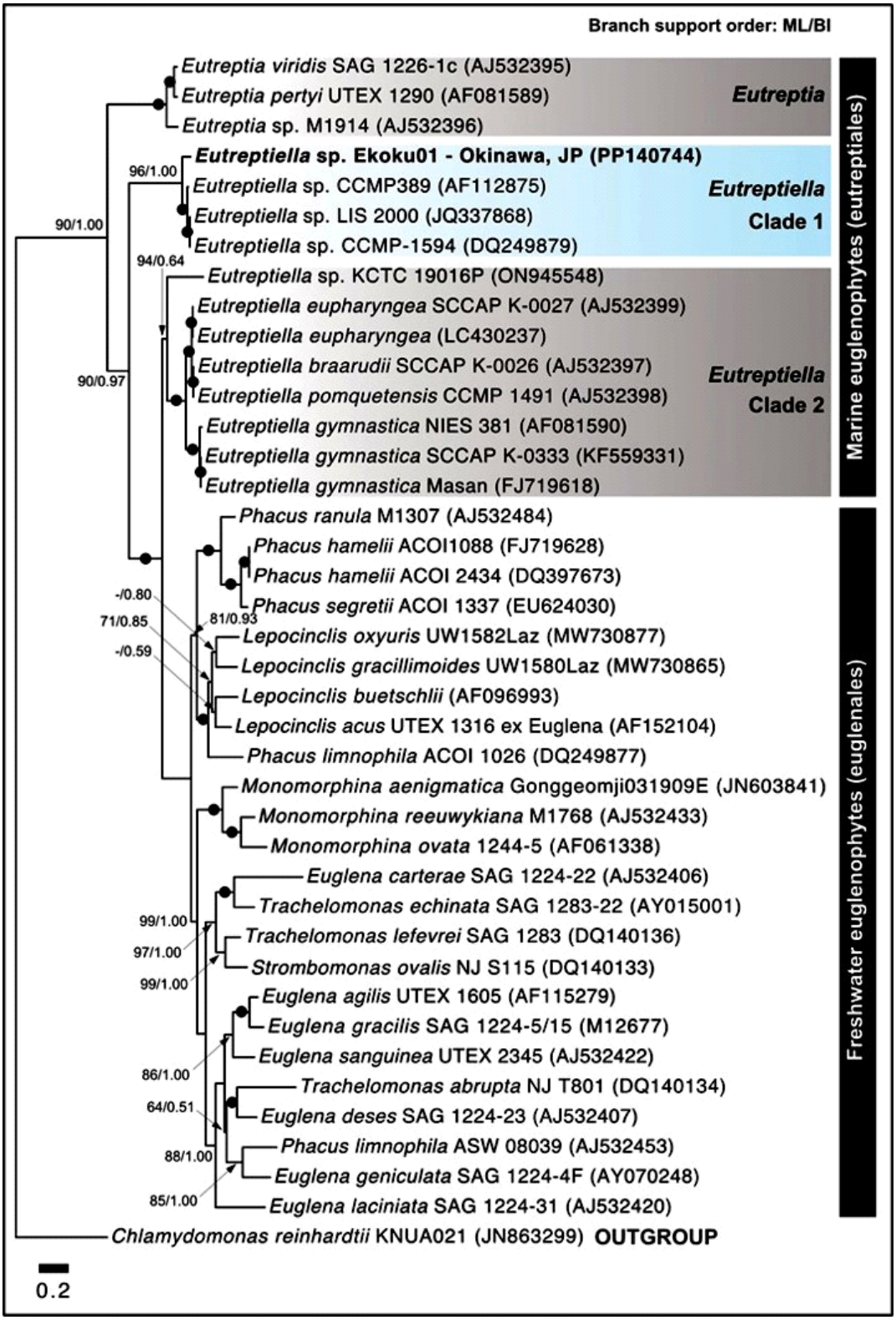


Figure 4. Maximum likelihood (ML; logL= -30675.620885) tree based on partial 18S rDNA (2210 sites, including introduced gaps) of euglenophytes (39 taxa) and one outgroup (*C. reinhardtii*) showing the position of strain EKoku01 from Okinawa, Japan (printed in bold). Branch supports values are shown in ML/BI order (bootstrap >50% / posterior probability >0.50). Black circles indicate full supports in both analyses. Scale bar = 0.2 nucleotide substitution per site. Due to space limitation, statistic data resulted from these analyses can be requested from the corresponding author.

