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Original Article

Caspian Sea's *Navicula salinicola* Hustedt 1939 and effect of the prolonged culture on its fatty acid profile

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Abstract: Diatoms are a potent source of polyunsaturated fatty acids. This study was conducted for screening a Naviculoid diatom strains from the southern Caspian Sea with analyzing its lipid production and accumulation potentials. The isolate was identified as *Navicula salinicola* strain IBRC-M 5083 based on micro-morphological characterization and analysis of 18S rRNA genomic region. *Navicula salinicola* were cultured in the f/2 medium under both normal and prolonged culture (21 days) conditions. Total lipid percentages of this strain were found to be 31.83% under normal condition and 43.72±1.4% in prolonged culture respectively on the basis of their dry cell weight (DCW). Also, the oil droplets were detected in 21 days' cells as shown by Sudan Black B staining experiments. Furthermore, the main fatty acids were found by Gas Chromatography analyses of this strain under prolonged condition to be Eicosapentaenoic acid (25.58%TFA). Such oil accumulation capabilities seem to be promising for performing further studies on this strain as a source of Omega-3 in aquafeed, pharmaceutical and biofuel industries.

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Introduction

Microalgae have many applications in different fields such as pharmaceutical, food industries, aquafeed, phytoremediation, sewage treatment and biofuel. There are several microalgae species which are able to accumulate lipids such as diatoms having a high potential of lipid production (Dunahay et al., 1998; Hildebrand et al., 2012). Therefore, they are potent and proper candidates for many biotechnological applications like biofuels (Merz and Main, 2014). Among them, the marine diatoms have been investigated more than others (Griffiths and Harrison, 2009) except the freshwater genus *Navicula* (Sheehan et al., 1998; Graham et al., 2012). Lipid in diatoms could be accumulated as intracellular droplets included TAGs (Triacylglycerol) (Yoneda et al., 2016; Yongmanitchai and Ward, 1991). The 16 or 18 carbon TAGs could be used as biodiesel sources because of its chemical similarity with aliphatic hydrocarbons in petrol (Yongmanitchai and Ward, 1991; Sivakumar et al., 2010). Furthermore, over 18 carbons such as C20:5ω3 (EPA) and C22:6ω3 (DHA) have also been found in diatoms which could be valuable sources for Omega3 (Brett and Muller-Navarra, 1997). Strong evidence of beneficial properties of Omega3 for human nutrition led to increase of interest in omega3 as nutritional supplements and aquafeed (Merz and Main, 2014).

Previous studies about *Navicula* sp. showed its proper level of C16:0, C16:1, C17:01, C18:0 (Matsumoto et al., 2010; Thajuddin et al., 2015), but many environmental variations such as nutrient

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deficiency can change its fatty acid composition (Lison, 1934; Mekhalfi et al., 2010). For instance, under nitrogen and silicon deficiencies in *N. salinicola*, the lipid content was induced up to 58% and 22-49%, respectively (Nagle and Lemke, 1990).

Nonetheless, prior to any lipid analysis, the target cells should be identified. Identification of diatoms is done using morphological characters such as the siliceous wall (frustules) ornamentations and protoplast features (Preisig and Andersen, 2005). For precise morphological identification of diatoms, it would be better to observe the frustules by Scanning Electron Microscope (SEM) (Crawford et al., 2001). Due to the presence of organic material on the frustule and cell contents, the characteristics of the valve are obscured thus it is necessary to clean diatom frustules from organic matter to provide a permanent slide and observe the frustule ornamentations under SEM. Recently molecular tools such as 18S rRNA gene sequence are used to confirm the morphological identifications (Dawson and Pace, 2002). The objective of this research was to identify the isolated cell and determine the effect of a prolonged culture period (21 days) on the fatty acid profile of *Navicula salinicola,* collected from the Caspian Sea, Iran.

