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Isolation of lipid-rich marine diatoms from the coastal waters of the Goto Islands, Japan

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Abstract Five diatom strains were isolated from the coastal waters of Goto Islands and their fatty acid content and composition were evaluated. Fatty acid composition of the isolates showed a similar tendency, and fatty acids of 14:0, 16:0, and 16:1 (*n-7*) accounted for the majority, comprising 7.4 to 27.2% of the total, 3.8 to 37.1% of the total, and 15.7 to 59.6% of the total, respectively. In addition, most isolates contained polyunsaturated fatty acid of 20:5 (*n-3*) (eicosapentaenoic acid, EPA) and docosahexaenoic acid (DHA), which are essential fatty acids in marine organisms. Among the isolates, a pennate diatom cf. Diploneis sp. contained the highest EPA. The potential of the pennate diatom for aquaculture feed, and the ecological significance of EPA and DHA content of diatoms in marine food webs in the vicinity of Goto Islands are briefly discussed.

Keywords: diatom, Goto Islands, isolation, PUFA

1. Introduction

Microalgae are notable for their rich lipid content, specifically polyunsaturated fatty acids (PUFAs) which regulate cell membrane fluidity and act as precursors to hormones, with *n-3* PUFAs being particularly beneficial for human health, and thus utilized in nutraceutical applications (Li et al. 2014). PUFA is known as essential fatty acid, which is transported through food webs from microalgae to higher trophic organisms that cannot produce PUFA (Brett & Müller-Navarra 1997). Since among PUFAs, 20:5 (*n-3*) (eicosapentaenoic acid, EPA) and 22:6 (*n-3*) (docosahexaenoic acid, DHA) are known to be important for the growth of various marine larvae, microalgae rich in EPA and DHA are being explored as excellent feed for larvae in aquaculture (Koven et al. 1989, Wacker et al. 2002, Patil et al. 2005, Waldock & Holland 1984).

Thus, EPA and DHA stand out as the most sought-after n-3 PUFAs, traditionally and currently sourced from fish oil. However, this method encounters several issues, including variable fish oil quality, concerns over the sustainable supply of fish oil, and the undesirable odor associated with fish products (Dulvy et al. 2003, Gerber et al. 2012). Furthermore, the production of EPA and DHA from fish oil is plagued by fluctuations in fish catches and resultant variability of prices. Consequently, there is a pressing need for cost-effective, alternative sources of DHA and EPA, prompting a search for new PUFA sources (Lenihan-Geels et al. 2013).

Microalgae emerge as a viable and sustainable alternative for *n-3* PUFA production, offering advantages such as a higher growth rate and greater biomass density compared to terrestrial crops. This has led to a growing interest in harnessing microalgae industrially for *n-3* PUFA production. In particular, diatoms are considered to be a suitable source of *n-3* PUFA (Dunstan et al. 1994). Yet, to date, only a limited number of diatom strains have been commercially exploited for producing high-value substances. Specifically, *Phaeodactylum tricornutum*, *Nitzschia laevis*, *Chaetoceros gracilis*, and some polar species are currently being explored on a laboratory scale (Hamilton et al. 2015, Katayama et al. 2020, Mao et al. 2020, Steinrücken et al. 2018)

Given the vast taxonomic diversity of diatoms, a broad variability in EPA and DHA production can be anticipated. This study aims to establish new strains with high EPA and DHA productivity from the coastal waters of the Goto Islands in west Japan, highlighting the potential of these organisms as sources of valuable PUFAs.

2. Materials and methods

2.1. Collection of water samples

Sampling was made in the coastal waters of the Goto Islands in Nagasaki prefecture, Japan in February and August 2017. The islands formed complex topography (Fig. 1) and were selected as an ecologically or biologically significant marine area (Ministry of the Environment, 2014). Seawater samples were collected using a bucket and then introduced into 500 mL bottles through a 180- μ m mesh to remove larger particles in Arikawa, Koteno-ura, Oso, Sangen-ya, Shirauo, and Taino-ura (Fig. 1). Diatoms were condensed on 0.45-μm membrane filters, and then cultivated in 12-well plates as enrichment cultures. Enriched f/2 medium with silicate was used throughout this study. To obtain various species, the serial dilution method was adopted (Throndsen 1978). The plates were incubated at 20 ± 1°C under an irradiation of 200 μmol m^{-2} s^{-1} with a 12L:12D cycle.

