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RESEARCH ARTICLE

THE CULTIVATION OF SOME DIATOM SPECIES (*CHAETOCEROS* SP. AND *NITZSCHIA* SP.) ISOLATED FROM YUMURTALIK BAY (NORTHEAST MEDITERRANEAN)

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ABSTRACT

The diatoms collected and isolated from the Yumurtalık Bay at Northeastern Mediterranean at Gulf of Iskenderun, and species were cultivated under laboratory conditions, identified using molecular techniques, and their growth and biochemical composition were investigated. Sampling was carried out using a plankton nets having 20 µm and 55 µm mesh size for 10 minutes at different depths, horizontally. Salinity, temperature and pH were measured with a YSI model salinometer at each station. The isolation of the required species were done by serial dilutions and applying agar plating methods. Isolated diatom species were cultured in the different media. To purify the species, antibiotic (Ampicillin sodium and Kanamycin) media were used at 5 different rates (200 mg/L, 400 mg/L, 600 mg/L, 800 mg/L, 1000 mg/L). At the same time, acid/base treatment (pH2, pH3, pH4, pH5, pH9, pH10 and pH11) were applicated to the samples. These cultures were maintained at room temperature (20±1°C) under 40 µmol photon $m^{-2}s^{-2}$ light intensity at 12:12-h light/dark cycle and were aerated continuosly. Isolates were identified morphologically. Isolated diatom species were detected following morphological and molecular identification. Genomic DNA extractions were performed with a DNeasy Plant Mini Kit (Qiagen, Tokyo, Japan) following the manufacturer's protocol. Then DNA isolated from the cells was and amplified by PCR. Identifications of the species were made with phylogenetic analyses depending on nucleotide sequences of small subunit of nuclear ribosomal DNA. Nucleotide sequencings were performed commercially by Macrogen Inc. (Korea) with the same primers used for PCR amplifications. To evaluate the phylogenetic relationships among isolates Neighbor-Joining (NJ) were used. Experiments were carried out by cultivation of 10^4 cells mL^{-1} of *Chaetoceros sp.* and *Nitzschia sp.,* in 1 L cylinder tubes containing sterilized Si-F/2 medium with a salinity of 30 ppt. The specific growth rate and biomass and biomass productivity, cell density, chlorophyll a, total carotenoid, protein, carbohydrate, lipid and lipid productivity, fatty acid methyl esters (FAMEs) and fucoxanthin contents were determined and showed significant differences ($p<0.05$) between these two species. The best growth and biochemical composition were obtained from the diatom *Chaetoceros* sp., while the highest amount of fucoxanthin was found in *Nitzschia* sp.

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INTRODUCTION

Microalgae ecologically represent an important group that carry out primary production in marine or freshwater environment. Microalgae, which are mostly photoautotrophic organisms, have an active role by being the largest producers of organic matter in marine and freshwaters. Due to microalgae significant importance in primary production, it is thought that a study pioneering natural environment works is found vital to carry out a research in laboratory or outdoor conditions. Our country is generally well suited for microalgae culture in terms of sunny days and mild climate.

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It is crucial to increase the number of studies on the utilization of algae and to transform the results obtained from these studies into products that will contribute the economy. However, our country's seas are extremely rich in microalgae diversity and there are many marine or freshwater species whose commercial importance is unknown yet. The microalgae culture studies in our country are carried out mostly with commercial species provided from abroad. The isolation of microalgae from the territorial waters from our region and determination of taxonomic locations of microalgae species in details by molecular phylogeny methods and in the future, cultivation of species under laboratory or outdoor conditions together with will provide resource for industrial production which will be the unique aspects of the researches. With reference to European Union's Blue Growth Policy which describes the further utilization of marine resources so the

OECD's similar policy namely Green Growth nevertheless considering that four thirds of the world is covered with sea and oceans and our country remain as a peninsula, could provide sufficient reason for best utilization of marine resources. Microalgae exist in almost every habitat in all over the world. Microalgae have the ability to use solar energy in combination with water and carbon dioxide to create biomass. Microalgae are able to double their weights daily, can easily be used in biotechnological processes, have low cost, contain many useful substances which have an economic value and their ability to resist against environmental factors are the reasons of their significance. Diatoms ecologically represent an important group of microalgae that carry out primary production in marine environment. Diatoms, which are mostly photoautotrophic organisms, have an active role by being the largest producers of organic matter in marine and fresh waters. Various studies have shown that these photo synthetically active organisms are responsible for 20–25% of total terrestrial primary production (Field *et al*. 1998) and approximately 40% of annual marine biomass production (Falkowski *et al*. 1998).

Due to diatoms significant importance in primary production, it is thought that a study pioneering natural environment works is found vital to carry out a research in laboratory conditions. In line with EU Blue Growth and OECD Green Growth Policy and bearing in mind that approximately 75% of the world is covered by seas, it has been thought that utilization of further marine resources should be diversified ad enhanced. Diatoms are unicellular, sessile, photosynthetic algae which compose silica cell wall. They are found in diverse habitats like freshwater, marine and brackish. They are important tools for determining ecological conditions and play an important role of the primary production in these habitats. Also, they play a vital role on the photosynthesis in aquatic habitats. Nowadays, interest in microalgal biotechnology has mostly been focused on the efficient algae culture technologies to produce valuable metabolites commercially from algal biomass.

