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Research Article

Improvement of *Thalassiosira weissflogii* as a high valuable nutritional feed

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Abstract

Recently, the rising demand for high-quality seafood has created a fresh look at the sustained and adequate aquafeed as a necessity. Considering the potential of microalgae cells, many companies are looking for practical methods to enhance the nutritional content of these microorganisms as valuable aquafeed. In this attempt, *Thalassiosira weissflogii* isolated from the Caspian Sea and identified with morphology and molecular characteristics. In order to improve lipid content, this strain was cultivated in normal and nitrogen deficiency F/2 medium for 18 and 30 days. The growth indices, total lipid, fatty acids profiles were measured in both cases.

Growth of *T. weissflogii* during nitrogen deficiency conditions was associated with a sharp decline in cell growth and significant rise in lipid production such as polyunsaturated fatty acids (PUFAs). Although the eicosapentaenoic acid (EPA) level was reduced by half under nitrogen deficiency condition (8.8 to 3.23 % TFA), the amount of docosahexaenoic acid (DHA) escalated during this situation (3.5 to 12.63 % TFA). Results showed that the concept of N-deficiency conditions along with prolonged culturing could improve PUFA n-3 content to provide highly valuable feed for shellfish and shrimp industries.

Keywords: Aquafeed, *Thalassiosira weissflogii*, nitrogen deficiency, EPA, DHA

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Introduction

Desirable aquaculture is dependent on various aspects, such as providing high-quality feed with the lowest price. Therefore, microalgae as the member of the first loop in the food chain can play a significant role in the hatchery systems. The valuable protein and lipid content found in microalgae make them an accessible source of aquafeed for aquatic animals. Proper microalgae strains can be used directly as live feeds in all growth stages of bivalve mollusks and some fish species or indirectly utilized to feed the zooplankton used in the aquaculture food chain. However, these microalgae should have different characteristics like proper nutrient composition, rapid growth rates, appropriate size for ingestion, and stability in hatchery systems and the absence of toxins that might enter to the food chain.

In order to meet the complex nutritional requirements of aquatic animals, many companies are seeking high nutritious microalgae strains and finding different ways to enrich their nutritional value. Providing feeds with good nutritional properties like unsaturated fatty acids can make healthier seafood and help reduce the consumption of wild omega-3 sources (fish liver oil) (Tredici *et al*., 2009; Tibbetts, 2018).

Today, several microalgae strains have been successfully used for feeding the larval, juvenile and bivalve mollusks, such as *Isochrysis sp.* (T.ISO), *Pavlova lutheri,* and *Chaetoceros calcitrans*. Previous studies indicated the importance of aquatic nutrition by microalgae strains that have a good levels of polyunsaturated fatty acids (PUFAs) like eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), arachidonic acid (AA) and their impact on the seafood's growth and content (Chauton *et al*., 2015; Norambuena *et al*., 2015). As a result, methods such as metabolic engineering have attracted a lot of interest to boost the amount of PUFA n-3 content in microalgae cells (Müller *et al*., 2013). These studies have been proved that nutrient limitation conditions like nitrogen stress makes restrictions in growth and enhancement in lipid productivity (Guschina *et al*., 2006; Chisti *et al*., 2007; Breuer *et al*., 2012; Draaisma *et al*., 2012; Boelen *et al*., 2013; Breuer *et al*., 2013). Cellular studies on microalgae cells under $NO3^{-1}$ depletion conditions illustrated a change in the direction of carbon flow from protein synthesis to lipid synthesis; however, many of the nutritional starvation effects on the biochemical composition are still obscure (Uauy *et al*., 2000; Milledge, 2011). Despite many unknown issues in nutrient restriction conditions, the aquaculture industry has still focused on microalgae strains that can produce high levels of PUFA n-3 (Breuer *et al*., 2012; Breuer *et al*., 2013).