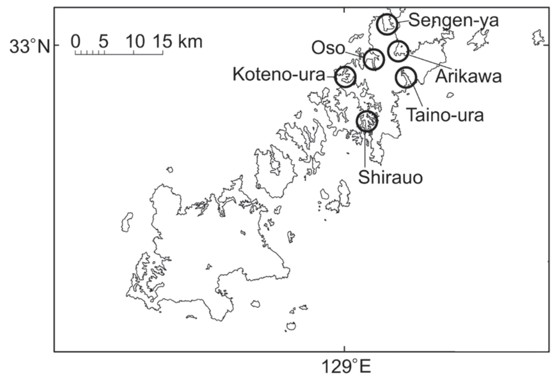

Fig. 1. Sampling locations in the Goto Islands.

2.2. Isolation and culture conditions

Diatoms were isolated using a micropipette under an inverted microscope. Each isolated cell was transferred to a well in 96-well plates and irradiated at 200 μmol m^{-2} s^{-1} with a 12L:12D cycle. Diatoms grown in 96-well plates were then transferred successively to a 12-well plate, 100-mL Erlenmeyer flask, and 500-mL Erlenmeyer flask. Isolates were maintained at 25 ± 1°C because they showed higher specific growth rates at 25 ± 1°C than at 20°C. Isolated diatoms were identified to species morphologically.

2.3. Fatty acid analysis

Cultures of each strain at the late logarithmic growth phase were harvested onto Whatman GF/F filters which were burned beforehand at 550℃ for 2 hours. After drying in a dryer (Lab-ware drying oven, Yamato Scientific) for 24 hours, dry weight (DW) was measured using ultra-microbalance (UMX2, Mettler Toledo). Wet biomass of each strain collected on a Whatman GF/F filter in triplicate was freeze-dried and stored at -80℃ (SANYO, MDF-C8V1) until extraction of fatty acids.

Fatty acids were extracted according to the protocol of Bligh & Dyer (1959) with some modifications. The stored GF/F filter was immersed in chloroform/methanol (1:2 v/v) mixture solution. Then, fatty acids were extracted by sonication, and the extract was dried using a dry thermo unit (DTU-1CN, TAITEC) in nitrogen gas as a blower. Then, 1 mL of acetyl chloride/methanol (5:100 v/v) was added to the dry extract, and heated at 100℃ for 1 hour. The reaction product was extracted three times with hexane, and the extraction was dried with nitrogen. Then, the dry matter was dissolved in 300-μL hexane.

Fatty acids were analyzed by gas chromatography-mass spectrometry (6890N GC/5973MS, Agilent Technologies) equipped with a DB-5 MS column (0.25 mm inner diameter, 30 m length, 0.25 μm film thickness, J&W Agilent Technologies). Helium gas was carried at 1.0 mL min^{-1}. The injector temperature was set at 310°C. Individual fatty acids were identified by matching with the mass spectrum of the standard substance and the retention time. Fatty acids were quantified from the ratio of the area of the mass fragment peak of the sample to that of the standard substance.

2.4. Data analysis

Results of GC/MS were analyzed using the software of Agilent MSD Productivity ChemStation for GC and GC/MS Systems Date Analysis. Tukey test was applied to evaluate significant differences (p<0.01).

3. Results

Five diatom species were isolated: a pennate diatom cf. Diploneis sp. (referred to as *Diploneis sp. hereafter*), *Cylindrotheca closterium*, *Bacterosira fragilis* from Koteno-ura, *Chaetoceros pseudocrinitus* from Taino-ura, and *Talassiosira subtilis* from Shirauo.