Materials and Methods

Isolation and culture conditions: The samples were collected from the Gohar-Baran port, the southern Caspian Sea, Iran (36°49'51.1"N, 53°11'38.4"E). The samples were left for 24 hrs to settle the organisms in IBRC microalgae laboratory and then cultivated in filter sterilized seawater enriched with Guillard f/2 medium ($NaNO_3$ 8.82104 M; KH_2PO_4 3.62105 M; $FeCl_3.6H_2O$ 1.17105 M; $Na_2EDTA.2H_2O$ 1.17105 M; $CuSO_4.5H_2O$ 3.93108 M; $Na_2MoO_4.2H_2O$ 2.60108 M; $ZnSO_4.7H_2O$ 7.65108 M; $CoCl_2.6H_2O$ 4.20108 M; $MnCl_2.4H_2O$ 9.10 107 M; thiamine HCl 2.96107 M; biotin 2.05 109 M; cyanocobalamin 3.691010 M, 100mg.L^{-1} Imipenem - all chemicals purchased from Sigma Aldrich Inc., St Louis, MO, USA) (Guillard, 1975; Presieg and Andersen, 2005). The pH of the medium was set at 7 and the sample was grown at 18±1°C under a light/dark cycle (12h: 12h) in f/2 medium that prepared from seawater under a low intensity of 50 μmol m^{-2} s^{-1} (Preisig and Andersen, 2005). First, the axenic colonies of diatom on the plate were transferred to the triple 150 ml flask after 7 days, subsequently they were inoculated with 10% (v/v) into triple 500 ml fresh f/2 medium over another 7 days and kept to grow for 7 days at the same condition (prolonged culture). Then, one of the triple sample of normal and prolonged culture were randomly chosen and centrifuged at a speed of 3,000 rpm for 30 min, then the supernatant were removed. The pellets were washed twice with Distilled Water and dried at 60°C to obtain their constant weight. Then, they were weighted and stored at 4ºC for lyophilizing step. Finally, before starting a lipid extraction process, the pellets were lyophilized and weighted.

Morphological identification: Morphological identification was carried out through micromorphology under a light microscope (LM microscope, Olympus, CX31) and SEM (VEGA 3, TESCAN). For precise morphological identification, the permanent slide was used with H_2O_2 treatment. Following Cox and Mann's method (Cox, 1990), the permanent slide was made with Naphrax and pictures were captured with a DIC and phase contrast microscope (Nikon eclipse 80i).

Molecular identification: Total genomic DNA from the cultured sample was isolated based on Liu method (Liu et al., 2000) and stored at -20°C for further analysis. The primers SSU1 5'-AACCTGGTTGATC CTGCCAGT-3' (Kociolek, 2013) and ITS1DR 5'-CCTTGTTACGACTTCACCTTCC-3' (Kociolek, 2013; Edgar and Theriot, 2004) and amplification of the target gene (18S rRNA) were done according to Kociolek protocol (Kociolek, 2013) by PCR (PCR Bio-Rad My Cycler Personal Thermal Cycler, USA). The genomic DNA and PCR product were separated by agarose gel electrophoresis (Bio-Rad Power Pac Universal power supply Electrophoresis, USA). PCR product was sequenced based on the Sanger method and edited by ChromasPro and compared with registered sequences at NCBI by using Basic Local Alignment Search Tool (BLAST).

Lipid analysis: Lipid of the normal and prolonged sample was qualitatively analyzed with modified

Table 1. The Fatty acid profile of *Navicula salinicola* extracts under prolonged culture (21 d) by GC

Strain	Fatty acid composition (% of total fatty acids)					
	C16:0	C16:1	C18:1	C18:2	C18:3 (n-6)	C20:5
Navicula salinicola	4.79	9.28	2.25	0.48	0.07	25.58

Sudan Black B staining method (Jape, 2014; Thakur et al., 1989). The procedure initiated by fixing the air dried slides and flooding the slide in Sudan Black B stain for 20 min at room temperature (prepared about 0.3% Sudan Black B in 70% ethanol). Finally, the slide was stained by Safranin (0.5% aqueous solution) for 5 to 8 seconds (Thakur et al., 1989). The lipid of the lyophilized samples (normal and prolonged) which were in late stationary growth phase were extracted via $CHCI_3$: MeOH (2:1 v/v) solvent mix method (Folch et al., 1957; Axelsson and Gentili, 2014). The samples were homogenized and the residues were removed by filtration after homogenization. 0.73% NaCl solution was added which led to the production of final solvent (2:1:0.8 chloroform: methanol: water (v/v/v)). Solvents were removed by evaporation and the dry extracts were weighted for determination of total lipid percentages in normal and prolonged condition.