One of those microalgae strains that are highly capable in PUFAs n-3 production (EPA and DHA) is *Thalassiosira weissflogii*, which is widely used in shrimp and shellfish larviculture industry. *Thalassiosira weissflogii*, a large centric diatom (4–32

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µm in diameter), is generally found in coastal waters and some inland rivers (Lee, 2012). This cylindrical diatom is considered as the single best microalgae for larval shrimp, feeding copepods and brine shrimp, and even be excellent feed source for the post-larval stage (200 microns and larger) of clams, mussels, and scallops (Kiatmetha *et al*., 2011; Guedes and Malcata, 2012). As noted above, various nutritional limitations such as nitrogen, nickel, and silica may cause limited growth and lipid production. According to prior investigations carried out on this diatom under various environmental stresses, it was found that this strain can accumulate a different levels of EPA (10.6 to 14.6 (%TFA)) and DHA (2.3 to 4.6 (%TFA)) (Ishida, 2000; Kiatmetha *et al*., 2011; Laws *et al*., 2013; Mocquet *et al*., 2013; Clark *et al*., 2014). These studies also reported that using microalgae like *T. weissflogii* as a live feed leads to development in egg production (10×higher) of copepod *Acartia tonsa* (Camus and McKinnon, 2009; Teixeira *et al*., 2010).

This study attempted to provide higher nutritional value aquafeed from *T. weissflogii* by improving its fatty acids content through nitrogen deficiency conditions with long-term cultivation.

Materials and method

Isolation and experimental culture set-up

Thalassiosira weissflogii was isolated from a water sample of the Galugah county, Mazandaran province, Iran (N: 36-47-25, E: 53-47-52.3). Isolation from water sample was undertaken by serial dilutions and culturing in f/2-enriched seawater agar plating **(**Lebeau and Robert, 2003**):** NaNO3 8.82104 M; KH2PO4 3.62105 M; FeCl3.6H2O 1.17105 M; Na2EDTA.2H2O 1.17105 M; CuSO4.5H2O 3.93108 M; Na2MoO4.2H2O 2.60108 M; ZnSO4.7H2O 7.65108 M; CoCl2.6H2O 4.20108 M; MnCl2.4H2O 9.10 107 M; thiamine HCl 2.96 107 M; biotin 2.05 109 M; cyanocobalamin 3.691010 M, 100 mg L^{-1} Imipenem (All chemicals were purchased from Sigma Aldrich Inc., St Louis, MO, USA**)**. When single colonies were formed, it was cultivated at pH 8 and 18 ±1˚C in batch cultures, under a light intensity of 70 µmol m^{-2} s^{-1} by cool white fluorescent lights. Growth experiments were conducted by 10% inoculum under two nitrogen regimes in F/2 medium (70 and 25 µMol NaNO3) for 18 and 30 days of cultivation. The inoculation was performed triplicate under normal nitrogen (NN-70 µMol NaNO3) and nitrogen deficiency (ND- 25 µMol NaNO3) in 500 ml flasks for 18 and 30 days in chamber room with above mentioned condition.

Morphological and molecular identification

The sample was recognized based on polyphasic identification with microscopic and molecular techniques. For an extensive study on frustules, the sample was observed by light microscope (Nikon Eclipse 80i, Japan) and SEM (VEGA 3, TESCAN, and Czech Republic). Generally, this

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cylinder shape cell has rounded flat valves and variation in cell size (4 to 32 µm) in winter and summer (Armbrust and Chisholm, 1990; Wehr *et al*., 2015). In addition, there are usually three or six fultoportulae with cellular arrangement as a marginal ring in the center of the valve plate. There is also a prominent rimoportula on the margin of the valve (Hasle *et al*., 1996; Wehr *et al*., 2015).

In continue, the DNA was extracted for Polymerase Chain Reaction (PCR) step (Liu *et al*., 2000; Stancheva *et al*., 2013). Finally, the amplified sequence of the sample was edited by ChromasPro, and the Blast result was compared for similarity of sequences in NCBI database. Phylogenetic relationships among *Thalassiosira weissflogii* specimens were analyzed based on SSU sequence data using the unweighted Pair Group Method with Arithmetic Mean (UPGMA) in the MEGA program (version 5.0).