Fatty acid composition was not variable among the isolates (Table 1). Fatty acids of 14:0, 16:0, and 16:1 (*n-7*) accounted for the majority, comprising 7.4 to 27.2% of the total, 3.8 to 37.1% of the total, and 15.7 to 59.6% of the total, respectively. In addition, most isolates tended to contain PUFA of 20:5 (*n-3*) except *C. pseudocrinitus*, occupying 15.2 to 19.5% of the total. Biomass-specific cellular content of 16:1 (*n-7*) and 20:5 (*n-3*) in *Diploneis* sp. was 20.3 mg g-DW^{-1} and 6.7 mg g-DW^{-1}, respectively, significantly higher than those of other isolates (p<0.01) (Fig. 2). This strain also contained 0.70 mg g-DW^{-1} of 18:3 (*n-6*), 0.08 mg g-DW^{-1} of 22:0, 1.02 mg g-DW^{-1} of 22:6 (*n-3*), and 0.92 mg g-DW^{-1} of 24:0 (Fig. 2).

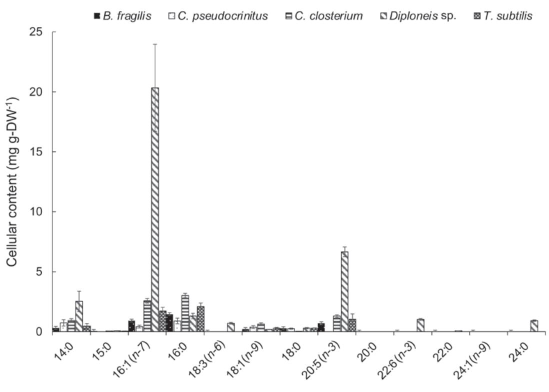

Fig. 2. Fatty acid content and its standard deviation (n=3) of *Bacterosira fragilis*, *Chaetoceros pseudocrinitus*, *Cylindrotheca closterium*, *Diploneis* sp., and *Thalassiosira subtilis*.

Table 1. Relative proportion of individual fatty acids (wt%) of isolated diatoms.

Fatty acid	*Bacterosira fragilis*	*Chaetoceros pseudocrinitus*	*Cylindrotheca closterium*	*Doploneis* sp.	*Thalassiosira subtilis*
14:0	7.9	27.2	10.9	7.4	7.7
15:0	1.3	0.0	0.7	0.2	0.9
16:0	37.1	33.1	35.2	3.8	35.1
16:1 (*n-7*)	23.2	15.7	30.1	59.6	28.9
18:0	7.1	9.5	0.4	0.9	5.1
18:1 (*n-9*)	5.6	14.4	7.5	0.5	5.0
18:3 (*n-6*)	0.0	0.0	0.0	2.1	0.0
20:0	0.0	0.0	0.0	0.0	0.0
20:5 (*n-3*)	17.8	0.0	15.2	19.5	17.4
22:0	0.0	0.0	0.0	0.2	0.0
22:6 (*n-3*)	0.0	0.0	0.0	3.0	0.0
24:0	0.0	0.0	0.0	2.7	0.0
24:1 (*n-9*)	0.0	0.0	0.0	0.0	0.0

4. Discussion

4.1. PUFA content in isolated diatoms

The isolates exhibited high ratio of 14:0, 16:0, 16:1 (*n-7*) and 20:5 (*n-3*) to total fatty acids, supporting the previous studies that diatoms contain high amounts of 14:0, 16:0, 16:1 (*n-7*) and 20:5 (*n-3*) (Servel et al. 1994, Viso & Marty 1993, Zhukova & Aizdaicher 1995).

Among the isolates, *Diploneis* sp. contained a large amount of PUFA, and its biomass-specific EPA and DHA contents were 6.66 mg g-DW^{-1} and 1.02 mg g-DW^{-1}, respectively (Fig. 2). DHA content of *Diploneis* sp. exceeded a reported high DHA content of 0.6 mg g-DW^{-1} in *Nitzschia frustulum* (Renaud et al. 1994). Thus, *Diploneis* sp. was the best producer of *n-3* PUFAs among the isolates in this study. However, the highest EPA content in diatoms is reported to be 62.3 mg g-DW^{-1} in *Phaeodactylum tricornutum* (Yongmanitchai & Ward 1991), which is almost one order of magnitude higher than that of *Diploneis* sp. in this study. Furthermore, higher *n-3* PUFA contents are known for other microalgae than diatoms (Ma et al., 2022). Therefore, the next step should be an examination of optimum culture conditions for better productivity of *n-3* PUFA, that is, both its content and algal growth rate of the isolates obtained in the present study (Ma et al., 2022).