As a final process, the dry extract of prolonged condition sample dissolved in dichloromethane for further quantitative analysis by Gas Chromatography (GC). In the following, the extracted oil was trans-esterified by two-step procedure (2-TE) (Cavonius, 2014) and the fatty acid profile was determined by GC- young lin 6000 with an oven temperature 145°C isothermal, the injector temperature 280°C, detector temperature 300°C, capillary column-length: 50 m, internal diameter: 0.25 mm, Varian-CP-Sil 88, 60 m. The GC analysis was performed at the Department of Food Science, Iranian National Standards Organization (INSO) Karaj, Iran, based on the national standard 4090 and 4091.

Results

Morphological and molecular analysis: Identification of the isolated *N. salinicola* from the Gohar-Baran port in the Caspian Sea were confirmed according to the morphological and molecular characters explained in Stoermer et al. (1999) and Kociolek (2005). In *N. salinicola*, the valves were linear-lanceolate, rounded ends with the length-width 7-20, 2-4.5 μm, respectively. Filiform raphe and striae features were slightly paralleled to radiate, convergent at the ends (Fig. 1B, D, E, F). The amplified sequence of small subunit 18S rDNA which BLASTed in NCBI revealed 99% similarity to *N. salinicola* (FR865499) identified from, San Francisco, California, USA. As a result, this strain was identified as *N. salinicola* strain IBRC-M 5083 in IBRC (Iranian Biological Research Centre) microalgae collection.

Lipid analysis: Determination of total lipids percentages of this strain were obtained 31.83% (DCW) in normal condition and 43.72±1.4% under prolonged culture. In addition, primary screening of lipid vacuoles of *N. salinicola* under prolonged condition by staining with Sudan Black B indicated blue–black color droplets (Fig. 1C). Finally, the fatty acid composition of prolonged cultivation of *N. salinicola* was demonstrated by GC analysis (Table 1). The result revealed the high level production of EPA (25.58% TFA) under prolonged culture in this strain.

Discussion

The diatoms could be an affordable and appropriate source for biotechnological applications, however, studies on diatoms are rare in Iran and need more attention. For this purpose, the isolated sample should be identified in the first step. Taxonomic of diatoms are based upon morphology characteristics such as frustules structure (Round et al., 1990) as well as ribosomal DNA sequencing (Kociolek et al., 2013). It needs to clarify that the ornamentations of frustule have enough information for diatom identification, however, the gene sequences of diatoms in databases