In recent years, an increasing interest in microalgal biotechnology was noticed due to the accumulation of cells metabolites in large amounts. Microalgae are used for various purposes mainly as food supplement, fertilizer for improving soil structure, in animal feed due to protein, vitamins, fatty acids, carbohydrates, minerals and pigments, hydrocarbons, polysaccharides, antibiotics, and for other metabolites (Becker, 2007; Milledge 2011; Yaakob *et al*., 2014). Microalgae are a diverse group of microscopic organisms with the wide range of biochemical compounds and contain up to 50-70% protein, 30% lipids, 20-40% carbohydrates, over 40% glycerol, up to 8-14% carotene and high concentration of aminoacids, fatty acids and vitamins (B1, B2, B3, B6, B12, E, K, D, etc.), compared with other plants or animals (Avagyan, 2008). Despite the fact that more than 50000 microalgae species of them are known, only 30000 have been researched (Mata *et al*., 2010). The diatom numbers of their genera and species are approximately 250 and 100,000, respectively (Lebeau and Robert, 2003; Guiry, 2012). Nowadays, there are numerous commercial applications of diatoms to produce valuable metabolites commercially from algal biomass. Microalgal biomasses have been produced industrially for applications in different fields such as food, aquaculture, pharmaceutical, nutraceutical, cosmetic, and animal feed industries. Industrial applications considered and pioneered for commercial use of diatoms consist of nitrogen-fixing biofertilizer, renewable energy, fluid fuel production, raw materials production, and naturally-occurring and industrial waste detoxification using biological refuse as substrate (Bozarth *et al.,* 2009). Besides these important aspects, which have been showed that reviewed recently (Kroth 2007), cell biological and molecular applications have been emphasized for diatoms in the last couple of years, revealing enthusiastic perspectives for new applications of diatom biotechnology. In many countries, lipid productions from microalgal biomass studies are subject to research. With regard to the benefits of using microalgal biotechnology, the major areas of progress have been in the fields of biodiesel production using microalgae. In many countries they are researching the stress conditions which stimulate the increase of the current lipid content in the cell and they are making serious efforts to specify the high growth rate of microalgal species and their lipid content. Diatoms were investigated that have higher lipid content than other algal species (Hildebrand *et al.,* 2012).

The high production of lipids in many species of diatoms has attracted great notice to the potential use of diatoms as a source for biofuels (Daboussi *et al.,* 2014). Lipids are the major metabolites of diatoms, being consisting of neutral lipids, polar lipids and traces of sterols (Chen, 2007). Several studies have shown that neutral lipids are their main stored lipids, contains of triacylglycerol (TAG), diacylglycerol (DAG) and monoacylglycerol (MAG), with the TAG content typically accounting for more than 60% of total lipids (Chen, 2007). The fatty acids of diatoms consist of those from lauric acid (C12:0) to lignoceric acid (C24:0). The contents of myristic acid (C14:0), hexadecanoic acid (C16:0), 9-hexadecenoic acid (C16:1) and eicosapentaenoic acid (EPA, C20:5 n-3) are generally higher than those of other fatty acids (Ackman et.al., 1964, Kates and Volcani, 1966; Chuecas and Riley, 1969; Wen and Chen, 2002). In addition to, some diatoms include docosahexaenoic acid (DHA, C22:6 n-3), which is important for many marine animals that appear to have a limited capacity for synthesising longchain polyunsaturated fatty acids, especially EPA and DHA, which are needed for good growth and survival (Reitan *et al.,* 1994; Renaud *et al*., 1991; Yongmanitchai and Ward, 1989). Several researches have been carried out concerning the effectiveness of different species of diatoms as a food source for culturing larvae of marine organisms. Therefore, alteration in the lipid composition of various diatom species under different environmental conditions is a topic worth investigating. Therefore, the number of studies on this issue can be increased.

Fucoxanthin, a carotenoid with potential to be used in pharmaceutical, food and cosmetics, not found in higher plants is an organic substance with known antioxidants and coloring properties is an important metabolite generated by diatoms within the cell. In many countries, lipid productions from microalgal biomass studies are subject to research and in this study lipid production capacity of chosen local species have been determined and defined molecular species will be registered for our country. To produce microalgal metabolites of high-value products specifically, using microalgae is a large income-generating industry the potential for substantial in many parts of the world growth. Having three of the four covered with seas and oceans around the world and in our country is surrounded on three sides by the sea, it should be considered as important reasons that we need to make better use of our marine resources. In this study; the diatoms collected and isolated from the Yumurtalık Bay at Northeastern Mediterranean at Gulf of Iskenderun, and two

species (*Chaetoceros* sp. and *Nitzschia* sp.) were cultivated under laboratory conditions, identified using molecular techniques, and their growth and biochemical composition were investigated. Finally, strain selection, isolation and purification techniques for diatom species, molecular identification, growth characteristics, large scale cultivation, and harvesting are explained and the possibilities of their improvement have been discussed and evaluated. The isolation of diatoms from the territorial waters from our region and determination of taxonomic locations of diatom species in details by molecular phylogeny methods and in the future, cultivation of species under laboratory conditions together with have provide resource for industrial production. In conjunction with the determination of biochemical contents of local diatom species in the region, industrial use possibilities of these species have be revealed. Thus, by utilizing the advantage of more efficient cultivation of local species in regional climatic conditions, new types of candidates for commercial use have been explored. Moreover, the phylogenetic records of diatom species identified in the area have been done which will permit to collect data that have contribute to the diversity of the scientific literature.