Biomass measurement

Triplicate concentrations of the diatom cells in both nitrogen regimes were obtained by OD measurements in 1 cm quartz cuvettes at 680 nm by a UV–spectrophotometer; model UV-1800 (Shimadzu, Japan). Moreover, the cell number was counted via haemocytometer slide. The samples were centrifuged at 5000 rpm for 15 min and washed twice with 0.5 M ammonium bicarbonate solution and the pellet was dried at 105°C for 48 h to measure dry weight (Zhu and Lee, 1997).

The specific growth rate (µ/day) was calculated by the equation as follows (Jena *et al*., 2012):

$\mu=\ln (W_2/W_1)/\Delta t$

Where W_2 and W_1 were the biomass concentrations (CDW) at the end and the beginning of batch culture, respectively. Δt was the cultivation time in days.

The Biomass Productivity (gL^{-1} day^{-1}) was calculated by the equation:

$P_{Biomass}$ ($gL^{-1}day^{-1}$) = $(X_2-X_1).\Delta t$

Where X_1 and X_2 were the biomass CDW concentrations ($g\ L^{-1}$) on days t_1 (start of cultivation) and t_2 (end of cultivation), respectively (Hempel *et al*., 2012).

The Generation Time (Tg) was calculated by the following equation (Rashid *et al*., 2015):

Tg = 0.6931 /µ .24 (hrs)

Lipid accumulation and extraction

The sample was stained through the Sudan black B method (Thakur *et al*., 1989) as lipid bodies screening and the cells were observed under phase contrast microscope (Nikon eclipse 80i, JAPAN) subsequently. In continue the biomass of NN and ND samples for 18 and 30 days' cultivation were collected via 5 min centrifugation (5000rpm). After twice washing with deionized water, the pellets were stored at −20°C overnight for the lyophilizing process. Lipid extraction was carried out by Bligh and Dyer method (Mubarak *et al*., 2015) from 0.5 g lyophilized samples (NN-ND). Based on the process, 0.5 g of each lyophilized samples were added to solvent with deionized water to attain 1:2:0.8 ratio (chloroform: methanol:

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water) and the mixture homogenized. After adding another part of chloroform and deionized water, the mixture homogenized again and the final ratio reached to chloroform: methanol solution (2:1, v/v) and 0.01% butylated hydroxytoluene (BHT) solution (Cavonius *et al*., 2014). The supernatants were washed with KCL: distilled water (1:1, v/v) after three repetitions of the extraction process. Finally, the cell debris was removed by filtration and the solvents were evaporated by rotary evaporator machine. The dried extracts were weighed and dissolved in dichloromethane for the next step. Determination of fatty acids profile of samples were analyzed by Gas Chromatography (GC) (Younglin 6000, South Korea) equipped with flame ionization detector (GC-FID) fused by silica capillary column (60m x 0.25 µm x 0.25µm). The total lipid (Dry weight %) content was determined gravimetrically and the specific yield of EPA/DHA (gL^{-1}) was calculated by the amount of EPA/DHA per gram of biomass which was obtained in GC analysis via the equation below (Cavonius *et al*., 2014):

$Y_{p/x} = dp/dx$ (27)

Where *P* stands for product mass (g) and *X* for biomass (g).

Statistical analysis

All the experiments were taken in 3 replications, and results were represented along with standard deviation. The data in the graphs have been represented along with standard errors.

Results

Identification of strain

Morphological and molecular characteristics were used as polyphasic identification. *Thalassiosira* is a centric diatom with fultoportulae (central tubular process surrounded by two or more satellite pores) and rimoportula (a tubular process through the valve of *Thalassiosira*) ornamentation, which plays a pivotal role in identifying species. A projecting rimoportula was observed on the valve, close to the edge of the cells. The general morphology of the centric diatom was determined by images obtained by light and SEM microscope (Fig.1).

The 18S rDNA sequence confirmed the morphological identification with 99% similarity to a sequence via GQ281043 accession number as *T. weissflogii* in NCBI. Furthermore, Phylogenetic trees for the SSU sequences are represented in Table 1.