supported *Eutreptiella* Clade 1 together with *Eutreptiella* sp. CCMP389, LIS 2000, and CCMP-1594. Interestingly, *Eutreptiella* Clade 1 was distinctly separated from the species of the genus *Eutreptia* and *Eutreptiella* Clade 2, of which all of them formed a sister link to the freshwater Euglenales (Figure 4). The sequence divergences between the strains in Clade 1 were relatively lower (2.0-7.0%) compared to the genetic distances between Clade 1 *vs*. Clade 2 (26-31%), Clade 1 *vs*. *Eutreptia* (36-39%), or Clade 2 vs. *Eutreptia* (32-40%). As a note, the DNA sequence of strain CCMP-1594 (DQ249879) belonging to Clade 1 was annotated as *E. braarudii* (Milanowski et al., 2006), however, the strain was identified as *E. gymnastica* by the National Center for Marine Algae and Protist (NCMA, Bigelow; *https://ncma.bigelow.org/CCMP1594)*, although its phylogenetic position was distantly related to any other *E. gymnastica* strains and *E. braarudii* strain SCCAP K-0026 (Figure 4). This confused the true identity of strain CCMP-1594, but also indicated that taxonomic reinvestigation should be performed for strains belonging to Clade 1. When contrasting to the phylogentic positions between EKoku01 and the freshwater euglenophytes, the separation between them were clear. The freshwater euglenophytes formed a monophyletic clade (*i.e.*, euglenales) that was distantly related to all marine euglenophytes (*i.e.*, eutreptiales) presented in Figure 4, thus conforming their taxonomic groupings based on the environmental condition of their habitat (*i.e.*, freshwater *versus* marine).

The polyphyletic positions of the Eutreptiales (*i.e.*, *Eutreptia*, Clade 1, and Clade 2) were also shown in previous investigations (Marin et al., 2003; Lukešová et al., 2020; Maciszewski et al., 2022; Kang et al., 2023). Considering the topology of the tree and the genetic distances between these three clades, strains belonging to Clade 1, including EKoku01, might actually represent a new or undescribed species, within the Eutreptiales. Unfortunately, the 18S rDNA sequences of the five remaining species of *Eutreptiella* summarized in Kang et al. (2023), including its type species (*i.e.*, *E. marina*), were not available in GenBank. Consequently, it is currently impossible to compare and discriminate our strain against all established species of this genus. Although morphological data of *Eutreptiella* species were available for comparison (Kang et al., 2023), it was extremely challenging to find precise matches between our strain and other *Eutreptiella* species by relying only on the cell dimension, the presence of a stigma, the number and shape of the chloroplasts, or the number of pellicular striae. Until the DNA sequences of all *Eutreptiella* species are available for comparison in a phylogenetic analysis, we refrained from further describing the detailed taxonomy and classification of EKoku01 and the species of Clade 1 within the Eutreptiales. Furthermore, we suggest that detailed morphological and ultrastructure observation using electron microscopes (*i.e.*, SEM and TEM) of strains in Clade 1 be conducted in subsequent studies. Nevertheless, at this stage, based on the morphological characterization and molecular phylogenetic analyses, we retained the identity of EKoku01 as *Eutreptiella* sp. It is also worth noting since its discovery in 2010 to the present report, to the best of our knowledge, no other species of *Eutreptiella* has been reported from Okinawa Island, Japan. Thus, EKoku01 represents the first recorded species of *Eutreptiella* from this region.