MATERIALS AND METHODS

Iskenderun Bay (Fig. 1) is located in the north-eastern. It has a length of 65 km and a width of 35 km giving surface area of 2275 km^2 and maximum depth is 100 m. The sea surface temperature varies from nearly 20.5 ^{0}C in spring, 27.5 ^{0}C in summer, 26 ^{0}C in autumn, 19 ^{0}C in winter. The salinity reaches a maximum of 37.5–38.5 ppt. Sampling was carried out using a plankton nets having 20 µm and 55 µm mesh size plankton nets for 10 minutes at different depths, horizontally. The samples were collected horizontally from the sampling stations shown in Fig. 1, seasonally. Firstly, depths of horizontal sampling were 70 m (35°48′45.1″E 36°43′41.3″N), 60 m (35°48′51.3″E 36°44′67.6″N), 50 m (35°48′34.0″E 36°44′39.2″N), 30 m (35°48′33.8″E 36°45′23.7″N) and 20 m (35°47′89.1″E 36°45′82.6″N). Salinity, temperature, pH measurements of each stations were made with a YSI model salinometer. Samples were placed in plastic jars and kept in ice in the dark plastic box and transferred to Algal Biotechnology Laboratory, Fisheries Faculty, Cukurova University within 2h. The isolation of the required species were done by serial dilutions and applying agar plating methods.

By agar-plating method: 1.5 % agar was added to 1 L of Si-Conway Medium (Walne, 1974) and Si-F/2 medium (Guillard and Ryther 1962, Guillard 1975) this agar solution was sterilized in an autoclave for 15 min under 150 lbs pressure and 120°C temperature. Then this medium was poured in sterilized Petri dishes. The inoculum was introduced using a wire loop which was first sterilized in a flame, then the loop was loaded with a small amount of sample, and parallel streaks of the species suspension were made. The plates were covered and sealed with parafilm, and incubate under low light (40µmol photon $m^{-2}s^{-2}$) at a constant temperature (20°C±1). The agar plates were incubated until colonies of cells appeared after 2 weeks approximately. Meanwhile the plates were regularly observed under microscope to make sure that a unialgal species was isolated. Cells were picked up with a loop and then rinsed in a liquid culture medium in 50 ml Erlenmeyer flasks. After isolation, stock cultures were establish in a Si-Conway and Si-F/2 medium under controlled laboratory conditions, at room temperature (20°C±1), continuously illuminated by fluorescent lamps providing 40 µmol photons $m^{-2}s^{-2}$, and manually shaken three time per day. The stocks were maintained by monthly subculturing them. The composition of Si-Conway Medium was as following (Walne, 1974) (1 ml Conway Medium/liter of seawater): Stock enrichment solutions: $NaNO_3$ (100 g); NaH2PO4 (20 g); Na_2EDTA (45 g); H_3BO_3 (33.60 g); $FeCI_3.6H_2O$ (1.30 g); $MnC_2.4H_2O$ (0.36 g) and $Na_2SiO_39H_2O$ (30g) added to 1 ml/L; Trace metal 1 ml*; Vitamin Mix 100 ml**, Distilled water 1 liter. *Trace metal solution: $ZnCl_2$ (2.10 g); $CoC_2.6H_2O$ (2.00 g); $(NH_4)6MO_7O_{24}$. $4H_2O$ (0. 90 g); $CuSO_4.5H_2O$ (2.00 g); Distilled water 100 ml. **Vitamin stock solution: B12 (10 mg); B1 (thiamin) (20 mg); Distilled water (200 ml). The cultures were grown in Si-F/2 medium (Guillard and Ryther 1962, Guillard 1975). The medium consist of following composition:75gL^{-1} $NaNO_3$, 5gL^{-1} $NaH_2PO_4H_2O$, 30gL^{-1} $Na_2SiO_39H_2O$ and trace metal solution (9.8gL^{-1} $CuSO_45H_2O$, 6.3gL^{-1} $Na_2MoO_42H_2O$, 22gL^{-1} $ZnSO_47H_2O$, 10gL^{-1} $CoCl_26H_2O$, 180gL^{-1} $MnCl_24H_2O$) were added to 1 ml/L, and the vitamin solution (1gL^{-1} biotin, 1gL^{-1} cyanocobalamin) was added 0.5 mL to 1L.

Serial dilution: Label tubes 10^{-1} to 10^{-10} indicating dilution factor. Aseptically add 1 ml of enrichment sample to the first tube (10^{-1}) and mix gently. Take 1 ml of this dilution and add to the next tube (10^{-2}), mix gently. Repeat this procedure for the remaining tubes (10^{-3} to 10^{-10}). Incubate test-tubes under controlled temperature and light conditions: Examine cultures microscopically after 2-4 weeks by withdrawing a small sample aseptically from each dilution tube. A unialgal culture may grow in one of the higher dilution tubes e.g. 10^{-6} to 10^{-10}.