The *Thalassiosira* genus (Thalassiosiraceae) has made two adjacent clades which include most of the species. All of the species except *cyclotella* (from Stephanodiscaceae family) are from the same family. The situation of *cyclotella* is not proper for the phylogram, which requires another extensive investigation. Our isolate (KhW9) is situated in *T. weissflogii* clade with high statistic support (100 bootstrap), which confirms strain identification. The *Lauderia* and

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Porosira genera are completely separated in a distinct clade. The *Melosira* from the melosiraceae family is chosen as an out-group (Fig. 2).

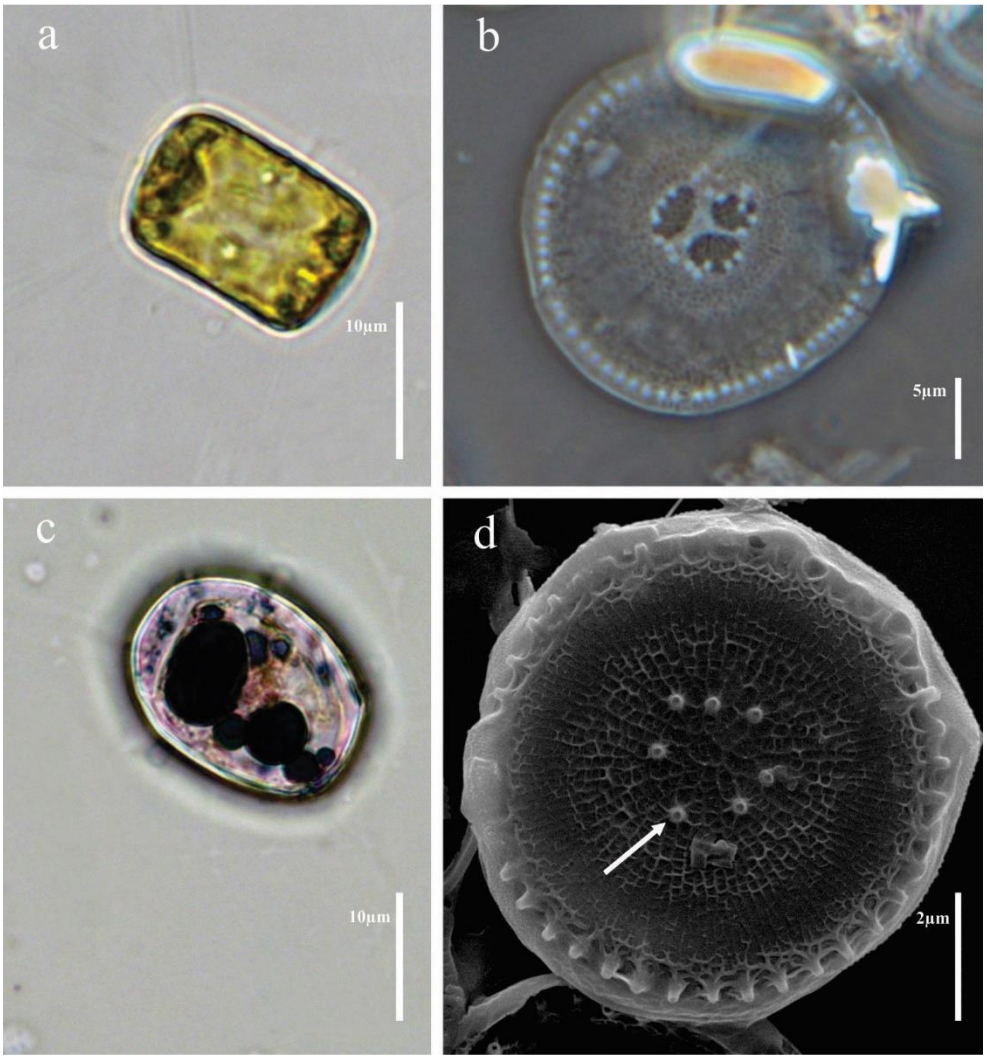

Figure 1: a *T.weissflogii* under light microscopy, b view of frustule and fultoportulae in permanent slide, c lipid drops staining via Sudan black B, d view of the fultoportulae under SEM microscopy.