## Growth Performances

The growth curves and growth rates of EKoku01 under five temperature treatments are presented in Figure 5. Our results suggested that EKoku01 grew well at all tested temperatures, except at 30 °C (Figure 5a). In all temperatures, no notable growth was observed within the first five days of culture. However, substantial changes in cell density were noticed starting from day 7 onwards for all EKoku01 cultures, except the one grown at 30 °C. The highest cell density was recorded by the Control (22 ± 1 °C) on day 25 with 9.4 x $10^5$ cells.mL$^{-1}$, while the lowest was at day 19 (0.2 x$10^5$ cells.mL$^{-1}$) generated by the strain at 30 °C. EKoku01 grown at 15 °C, 20 °C, and 25 °C showed the highest cell densities at day 25 (6.3 x $10^5$ cells.mL$^{-1}$), day 31 (10.4 x $10^5$ cells.mL$^{-1}$), and day 21 (2.5 x $10^5$ cells.mL$^{-1}$), respectively, either at the end of the logarithmic or during the stationary phases (Figure 5a). The maximum growth rate (0.17 ± 0.037 day$^{-1}$) of EKoku01 was estimated at 20 °C and the lowest was at 30 °C (0.03 day$^{-1}$). Growth rate at the Control (0.16 ± 0.029 day$^{-1}$) was not significantly different from the experimental treatment at 20 °C, suggesting that the optimum growth performance of EKoku01 fell within temperature range of 20-23 °C. However, its growth rate substantially differed from the treatments at 15 °C (0.15 ± 0.057 day$^{-1}$), 25 °C (0.12 ± 0.051 day$^{-1}$), and 30 °C (0.03 ± 0.002) (Figure 5b).

The growth performances of EKoku01 at different temperatures were similar to *Eutreptiella eupharyngea* reported by Yamasaki et al. (2019), suggesting that *E. eupharyngea* performed well at temperature range of 10-25 °C, but failed at 30 °C. However, the maximum growth rate of *E. eupharyngea* (0.99 ± 0.10 day$^{-1}$) that was recorded at 10 °C and a salinity of 20 PSU (Yamasaki et al., 2019), surpassed the maximum value of our EKoku01 by up to five times. Moreover, Kang et al. (2023), reported that *Eutreptiella* sp. strain KCTC 19016P grown at 23 °C reached a maximum growth

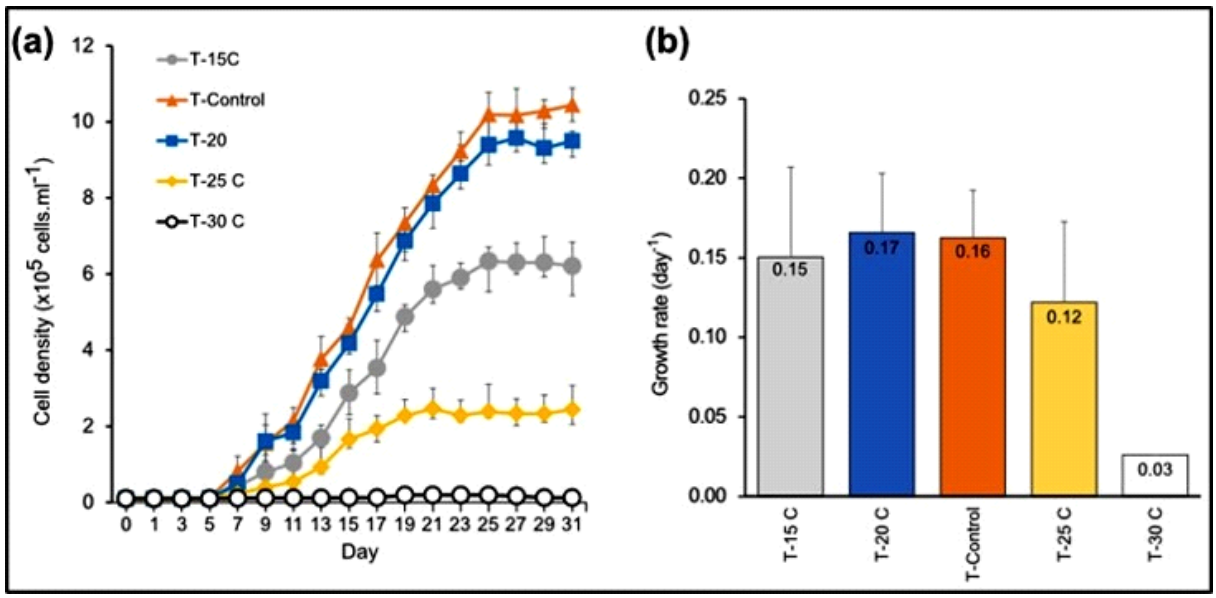


Figure 5. Growth performance of EKoku01 under five temperatures treatments as indicated by the growth curves (a) and growth rates (b).

rate of 0.50 ± 0.037 day$^{-1}$, almost three times the growth speed of EKoku01, indicating that the strain from Okinawa, Japan was a slow grower. However, it is important to note, that these growth rate comparisons were restrained by the difference of culture conditions used in the respective studies. As the growth of microalgae could be enhanced by altering and combining various physical and chemical properties of the culture conditions (*e.g.*, light intensity, temperature, salinity, media, *etc*.), there is a possibility that EKoku01 can exhibit better performance when the right culture setup is defined. Nonetheless, our growth experiment suggested that EKoku01 preferred lower temperatures (<25 °C) for the strain to grow well, thus sampling strategy for this euglenophyte should probably be conducted when the water of Kokuba River reached the above temperature range.