Isolation of diatom and then pure culture: Antibiotic (Ampicillin sodium and Kanamycin) media were prepared at 5 different rates (200 mg/L, 400 mg/L, 600 mg/L, 800 mg/L, 1000 mg/L) after sterilization of the agar medium in the autoclave. Into 350 ml Si/F2 medium; 200 mg/L for 0.35 mL Antibiotic (Ampicillin sodium and Kanamycin), 400 mg/L for 0.7 mL Antibiotic (Ampicillin sodium and Kanamycin), 600 mg/L for 1.05 mL Antibiotic (Ampicillin sodium and Kanamycin), 800 mg/L for 1.4 mL Antibiotic (Ampicillin sodium and Kanamycin), 1000 mg/L for 1.75 mL Antibiotic (Ampicillin sodium and Kanamycin) were added. Proportions of antibiotics were prepared by adding the proportions. It was then poured separately into agar plates (about 15 mL) and waited for cooling. Using four different types of loops, the agar plate were streaked in a line-by-line sequence. After streaking, the agar plates were incubated until colonies of cells appear. When colonies, each origatining from a single isolated cell, are present, these were isolated from the agar plate. Every day was checked agar plates, regularly. Cleaner to completely purify and single colonies were re-streaked where single colony-microalgae species were better developed. Formed colonies transferred to test tubes and inoculated to test tube with broth media. After 1 week coloring in the test tubes started. Every day mixed test tubes with vortex. And then, transferred to new flask, that to increase the cell density. Algae transferred to new flask were continuously mixed and illuminated with shaker. As the number of cells increases were applicated acid/base treatment. Seawater prepared 7 treatments including pH2, pH3, pH4, pH5, pH9, pH10 and pH11 for acid/base treatment. 1 ml of sample was taken from the flask. And eppendorf tubes were centrifugeted 3500 rpm, 5minute with mini scanspeed. Upper leaved the liquid. Eppendorf tubes were mixed artifical sea water (pH2, pH3, pH4, pH5, pH9, pH10 and pH11) taken 1 ml

and rested for 5 min. Again eppendorf tubes were centrifugeted 3500 rpm, 5minute and upper leaved the liquid. This application was repeated 5 rounds. The algae sample that was deposited at the bottom of the eppendorf tube was again streaked with agar.

Morphological and molecular identification: The identification of the isolates was established under microscope based on the morphological characters following Tregouboff and Rose (1957), Cupp (1977), Richard (1987), Delgado and Fortuno (1991), Hartley (1996) and Tomas (1997). Isolated diatom species were detected following morphological and molecular identification. Genomic DNA extractions were performed with a DNeasy Plant Mini Kit (Qiagen, Tokyo, Japan) following the manufacturer's protocol. Then DNA isolated from the cells was and amplified by PCR. Fifteen microliters of PCR master mix (0.5 U Taq polymerase (Promega Corp.), 0.25 mM each of all 4 dNTPs, 5 × reaction buffer, and 1.5 mM MgCl2) including 20 pmol of each primer were added to the PCR tube containing approximately 5 µl of the crude lysate at 4°C. Thermocycling was as follows: initially 95°C for 5 minutes, followed by 40 cycles of 95°C for 20 seconds, 55°C for 30 seconds, and 72°C for 60 seconds. Extension was facilitated at 72°C for 6 minutes. The amplicons were run at 1.5% agarose gel prepared in 1X TBE buffer, then were stained with ethidium bromide, and were then purified using a QIAquick PCR Purification Kit (Qiagen, Germany) as recommended. The PCR products were electrophoresed on 1% agarose gel (Amresco, Solon, OH, USA) prepared in 1X Tris-borate-EDTA (TBE) buffer. An MGW-Biotech thermal cycler was used for the amplifications in this study, and the electrophoresis gels (stained with ethidium bromide) were visualized with the GeneGenius Bio imaging system (Syngene, Synoptics Group, Cambridge, UK). Nucleotide sequencing of 28S LSU was performed commercially by Macrogen Inc. (Korea) with the same primers used for PCR amplifications. The SeqMan II software module of the LASERGENE 99 system (Applied Biosystems) was used to assemble nucleotide sequencings. Identifications of our samples were made with phylogenetic analyses depending on nucleotide sequences of small subunit of nuclear ribosomal DNA. Nucleotide sequencings were performed commercially by Macrogen Inc. (Korea) with the same primers used for PCR amplifications. Multiple data sets were aligned using ClustalX (Thompson *et al*. 1994) and then edited manually with BioEdit (Hall 1999). An initial redundant analysis was carried out by the software program DnaSP v.5. To evaluate the phylogenetic relationships among isolates Neighbor-Joining (NJ) were used.

Experimental Culture Conditions: Cultures were maintained at room temperature (20±1°C) under 40 µmol photon $m^{-2}s^{-2}$ light intensity on a 12:12-h light/dark cycle and were aerated continuosly. Experiments were carried out by cultivation of 10^4 cells mL^{-1} of *Chaetoceros* sp. and *Nitzschia* sp., in 1 L cylinder tubes containing sterilized F/2 medium with a salinity of 30 ppt.

Analytical Methods: Samples were taken daily for analyses of the specific growth rate, cell density, biomass and biomass productivity, chlorophyll *a*, total carotenoid. The specific growth rate (µ) was calculated from the slope of the linear regression of time (days) and cell density (cell ml^{-1}) according to eq. (1) (Wood *et al*. 2005):

$$\mu=(\ln_{Nt}-\ln_{N0})/\Delta t\mu=(\ln_{Nt}-\ln_{N0})/\Delta t \quad (1)$$

where µ (day^{-1}) is the specific growth rate in log phase, N_0 is the cell density at the beginning of log phase and N_t is the cell density at late log phase.