Growth rate and biomass production

The maximum cell density of *T. weissflogii* in NN and ND were detected as 440×10^3 and 50×10^3 cells mL^{-1}, respectively. The maximum growth rate in NN was reached on the 12^{th} day, but in the nitrogen starvation condition, the maximum growth rate was on the fifth day. Regarding the present study results, the lag phase duration of this strain in NN and ND conditions lasted for 2 and 3-4 days, respectively. The exponential phase in NN and ND condition on the 8^{th} -12^{th} and 5^{th} day respectively and the stationary phase were on the 13^{th} -18^{th} and 8^{th} -12^{th} for NN and ND condition. Triplicate absorption of NN and ND showed equitation Y=0.02239*X+0.03770 and Y=0.007856*X+0.007196 respectively through using spectrophotometer method (Fig. 3). The statistical analysis

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of these results indicated that the *p*-alue of both nitrogen conditions was significant ($p<0.05$).

Table 1: List of the *Thalassiosira* strains investigated from NCBI.

***Thalassiosira* strains**	**Culture Collection**	**GenBank Accessions number**	**Locality**
Thalassiosira weissflogii	CCAP	GQ281043	Atlantic and Pacific Oceans
Thalassiosira oceanica	CCMP1005	AF374479	The Sargasso Sea is a region of the North Atlantic Ocean
Thalassiosira weissflogii	Unpublished	HM991702	Unpublished
Thalassiosira guillardii	CC03-04	DQ514869	Clam Creek, GA, USA
Thalassiosira gessneri	AN02-08	DQ514864	San Joaquin River, Antioch, CA, USA
Thalassiosira fluviatilis	Unpublished	AJ535170	Unpublished
Thalassiosira pseudonana	CCAP 1085/12	KU900218	Moriches Bay, Forge River, Long Island, New York, USA
Thalassiosira pseudonana	Unpublished	HF565135	Unpublished
Cyclotella cryptica	Unpublished	FR865514	Unpublished
Cyclotella meneghiniana	Unpublished	AB430591	Unpublished
Lauderia annulata	CS30	DQ514849	Pacific Ocean, La Jolla, CA, USA
Porosira pseudodenticulata	Unpublished	X85398	Unpublished
Porosira glacialis	CCMP1099	DQ514847	Southern Ocean, Antarctica
Melosira varians	Unpublished	KT072969	Unpublished

Thalassiosira weissflogii samples produced ~400 mg L^{-1} (DW) biomass in normal conditions and ~300 mg L^{-1} (DW) under nitrogen starvation. The maximum dry weight biomass for both conditions was displayed on day 18th (Table 2).

Fatty acids analysis and lipid production

The primary screening of lipid bodies (LBs) in *T. weissflogii* was shown by Sudan Black B staining. In this method, the oil drops of microalgae cells appeared in blue-dark color (Fig. 1- B, C Oil drops of *T. weissflogii*). The fatty acid profiles under different nitrogen regimes and culturing periods displayed a high level of saturated fatty acid in NN (30 d), and mono-saturated fatty acid and polyunsaturated fatty acid in ND condition during 18 and 30 days cultivation (Fig. 4).

A significant amount of EPA was observed in NN condition (8.8 (TFA %)), while DHA was increased sharply after 30 days' cultivation under ND condition (12.63 (TFA %)) (Table 3).