Yongmanitchai and Ward (1991) investigated optimal culture conditions to maximize the EPA production of P. *tricornutum* and increase the EPA content by increasing salinity in the medium. A similar observation was made in *Nitzchia laevis*, which showed elevated EPA content presumably as a result of decreasing cell membrane fluidity to withstand high salinity (Chen et al. 2008). In addition, light, temperature, and growth stage are known to be effective environmental factors that promote EPA accumulation (Li et al. 2014). In the present study, while an optimal culture condition for EPA accumulation of *Diploneis* sp. was not examined, it is expected to increase EPA content of *Diploneis* sp. by suitable combination of the above factors.

4.2. Application of *Diploneis* sp. to aquaculture

Benthic diatoms are known to be fed by a variety of benthic marine organisms (Araújo-Castro & Souza-Santos 2005, Gerdol & Hughes 1994, Kanaya et al. 2005, Kawamura et al. 1998, Xing et al. 2007). In aquaculture, benthic diatoms are used as feed for the larvae of abalone, sea urchin, and harpacticoid copepod *Tisbe biminiensis,* and EPA and DHA in diatoms promote their growth, survival, settlement, reproduction and development (Araújo-Castro & Souza-Santos 2005, Gordon et al. 2006, Liu et al. 2007, Pinto et al. 2001, Xing et al. 2007, 2008). In addition, since *T. biminiensis* has a relatively low DHA content compared with other harpacticoid copepods, it is necessary to increase its DHA content before feeding it to fish larvae (De Lima et al. 2013, Liu et al. 2007). For this purpose, *Diploneis* sp. isolated in the present study containing relatively high EPA and DHA content can be used as a food for *T. biminiensis* as well as other benthic organisms.

4.3. Ecological significance of diatoms near the Goto Islands

Diatom blooms are recurrent every spring in the waters around the Goto Islands, followed by the increase of copepod abundance from spring to summer (Enomoto 1957, Enomoto & Hamada 1962). The dominance of calanoid and oncaeidae copepods was reported from August to September in the vicinity of the Goto Islands (Tanaka et al. 2006). Copepods contain fatty acids in wax esters that are mainly derived from phytoplankton feeding (Sargent & Peterson 1988). Calanoid copepods contain a large amount of C16 and 20:5 fatty acids, which are known as characteristic fatty acids of diatoms (Katter 1989). According to the feeding experiment of *Acartia*, high levels of 16:1 (*n-7*) and EPA were found in their bodies after feeding diatoms (Dalsggard et al. 2003). Moreover, when *Calanus helgolandicus* feeds diatoms, fatty acid composition is quite similar to that of the fed diatoms (Dalsgaard et al. 2003, Graeve et al. 1994). These observations indicate that calanoid copepods obtain 16:1 (*n-7*), EPA and other fatty acids directly by feeding diatoms. EPA is strongly correlated with the egg production rate of calanoid copepods (Brett & Muller-Navarra 1997). Egg production of *Acartia tonsa* fed by diatoms increases 10 times higher than that fed by ciliates, and the diatom-eating copepods and their eggs contain EPA predominantly (Brett & Muller-Navarra 1997). In general, marine animals uptake EPA directly by feeding, and convert it to prostaglandins for their growth and reproduction (Dunstan et al. 1994). The isolated diatoms in the present study contained EPA with variable contents ranging from 0.70 to 6.7 mg g-DW^{-1}, indicating that these diatoms contribute to egg production of calanoid copepods. In the waters around the Goto Islands, *Thalassiosira subtilis* was dominant from January to March, followed by copepods (Enomoto 1957), suggesting *T. subtilis* containing EPA supports the population growth of copepods (Fig. 2).

5. Conclusion

In this study, five diatom strains were isolated from Goto Islands and their fatty acid composition was evaluated. Among the isolates, Diploneis sp. contained the highest EPA and DHA, suggesting its potential for food for larvae in aquaculture. In order to exploit this possibility, an examination of optimum environmental factors for higher EPA and DHA content as well as for growth rate is the next step. In addition, since it was indicated that diatoms contribute to the egg production of zooplankton around the Goto Islands, the ecological implication of PUFA contents in diatoms is an interesting subject to understand planktonic dynamics in this area.

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