The growth rate of *Chaetoceros* sp. and *Nitzschia* sp. were characterized based on cell counts using a haemocytometer or via optical density measurements at 700nm by a UV-visible spectrophotometer (Shimadzu, UV mini, 1240 model, Japan). Biomass was determined according to the method developed by Boussiba *et al*. (1992) with 10 mL of microalgae culture through glass fiber filter (Whatman GF/C, 1.2 µm, UK). Algal biomass on the filter was dried at 105 °C for two hours and weighed (Boussiba *et al*., 1992). The biomass productivity was calculated according to eq. (2) (Song *et al*. 2013).

$$\text{Biomass productivity } mgL^{-1}day^{-1}=\mu\text{Xbiomass} \quad (2)$$

For pigment analyses, 10 mL samples were centrifuged at 3500 rpm for 10 min, and the pellet extracted with 5 mL acetone (Parsons and Strickland, 1963). The extracts were centrifuged again and chlorophyll *a* and total carotene were measured spectrophotometrically, recording the absorption at 665, 645, 630 and 480 nm and using the equations of Parsons and Strickland (1963). All measurements were repeated in five replicates. For lipid and lipid productivity, fatty acid methyl esters and protein analyses, samples of microalgae were collected at the stationary phase of the growth. *Chaetoceros* sp. and *Nitzschia* sp. cells were separated from the medium by centrifugation at 7500 rpm for 10 min, using the centrifuge model of Hereaus Supragufe 22. However, biomass was dried at 55°C for 2 h, pulverized in a mortar and stored at -20°C for later analysis. Dry extraction procedure according to Zhu *et al*. (2002) with a modification of the wet extraction method developed by Bligh and Dyer (1959) was used to extract the lipid from microalgae cells. Cells were harvested by centrifugation at 7500 rpm for 10 min. After drying, the samples were pulverized in a mortar and extracted using a mixture of chloroform: methanol (2:1, v/v), overnight. About 120 mL of solvents were used for every gram of dried sample in each extraction step. The solid phase was separated carefully using filter paper (Advantec filter paper, no. 1, Japan) in which two pieces of filter papers were applied twice to provide complete separation. The solvent phase was evaporated in a rotary evaporator by vacuum at 60°C. Total lipids and lipid productivity were calculated according to eqs. (3–4) (Song *et al*. 2013).

$$\text{Total lipid content}=(\text{Total Lipids/biomass})\text{x}100 \quad (3)$$

$$\text{Lipid productivity}=\text{Lipid content x Biomass productivity} \quad (4)$$

The amount of total protein (Nx6.25) was determined by Kjeldahl method (AOAC, 1995). FAMEs of algal lipids were prepared following the method of Prevot and Mordret (1976). Briefly, an aliquot of lipid (~9–12 mg) was added to 2 ml of 0.5 M CH_3ONa and was incubated at 55 °C for 30 min. Then, 2 ml of distilled water and nhexane were added and gently mixed to recover the upper nhexane layer containing FAMEs, which was neutralized by washing with distilled water many times and was finally purified by silica gel column chromatography using a 5% mixture of diethyl ether in hexane. The resulting FAMEs were analysed by GC-FID (7890 A, Agilent Technologies, USA) equipped with a DB-Wax column (127-2012, Agilent Technologies, USA). The oven temperature was set at and held at 170 °C for 2 min, then was

raised 5 °C min^{-1} to 240 °C where it was held for 14 min. The injector and detector temperatures were set at 250 °C. FAME components were identified by comparisons with retention times of FAME standards (37 FAME standards C4-C24, Supelco, USA). The carbohydrate content was determined as described by Liang *et al*. (2013). The total carbohydrate (CT) concentration was represented by the following equation: CT ¼ 0:9CR. Fucoxanthin content was analyzed by high performance liquid chromatography (HPLC), and purified fucoxanthin was determined by Terasaki *et al*., 2009. The freeze dried sample (100 mg) was mixed with methanol (15 ml) and stirred using a magnetic bar for an hour at room temperature. Solids were removed using a Whatman No.1 filter paper (Whatman GF/C, UK). Hexane (15 ml) and water (15 ml) were added to the methanol extract and vortexed for 1 min and left to partition into two distinct layers using a separation funnel. The upper phase was discarded and the lower phase was vortexed for 1 min with 10 ml of chloroform to extract fucoxanthin. The aqueous phase was removed, leaving the organic layer undisturbed. The organic phase was then dried at 30 C using a rotary evaporator. Methanol (5 ml) was added to the dried extract. Stored at -20°C samples for taken in 1ml transferred to GC tubes. Fucoxanthin concentration were separated 10mg/l for 1ml. The samples concentrated with methanol were re-filtered and transferred to new vials by filtration through glass syringe with methanol.

Fucoxanthin content was analyzed by high performance liquid chromatography (HPLC), as described previously. The HPLC analysis was carried out with an Agilent-1260 HPLC system equipped with a pump (1260 quat pump VL), an autosampler and a detector (UV), and online analysis software (HPLC-1260 online). The analyses were carried out at room temperature using a reversed-phase column (Zorbax Eclipse Plus C18,, 250 4.6 mm i.d., 5.0 μm particle size) protected with a guard column having the same stationary phase. The mobile phase was methanolacetonitrile (70:30, v/v), temperature 25 ^{0}C, the flow rate was 1.0 mL/min and the sample injection volume was 10 ml/min. Fucoxanthin was detected at 450 nm, and its content was estimated by the standard calibration curve using purified fucoxanthin. Each fucoxanthin standard curve set was injected (Fucoxanthin standart, FLUKA 16337-1). And then, 1 hours oven 60 ^{0}C dried. Cooled in a desiccator and weighted. Results were expressed in milligram fucoxanthin per gram biomass dry weight (mg FX•g^{-1} DW). The results were expressed as the mean values ± standard deviation. Comparisons of the means were conducted by oneway analysis of variance (ANOVA), followed by a Duncan's multiple range test to determine significance. In all cases, comparisons that showed a p value < 0.05 were considered significant. SPSS statistical package programme was used to compare means (Version 12.0, SPSS, Chicago, IL) (Zar, 1999).