When looking at the ecological aspect, a water sample containing EKoku01 cells was originally acquired during the Okinawan winter season when the water temperature of Kokuba River ranged 18-19 °C. This was in accordance with previous reports suggesting that blooms of *Eutreptiella* species frequently occurred in regions located in mid to high latitudes, where average water temperatures are relatively lower throughout the year, such as in the coastal waters of Norway (Throndsen, 1969), Japan (Okaichi, 1969; Iwasaki, 1971; Yamochi, 1984; Stonik, 2007; Yamasaki et al., 2019; Morimoto et al., 2024), and Korea (Lee & Lim, 2006; Kang et al., 2023), Russia (Stonik, 2007), Baltic Sea (Olli et al., 1996), and New Zealand (Norris, 1964). This might also explain why any euglenophyte cells could not be found in our sampling efforts during the Okinawan summer when the water temperature reached up to 31 °C. Until now, there is only two cases of *Eutreptiella* species blooms reported from the warm Banderas Bay in Mexico that caused massive fishkill (Cortés-Lara et al., 2010) and in shrimp farms in the south-west coast of India (Fernandes et al., 2019). Annual temperature records between 2010 to 2020 for Okinawa indicate stable temperature patterns fluctuating from 10°C (winter) to 34°C (summer), with a range of 11-28°C for the month of December (Weather Spark - https://weatherspark.com/). The specific impact of these natural temperature variations on EKoku01 cells remains undisclosed as the strain was only found once during the routine microalgae sampling conducted in Okinawa. Most likely, temperature alone may not exclusively influence the presence of this species in the Kokuba River. Other environmental factors like light intensity, nutrient availability, river dynamics, and others, are conjectured to significantly contribute to the occurrence and proliferation of Koku01 in this specific sampling locale. Further study to reveal the survival mechanism undertaken by *Eutreptiella* sp. during higher temperature seasons in Okinawa might be interesting to be pursued in the future.

## Phototaxis Response

Regarding the phototaxis observation, the initial cell density of EKoku01 was ~4.3 x 10$^6$ cells.mL$^{-1}$ (Figure 6). After 5 min, no color difference in the culture was observed in the tube, but a slight increase in cell density (~4.5 x 10$^6$ cells.mL$^{-1}$) was reached near the LED

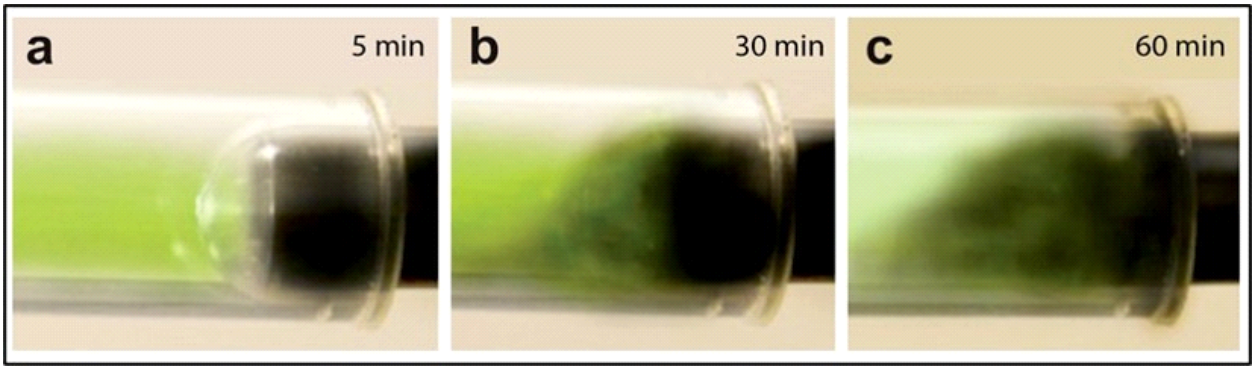


Figure 6. Time series photographs showing the accumulation of EKoku01 cells after 5 min (a), 30 min (b), and 60 min (c).