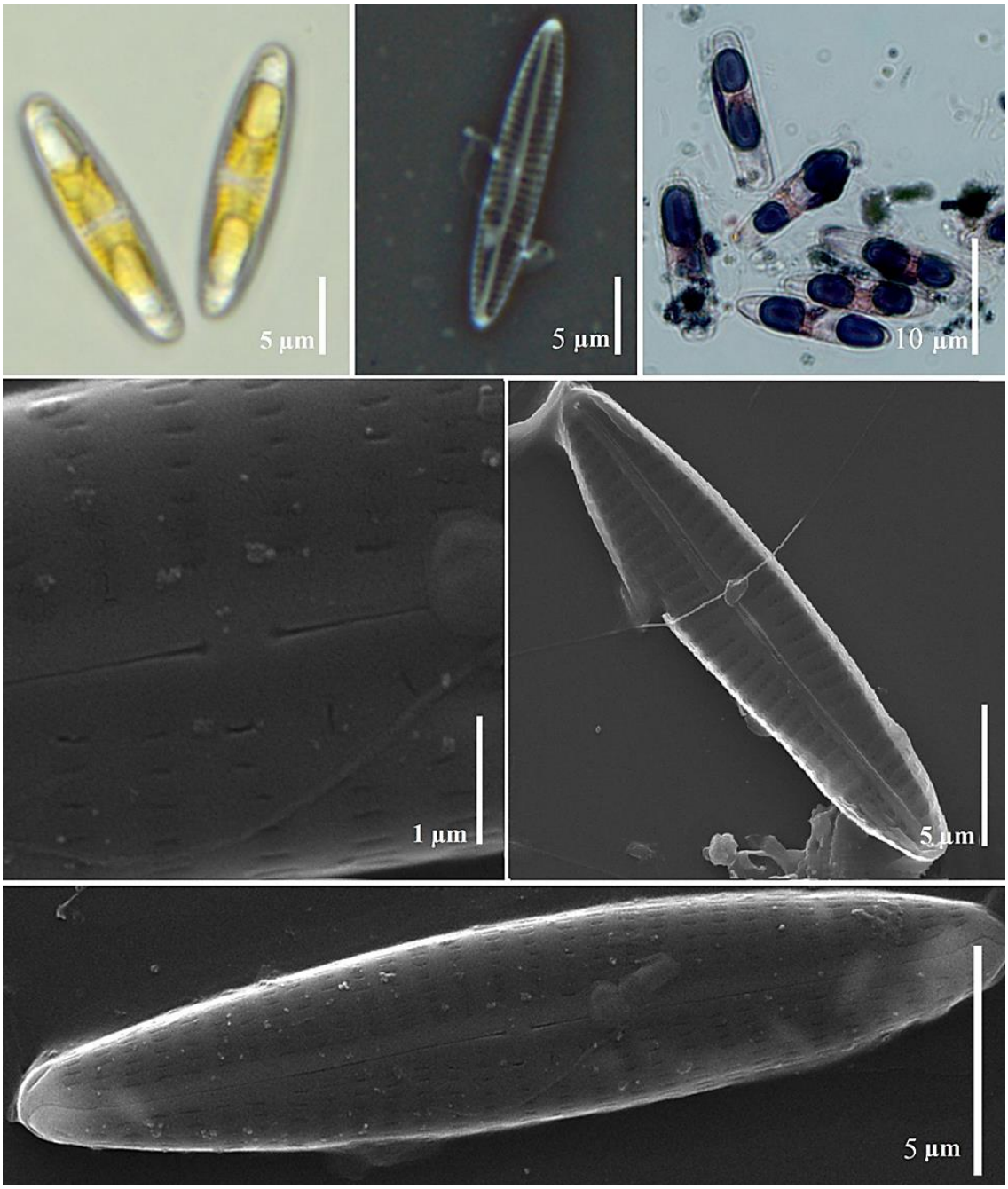

Figure 1. (a) *Navicula salinicola* under LM, (B) frustule features in P ermanent slide, (C) appearance of lipid droplets via Sudan Black B staining, (D) striae slightly parallel, (E) a filiform raphe in *N. Salinicola*., and (F) linear-lanceolate and rounded ends valves.

are not complete yet and in many cases, it is not feasible to compare the sequence of an isolate with the sequence information in NCBI. The results of this study revealed that the isolate algae is *N. salinicola* based on morphological and DNA data.

Algae produce carbohydrates, lipids and protein in photosynthesis process that depend on environmental factors (Juneja et al., 2013). Environmental conditions like nutrient availability are closely effective on quantity and content of carbon fixation and they lead to change of fatty acids composition as well (Juneja et al., 2013; Sharma et al., 2012). A study about evaluation of the nutrients in culture medium on lipid metabolism has shown that limitation of macronutrients such as nitrogen, silicon, phosphate can effect on lipid metabolism and contents of neutral lipids (TAGs) (Hu et al., 2008). It should be noted that determining the algae products under variable

environmental factors could be important for understanding how influence these factors on algae. Under prolonged incubation time of microalgae like diatoms, environmental factors could be altered and these changes effect on fatty acid composition and enhance TAGs during stationary phase in many algal species (Hu et al., 2008); for instance, the total lipid content of *Nacicula* sp. under normal condition was determined as 41.10 (% DCW) which could be improved to 60.28±4.92 (% DCW) under nitrogen starvation (Thajuddin et al., 2015). According to previous study, lipid content of *N. salinicola* was commonly induced up to 58% under nitrogen deficiency and to 22-49% under silicon deficiency (Nagle and Lemke, 1990). Based on the results, the total lipid content of isolated *N. salinicola* (IBRC-M 5083) was 43.72±1.4 (% DCW) which was higher than the total lipid content of *Nacicula* sp. under normal condition but it was lower than under nitrogen limitation conditions.