RESULTS AND DISCUSSION

The specific growth rate and biomass and biomass productivity, cell density, chlorophyll *a*, total carotenoid, protein, carbohydrate, lipid and lipid productivity, fatty acid methyl esters (FAMEs) and fucoxanthin contents determined and showed significant differences ($p< 0.05$) between these species. The growth was continued to 8 days for *Chaetoceros* sp. and 10 days for the *Nitzschia* sp .The best growth and biochemical composition were obtained from the diatom *Chaetoceros* sp., while the highest amount of fucoxanthin was found in *Nitzschia* sp. These species growth and biochemical composition are summarized in Table 1. These species fatty acids are summarized in Table 2 and 3. The major fatty acids of the *Nitzschia* species were 14:0, 16:0, 16:1(n-7), 16:3(n-4) and 20:5(n-3). The major fatty acids of *Chaetoceros sp.* were 14:0, 16:0, 16:1. The lipid and lipid productivity of *Chaetoceros sp*. was 19.74±2% and 15.32±1.35 $mgL^{-1}day^{-1}$ determined, respectively. The lipid and lipid productivity of *Nitzschia sp.* was 15.71±3% and 9.54±1 $mgL^{-1}day^{-1}$ determined, respectively. Despite the advances in algal biotechnology, there are some difficulties about in the microalgae culture. Basically, the main objective of the production of phototrophic organisms is to provide the continuous culture. It is known that the microalgae species are affected from changing environmental factors. The microalgae cells react to these changing factors, continuously. The biochemical composition of biomass depends on growth conditions such as nutrient medium, temperature, salinity, pH, and light (Sukenik, 1991; Cohen *et al*., 1988; Brown *et al*., 1989; Roessler, 1990, Lourenço *et al*., 1998; Hu, 2004). Many studies have shown that, the quantity and quality of lipids within the cell can vary as a result of changes in growth conditions such as temperature and light intensity, high salinity, or nutrients, especially concentrations of nitrogen, phosphates and iron (Illman *et al*., 2000; Liu *et al*., 2008; Xin *et al*., 2010).

In this study, the optimum conditions for isolated diatom species were determined and their growth and biochemical composition were investigated. The best growth in *Chaetoceros* sp. and *Nitzschia* sp. were observed in the Si-F/2 medium. This suggests that the microalgae has been adapted to Si-F/2 medium concentration. The highest spesific growth rate, biomass and lipid productivity values were found for *Chaetoceros* sp. The best growth and biochemical composition were obtained from the diatom *Chaetoceros* sp., while the highest amount of fucoxanthin was found in *Nitzschia* sp. In another study; the effect of temperature from 10 ^{0}C to 35 ^{0}C on the growth, total lipid content, and fatty acid composition of marine microalgae, *Isochrysis* sp., *Nitzschia closterium*, *Nitzschia paleacea* and the Tahitian *Isochrysis* sp. were investigated (Reneaud *et al.,* 1995). They reported that; species of *Nitzschia closterium, Isochrysis* sp. grew very slowly at 35 ^{0}C and *Nitzschia paleacea* was low-temperature tolerant, with cells growing slowly at 10 ^{0}C. *Nitzschia paleacea* determined that the highest percentage of lipids at 10 ^{0}C, while the other species observed that maximum amounts of lipid at 20 °C. In this study; both species (*Chaetoceros* sp. and *Nitzschia* sp.) were determined to growth and biochemical composition at 20 ° C (Table 1-2-3). According to the researches; the maximum growth rate determined that 20–25 ^{0}C in the case of *Phaeodactylum tricornutum* (Ak *et al.,* 2015; Uslu *et al.,* 2014; Kudo *et al.,* 2000; Sanchez *et al.,* 1995; Yongmanitchai and Ward 1991), however a wide range of temperatures at 20–35 ^{0}C for *Chaetoceros muelleri* (McGinnis *et al.,* 1997). In another study; De Castro and Garcia (2005), investigated that temperature (20, 25, and 30 ^{0}C), salinity (25 and 35 ppt) effects on growth and biochemical composition of *Chaetoceros cf. wighamii* under laboratory conditions. They determined that lipid and carbohydrate content were higher at lower temperatures (20 and 25 ^{0}C) while protein was unaffected. According to other research; for EPA production, optimum temperatures were 18.0 ^{0}C for *Nitzschia laevis* (Wen and Chen 2002). Blanchemain and Grizeau (1999) determined that the optimal growth temperature was at 20 ^{0}C for *Skeletonema costatum*, while the EPA content was higher at a