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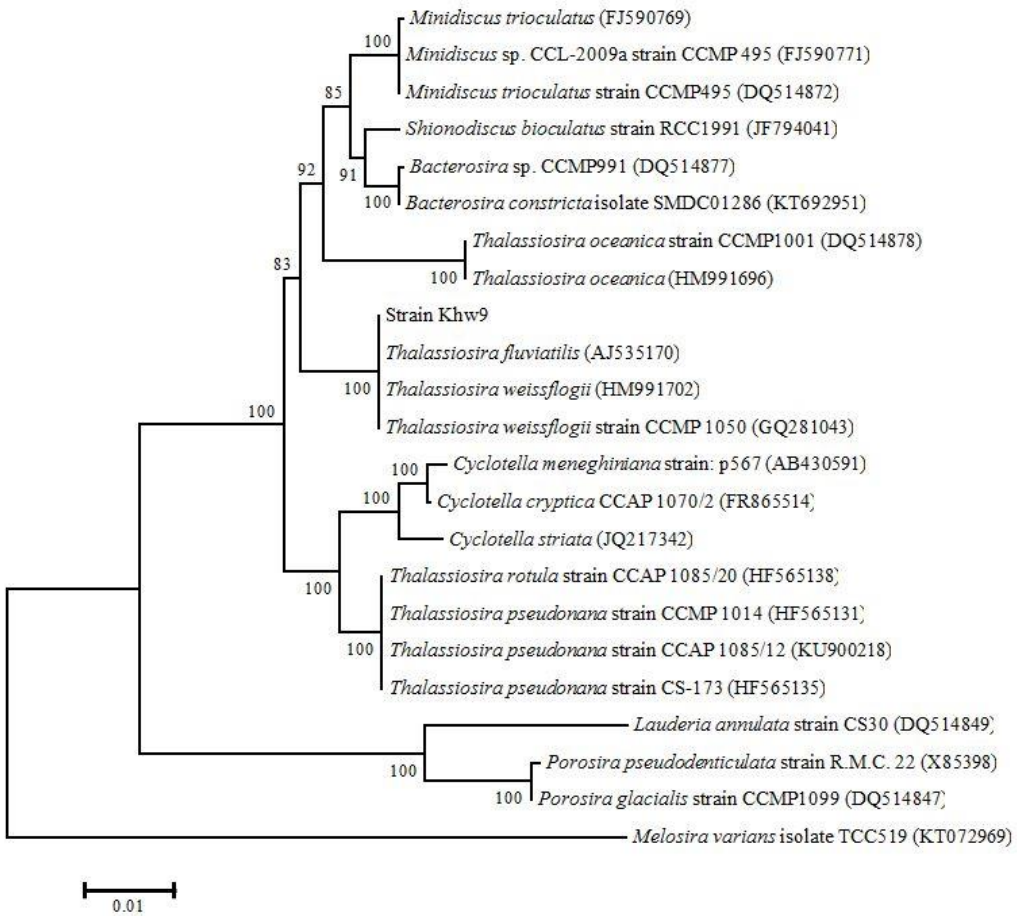

Figure 2: Evolutionary relationships of taxa.

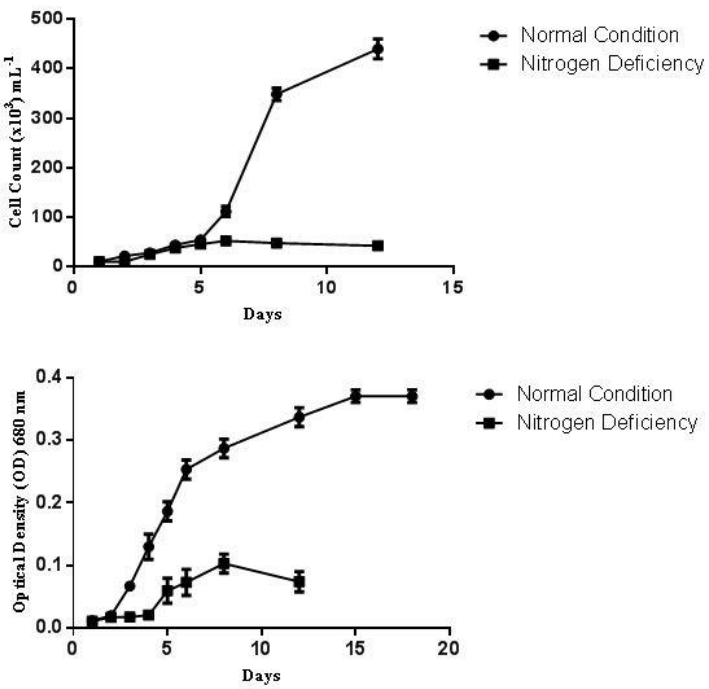

Figure 3: The cell count and optical density of *T.weissflogii* under NN and ND condition.