The content of lipids e.g. neutral lipids in the biomass like TAGs are important for biotechnological applications (d'Ippolito et al., 2015) such as aquafeed, Omega3 production and biofuel sources. For example in biofuel sources, linolenic acid and four double bonds in fatty acid methyl esters should not increase to 12% and 1%, respectively according to biodiesel standard EN14214 methods (Juneja et al., 2013). The GC analysis data in our work showed that the amount of linolenic acid in *N. salinicola* (IBRC-M 5083) is lower than 12%, which encounters the requirements of the European Standard EN 14214 for biodiesel. The above reports and absence of C18:3 demonstrated that *N. salinicola* (IBRC-M 5083) could be an appropriate source of biodiesel (Matsumoto et al., 2010; Thajuddin et al., 2015).

In addition, *Nacicula* sp. has suitable level of the other fatty acids such as C16:0, C16:1, C17:01 and C18:0 (Matsumoto et al., 2010; Thajuddin et al., 2015). The pervious works showed C16:0 (21.4% TFA) and C16:1 (25.1% TFA) under 200 hrs incubation in *Nacicula* sp. (Matsumoto et al., 2010) and C17:01 (29.36% TFA) and C18:0 (26.48% TFA) in *Naviculoid* for 336 hrs (14 days) incubation period under normal condition (Thajuddin et al., 2015).

The valuable polyunsaturated fatty acids, especially Omega 3 is one of the valuable products of microalgae. Studies revealed that Omega3 such as Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA) have proper effect on human health (Simopoulos, 1991). The results of this research demonstrated that the prolonged culture of *N. salinicola* produces a high level of EPA (25.58% TFA) after 21 days which agrees with the previous studies (Kitano et al., 1997; Khatoon, 2006; Zheng et al., 2007). However, the level of Omega3 were lower than the results of this study (Kitano et al., 2007; Khatoon, 2006; Zheng et al., 2007). It was reported C20:5 (8% TFA), C22:6 (2% TFA) (Khatoon, 2006), C20:5 (21.52% TFA) and C22:6 (3.64% TFA) in *Navicula* sp. (Zheng et al., 2007).

Our findings indicated that *N. salinicola* contains considerable lipid vacuole and it seems not only suitable for biofuel but also it is an appropriate source of Omega3 in prolonged culture conditions. By further study such as inducing genetic and physiologic variations on this strain for improving the efficiency, it is hoped to accelerate commercialization of such investigations.

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چکیده فارسی

جداسازی *Navicula salinicola* (Hustedt 1939) از دریای خزر و بررسی تاثیر کشت طولانی مدت بر محتوای اسید چرب آن

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چکیده:
دیاتوم‌ها یکی از منابع دارای پتانسیل تولید اسیدهای چرب غیر اشباع طبیعی محسوب می‌شوند. در این تحقیق دیاتوم ناویکلوئید از بخش جنوبی دریای خزر جداسازی و توانایی تولید و تجمع لیپید در آن مورد ارزیابی قرار گرفت. آرایه جداسازی شده به کمک مشاهده صفات ریخت‌شناسی و آنالیز مولکولی منطقه ژنومی 18S rRNA به‌عنوان *Navicula salinicola* مورد شناسایی قرار گرفت و با شماره دسترسی IBRC-M 5083 در مرکز ملی ذخایر ژنتیکی و زیستی ایران نگهداری شد. پس از کشت و اعمال تیمارهای کشت نرمال و کشت طولانی مدت (۲۱ روز) این سویه در محیط کشت f/2، میزان تولید چربی کل به ترتیب ۳۱،۸۳ و ۴۳،۷۲ درصد وزن خشک سلول بدست آمد. همچنین تولید ذرات لیپیدی توسط رنگ آمیزی سودان بلکB در این سویه مشخص شد. سنجش محتوای اسید چرب کشت طولانی مدت این سویه با استفاده از روش کروماتوگرافی گازی، میزان ۲۵/۵۸ (درصد کل اسید های چرب) ایکوزاپنتانوئیک اسید را نشان داد. با توجه به توانایی تجمع لیپید و محتوای مناسب امگا ۳ در *N. salinicola*، می‌توان با تحقیقات بیشتر بستر مناسبی جهت استفاده از این سویه در صنایع خوراک آبزیان، دارویی و همچنین سوخت زیستی فراهم نمود.

کلمات کلیدی: جلبک، امگا۳، ایکوزاپنتانوئیک اسید، دوکوزاهگزانوئیک اسید.