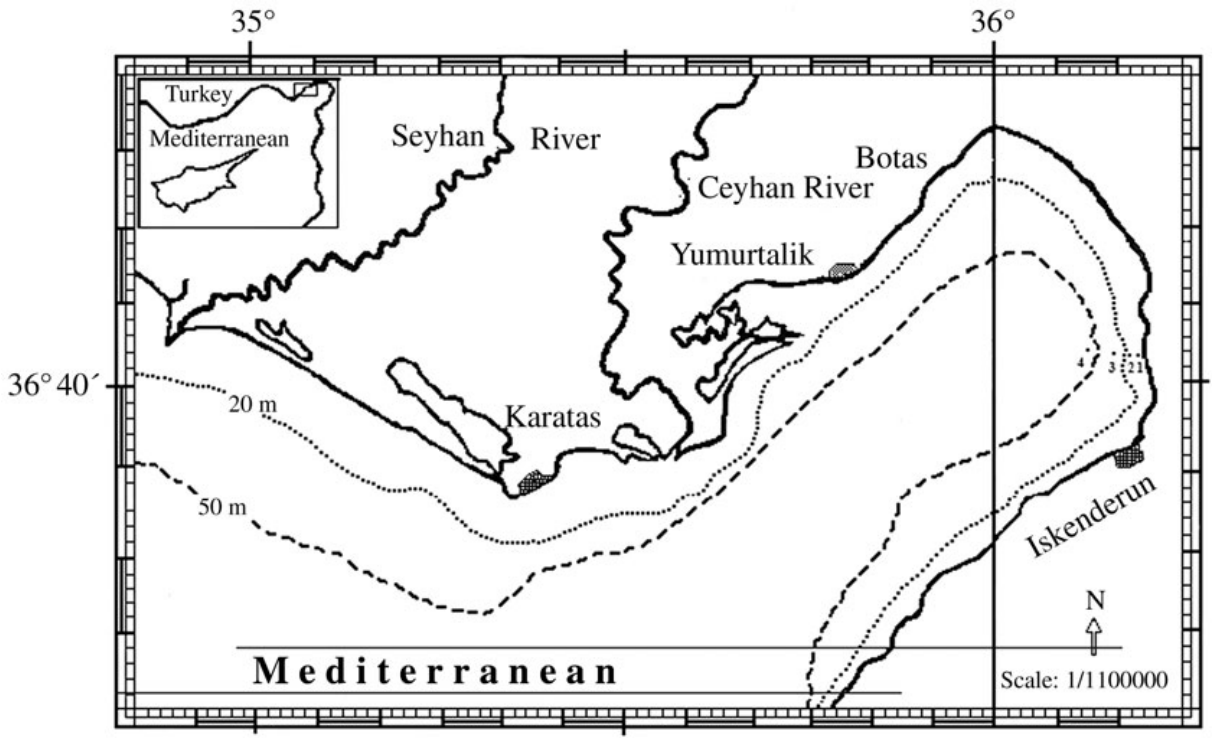

Figure 1. İskenderun Bay

Table1. Main parameters of growth rate and biomass and biomass productivity, cell density, chlorophyll *a*, total carotenoid, protein, carbohydrate, lipid contents of *Chaetoceros* sp. and *Nitzschia* sp

Parameters	*Chaetoceros* sp.	*Nitzschia* sp.
Biomass (mgL^{-1})	1.014 ± 0.001^{a}	0.970 ± 0.007^{b}
Growth rate (day^{-1})	0.765 ± 0.009^{a}	0.626 ± 0.004^{b}
Biomass productivity (mgL^{-1} day^{-1})	0.776 ± 0.007^{a}	0.607 ± 0.02^{b}
Chlorophyll *a* (μGl^{-1})	1.658 ± 0.025^{b}	1.780 ± 0.02^{a}
Total carotene(μgL^{-1})	2.181 ± 1^{b}	2.293 ± 2^{a}
Lipid (%)	19.74 ± 2^{a}	15.71 ± 3^{b}
Protein (%)	12.16 ± 0.001^{a}	11.65 ± 0.001^{b}
Carbohydrate (%)	37.48 ± 0.002^{a}	24.43 ± 0.001^{b}
Fucoxanthin (mg /L)	3.5 ± 0.3^{b}	4.8 ± 0.5^{a}

Means values, n=5; *Different letters between the columns indicate significant differences ($p<0.05$).

lower temperature 15 ^{0}C. In another study; they reported that lipid production by *Navicula saprophila* was optimum at 10 ^{0}C, while *Nitzschia* sp. EPA production was higher at around 20^{0}C (Kitano *et al*. 1997). Saravanan et. al. (2015), investigated that species of isolated *Amphora* sp. and *Gyrosigma* sp. were grown under different light intensities showed a significant growth effect on cell growth from Day 0 to Day 13. They results obtained that *Gyrosigma* sp. a high growth rate of 0.3067 d^{-1} (at 40 µmol $m^{-2}s^{-1}$), whereas *Amphora* sp. was growth slower than *Gyrosigma* sp. In this study; *Chaetoceros* sp. and *Nitzschia* sp. were cultured at a light intensity of 40 µmol photon $m^{-2}s^{-2}$ and growth were determined. According to the results; the best growth and biochemical composition were obtained from the *Chaetoceros* sp. Many previous studies reported that salinity stress cells have lower protein synthesis capacity, increasing lipid and carbohydrate metabolism for microalgae species (Fabregas *et al*., 1985; Richmond, 1986; Ben-Amotz *et al*., 1987). Other investigation showed that the visible increase in other components in *Chaetoceros* can be o related to cellular adjustments to osmotic stress due to the high salinity (Richmond, 1986). According to results in this study, a salinity of 30 ppt appears more adequate to the growth and biochemical composition for *Chaetoceros* sp. and *Nitzschia* sp.

Many studies have been investigated on pigment and lipid production from diatoms in different countries (Kim *et al*., 2012; Xia *et al*., 2013; D'Ippolito *et al*., 2015; Stonik and Stonik, 2015; Wang *et al*., 2011). Biodiesel production from microalgal lipid and especially diatoms is the field of research in many countries (Levitan, 2014; Hassan *et al.,* 2013; Hildebrand, 2012; Demirbas and Demirbas, 2011; Mata *et al.,* 2009; Griffiths *et al.,* 2009). The most important diatom species that are used for biofuels are *Chaetoceros calcitrans, Nitzschia closterium, Phaeodactylum tricornutum, Skeletonema costatum, Thalassiosira pseudonana* etc. In this study; the lipid and lipid productivity of *Chaetoceros* sp. was 19.74±2% and 15.32±1.35 $mgL^{-1}day^{-1}$ determined, respectively.