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Table 2: The exponential phase, generation time, biomass productivity, dry weight and the growth rate of *T. weissflogii* under two nitrogen regimes

Nitrogen Condition	The Exponential phase (Days)	The Generation Time (T_g) hrs	The Biomass Productivity (gL^{-1} day^{-1})	Dry Weight Biomass (g/L^{-1})	The growth rate (μ)
NN (18 days)	8-12th	32.61	0.022	0.41	0.51±0.1
ND (18 days)	5th	38.50	0.018	0.33	0.018±0.03

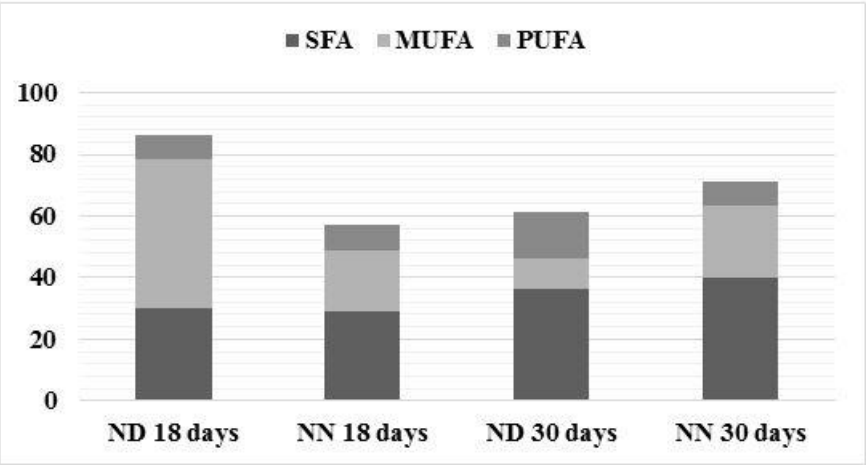

Figure 4:The level of saturated, mono-unsaturated and poly-unsaturated fatty acid of *T. weissflogii* under NN and ND condition for 18 and 30 days cultivation.

Table 3: The total lipid, amount of EPA-DHA, specific EPA-DHA yield of *Thalassiosira weissflogii* under 18 and 30 days cultivation.

Nitrogen Condition	Total lipid (Dry weight %)	EPA (TFA %)	DHA (TFA %)	The specific EPA yield (g/L^{-1})	The specific DHA yield (g/L^{-1})
NN 18 days	37.17	0.68	7.26	0.001	0.010
ND 18 days	37.84	0.42	7.6	0.0005	0.009
NN 30 days	39.46	8.8	3.5	0.018	0.007
ND 30 days	42.11	3.23	12.63	0.005	0.020

Discussion

The present work identified the isolated sample based on the morphology and molecular characteristics which were shown with titled Khw9 in phylogram of the *Thalassiosira* clades (Fig. 1). According to the literature, environmental stress, such as nutrient limitation, plays a key role in promoting microalgae to generate lipid (Benavente-Valdés *et al*., 2016). These limitations, such as nitrogen, effectively affect lipid production and fatty acid composition in microalgae (Cho *et al*., 2011). Generally, cells would be forced to make essential enzymes and structure with the rest of their nitrogen under nitrogen limitation (Armbrust and Chisholm,

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1990; Wehr *et al*., 2015). Therefore, carbon dioxide fixation would go toward producing lipid or carbohydrates rather than proteins (Fakhry and El Maghraby, 2015; Tan and Lee, 2016; Cheng *et al*., 2017).