source (Figure 6a). Noticeable cell accumulation (9.4 x $10^6$ cells.$mL^{-1}$) was observed after 30 min of illumination (Figure 6b). Finally, after 60 min of illumination, there were ~13.3 x $10^6$ cells.$mL^{-1}$ of EKoku01 cells near the LED end of the tube (Figure 6c). These findings were quite intriguing because previous studies have not mentioned the phototaxis responses of *Eutreptiella* spp. in their investigations (*e.g.*, Yamasaki et al., 2019; Kang *et al*., 2023). Nonetheless, it would be interesting to investigate the optimum light intensity to effectively concentrate the cells for harvesting the microalgal biomass in future studies of any microalgal species exhibiting phototaxis.

## Fatty Acids Composition and Potential Application of EKoku01

We identified at least 5 groups and 13 individual fatty acids (FAs) from the EKoku01 culture that was grown at the optimum temperature of 20°C (Table 1). The predominant FA group was saturated fatty Acid (SFA) (69.82%), which comprised four components, namely palmitic acid (C16:0; 47.86%) as the highest, followed by stearic acid (C18:0), myristic acid (C14:0), and lignoceric acid (C24:0) at 20.27%, 1.08%, and 0.61%, respectively (Table 1). The second largest group was the polyunsaturated fatty acid (PUFA) (15.1%), which included essential FAs in the forms of omega-3 (eicosapentaenoic acid (EPA; 3.03%) and alpha-linolenic acid (ALA; 4.09%)) and omega-6 (gamma-linolenic acid (GLA; 4.11%)). The remaining FAs were grouped into monounsaturated fatty acid (MUFAs) (3.5%), branched FA (4.42%), and unidentified FAs (7.08%) (Table 1). Most of the detected FAs in EKoku01 were also found in other *Eutreptiella* species, albeit lesser in the number of FA components and their proportions. For example, Kang et al. (2023) detected a total of 19 FAs in *Eutreptiella* sp. strain KCTC 19016, 17 FAs in strain NIES-2305, and 16 FAs in both *E. gymnastica*, and *Euglena gracilis*. While, a total of 22 and 24 FAs were identified in *E. eupharyngea* (Yamasaki et al., 2019) and *Eutreptiella* sp. strain CCMP3347 (Kuo et al., 2021), respectively.

The presence of omega-3 and omega-6 FAs, albeit in lower concentrations, placed EKoku01 as a potential candidate for nutraceutical, pharmaceutical, food, and feed applications, including aquaculture (Yamasaki et al., 2019; Maltsev & Maltseva, 2021; Gu et al., 2023). Microalgae with substantial quantities of SFAs, such as EKoku01, have the potential to serve as a substitute for the synthesis of hydrogenated vegetable oils (Los 2014). Moreover, high SFA content in microalgae suggest early potential for the species to function as a substantial lipid reservoir for biodiesel generation (Maltsev et al., 2021). However, practical implementation of SFAs for biodiesel necessitates supplementing with proportionate quantities of monounsaturated fatty acids (MUFA) to enhance biodiesel fuel fluidity, particularly under cold conditions (Hoekman et al., 2012; Knothe 2012). Subsequently, our toxicity assessment using *Artemia* bioassay observed no brine shrimp mortality at different concentrations of EKoku01 extract up to 48 hours of exposure. This suggested that EKoku01 is safe for dietary consumption in animals, although more rigorous testing is required for human application. Furthermore, similar to the growth performance, the production of fatty acids in microalgae is affected by numerous factors (*e.g.*, temperature, light, salinity, nutrients, culture duration, stress, *etc*.) (Maltsev & Maltseva, 2021). Therefore, culture condition optimization for EKoku01, and other potential microalgal species, to achieve the desired bioactive compounds and FAs contents should be determined accordingly.