The lipid and lipid productivity of *Nitzschia* sp. was 15.71±3% and 9.54±1 $mgL^{-1}day^{-1}$ determined, respectively. Pigments are the most important metabolites in recent years (Wijffels 2007; Del Campo *et al*., 2007). The fucoxanthin producing diatoms were determined in the species such as *Cylindrotheca closterium (*5.23 mg/g^{-1}), *Chaetoceros gracilis (*2.24mg/g^{-1}), *Odentalla aurita* (21.67 mg/g^{-1}), *Phaeodactylum tricornutum(* 2.24 mg/g^{-1}) *and Nitzschia sp.* (4.92 mg/g^{-1}) etc (Pasquet *et al*., 2011; Kim *et al*., 2012; Xia *et al*., 2013).

Table 2. Fatty acid content in *Chaetoceros* sp. (%)

C14:0	16.45 ± 0.2
C15:0	1.02 ± 0.5
C16:0	29.15 ± 0.7
C16:1	37.21 ± 0.12
C16:2	1.83 ± 0.9
C16:3	1.02 ± 0.2
C18:0	2.71 ± 0.3
C18:1	3.20 ± 0.1
C18:2	1.22 ± 0.1
C18:3	0.99 ± 0.4
C20:4	2.16 ± 0.5
C20:5	3.04 ± 0.6
C22:6	n.d. ± 0.7
SFA	49.33 ± 0.9
UFA	50.67 ± 0.11
MUFA	40.41 ± 0.12
PUFA	10.26 ± 0.13

Table 3. Fatty acid content in *Nitzschia* sp (%)

C14:0	7.0 ± 0.2
C16:0	38.9 ± 1.2
C16:1	40.2 ± 0.2
C18:1	1.7 ± 0.2
C18:2	0.9±0.1
C18:3(n-3)	5.4 ± 0.2
C20:4(n-6)	1.3 ± 0.3
C20:5(n-3)	4.3 ± 0.3

In this study; fucoxanthin content of *Chaetoceros* sp. and *Nitzschia* sp. were 3.5±0.3 mg/L and 4.8±0.5 mg/L determined, respectively. Many studies have been showed that fatty acid composition is similar to that determined for other diatoms with the exception that the C16 fatty acids constitute approximately 70% of all fatty acids (Good *et al.,* 1988). The major fatty acids are C14:0, 16:1, 16:0, 18:0, and 20:5. Fatty acids that are available in minor amounts are iso-14:0, iso-15:0, 15:0, 17:0, 18:4, 18:2, 18:1, 19:0, 20:0, 22:0, and 23:0 (Ackman et.al., 1964; Chuecas and Riley, 1969; Kates and Volcani, 1966; Wen and Chen, 2002). In this study; the major fatty acids of the *Nitzschia* sp. was 14:0, 16:0, 16:1(n-7), 16:3(n-4) and 20:5(n-3) and the major fatty acids of *Chaetoceros* sp. was 14:0, 16:0, 16:1 (Table 2-3). In another study; they investigated that the lipid class and total fatty acid compositions of 14 species of diatom (Bacillariophyceae).

They reported that the major fatty acids in most species were 14:0, 16:0, 16:1 (n-7) and 20:5(n-3) and the polyunsaturated fatty acids 16:2(n-4), 16:3(n-4), 16:4(n-1), 18:4(n-3) and 22:6(n-3) (Dunstan *et al*., 1993). Opute (1974) studied that the lipids and fatty acids of two freshwater diatoms *Nitzschia palea, Navicula muralis* and one marine species, *Navicula incerta.* Researcher determined that the major lipid components in all species were triglycerides, monogalactosyl, digalactosyl and sulphoquinovosyl diglycerides, phosphatidyl glycerol, phosphatidyl choline (lecithin), and phosphatidyl ethanolamine; while palmitoleic, palmitic, eicosapentaenoic and eicosatetraenoic acids were the major fatty acid constituents. In conclusion; diatom species isolated from different stations at Yumurtalık Bay were determined in this study. In conjunction with the determination of biochemical contents of local diatom species in the region, industrial use possibilities of these species have be revealed. Thus, by utilizing the advantage of more efficient cultivation of local species in regional climatic conditions, new types of candidates for commercial use have been explored.

Moreover, the phylogenetic records of diatom species identified in the area have been done which will permit to collect data that have contribute to the diversity of the scientific literature. We continue our culture studies of diatoms isolated and, the amount of fucoxanthin and lipid of the local diatom species in the region will be determined and compared with the results of previously cultured commercial diatom species. The studies about lipid production from microalgae are mostly conducted in the controlled conditions in the laboratory environment whereas; the success is able to transfer of the results to the practice. It means the achievement is the high production volume economically. Finally, it is considered that the results obtained from this study showed that high lipid content of diatom contribute to biodiesel industry and the supply facilities. In addition to this expected to these types of biodiesel production in the Mediterranean Region with the aim of achieving a high level of lipid will supplemented for large commercial companies. In our country, also we have to study about isolation of the microalgae contained high lipid in our seas. Our country has planty of sunlight and subtropic climate, and the photosynthetic microalgae biomass can produce easily.

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