Another potential application of EKoku01 was its strong phototaxis response towards a light source (Figure 6) that can be adopted to develop an innovative light-dependent harvesting method for microalgal cells. Previously, various harvesting methods have been developed and applied for obtaining microalgal biomass,

Table 1. Fatty acid (FA) composition of *Eutreptiella* sp. strain EKoku01

| Group | FA components | (%) of total FA |
|---|---|---|
| Saturated (SFA) (69.82%) | C14:0 | 1.08 |
| | C16:0 | 47.86 |
| | C18:0 | 20.27 |
| | C24:0 | 0.61 |
| Monounsaturated (MUFA) (3.6%) | C16:1 n-7 | 1.35 |
| | C16:1 n-9 | 0.73 |
| | C17:1 | 0.79 |
| | C20:1 n-9 | 0.73 |
| Poly-unsaturated (PUFA) (15.1%) | C20:1 n-7 | 3.87 |
| | C18:3 n-6 (GLA) | 4.11 |
| | C18:3 n-3 (ALA) | 4.09 |
| | C20:5 n-3 (EPA) | 3.03 |
| Branched FA (4.42%) | C18:0 anteiso | 4.42 |
| Unidentified FA (7.08%) | – | 7.08 |

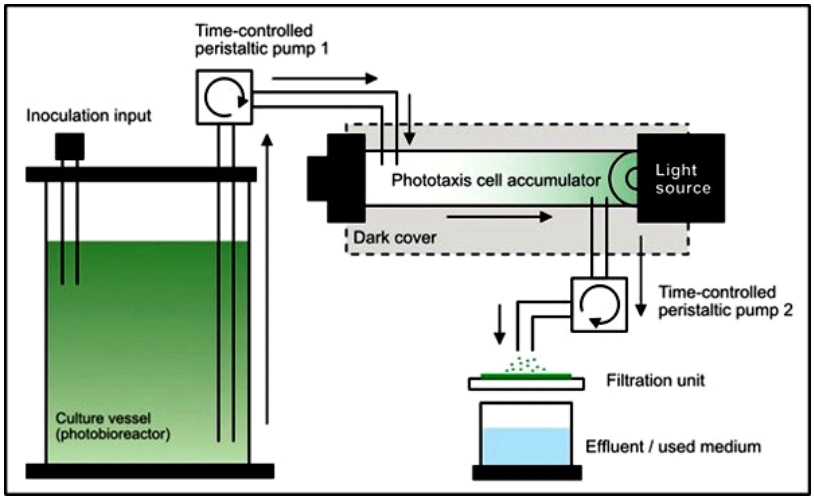


Figure 7. Conceptual design of an integrated phototaxis-based microalgal cell accumulation/harvesting system.

implementing filtration, centrifugation, flocculation, and flotation principals (Yin et al., 2020), sometimes with the combination of two or more of these methods. Thus, selecting the most appropriate cell harvesting method for microalgae mainly considers the following aspects: the nature of the microalgae species being cultivated (*e.g.*, cell size, specific characters, physiology, etc.), the desired end product, cost, and how the used medium is treated (Deepa et al., 2023). It is also possible to develop a specific harvesting method for certain microalgae species possessing unique characteristics. Nakajima and Takashi (1999) observed the phototaxis response of *E. gracilis* and developed a continuous culture system based on the principle of solid-liquid separation mechanisms combined with the phototaxis response of this freshwater euglenophyte. Here we propose a conceptual design of a similar system for EKoku01 (Figure 7). However, instead of using the cell accumulator to continuously return the cells to the culture vessel (*i.e.*, photobioreactor) as in Nakajima and Takashi (1999), our concept is to utilize the phototaxis-based cell accumulator (Figure 2) as an automated and time-scheduled cell harvesting tool that can be integrated into the culture vessel system (Figure 7). Although not yet tested, the potentiality of such an integrated device to harvest microalgae, particularly species exhibiting strong positive phototaxis response, should be explored in future studies.

## Conclusion

Our investigation established foundational insights into the identity and potential applications of *Eutreptiella* sp. strain EKoku01 isolated from Okinawa Prefecture, Japan. Given that the optimal synthesis of FAs and other bioactive compounds in microalgae cultures, including EKoku01, can be influenced by the cultivation

conditions, forthcoming studies should evaluate growth performance and biochemical production under diverse physical and chemical parameters (*e.g.*, light exposure, temperature, salinity, culture media, *etc*.) to pinpoint the most conducive culture settings. Furthermore, considering the high diversity of microalgae, we propose that ongoing efforts to sample, isolate, characterize, and identify novel microalgal species or strains in diverse ecosystems, seasonal, and climatic conditions are required to unveil their untapped capabilities.

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## Supplementary Materials

Supplementary materials is not available for this article

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