In this work, the sample's variable patterns of growth and fatty acid composition were investigated under different nitrogen regimes and cultivation times. The results showed that nitrogen limitation (ND) led to cease cell division through the lag phase in way *T. weissflogii* left the log phase 24-48 h later than it was under the normal condition (NN) (Fig. 3). This happened for cell freezing in G1 phase, which is responsible for DNA synthesis preparation, growth, and cell size and synthesize of the girdles (Berges *et al*., 1996; Flynn and Jézéquel, 2000; Xin *et al*., 2010; Yang *et al*., 2013; Fazeli Danesh *et al*., 2018). In accordance with previous research, our results also indicated that the cell count, dry weight biomass and growth rate in *T.weissflogii* were impacted and declined under nitrogen-deficient condition (ND) (Fig. 3, Table 2) (Fidalgo *et al*., 1995; Xin *et al*., 2010).

Color shift was another physiological change that was observed in ND culture medium (brownish to yellowish) which approves the effect of nitrogen limitation on the pigment´s level like fucoxanthin (Henriksen *et al*., 2002; Xin *et al*., 2010; Nagao *et al*., 2014). As we know, nitrogen deficiency condition has the highest impact on photosystem II (Soler *et al*., 2010), and this strain with two-fold photosystem II (PSII: PSI ratio of 2:1) than photosystem I centers (Berges *et al*., 1996) is extremely susceptible to this condition. Thus, this color shift is probably related to the effect of nitrogen deficiency on photosystem II and the association of this photosystem on the fucoxanthin level; however, the interaction of nitrogen limitation on photosystem II and Fucoxanthin is still unknown (Soler *et al*., 2010).

Usually, microalgae cells attempt to survive under nitrogen limitation through reserving lipids (Soler *et al*., 2010) which is ordinarily obvious in cell appearance. Lipid body formation (LBs) is a common change in microalgae cells under nitrogen stress conditions. (Olmstead *et al*., 2013). Monitoring of these bodies includes triacylglycerides (TAG), and fatty acids (FA) (90% TAG and 10% free fatty acids (FFA), which is considered as an early lipid production screening (Wang *et al*., 2009).

In this study, cells in a defective condition appeared LBs earlier than the same in NN condition (Fig 1-c). Early lipid bodies' formation might occur to cells for the early arrival of the *T.weissflogii* into the stationary phase and the conversion of membrane lipids to triacylglycerol.

Triacylglycerol synthesis in microalgae cells is an active response to tolerate this unfavorable stress condition. It makes a variation in lipid metabolism in terms of storage more neutral lipids instead of membrane ones (Hu *et al*., 2008; Yang *et al*., 2017). This might be related to up-regulating of Acetyl-CoA carboxylase and the linear relation between the lipid content and

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expression of accD under nitrogen limitation conditions (Fan *et al*., 2014; Li *et al*., 2015).

Cultivation time is another parameter that effects on the total lipid and biomass concentration (Huang *et al*., 2015). Our results showed a 1.7% increase in total lipid (Dry weight %) of this diatom under ND condition for 18 days cultivation. This rate rose to 6.29% after 30 days of cultivation under the same condition. It seems that cultivation of this strain under nitrogen limitation with the prolonged culturing method can recover total lipid yield near to 50 percent of its dry weight (Table 3), which agrees with other studies (Boyle *et al*., 2012; Li *et al*., 2012).

Nitrogen deficiency also causes variation in fatty acid profiles of *T. weissflogii*, which led to growth in (SFA) and (MUFA) levels during prolonged cultivation (30 d). Our research also defined that SFA accumulation in this diatom during both nitrogen conditions was higher than other fatty acids types. Regarding the amount of PUFAs produced in this strain, the highest amount of PUFA was only supported by prolonged culturing of this diatom under ND conditions (Fig. 4).

Fatty acid profiles under both nitrogen regimes presented that this strain had a tangible increase in C16:0 and sharp growth in C18:1 level from 17.5 (%TFA) to 26.34 (%TFA) under ND condition. The high expression of Acyltransferase can explain this growth of SFA as one of the key enzymes involved in generating the free fatty acids (Li *et al*., 2020; Hu *et al*., 2008; Boyle *et al*., 2012). It seems that genetically alterations under nitrogen limitation lead to overexpression of *DGAT1*, *DGTT1* and *PDAT1* genes responsible for encoding of acyltransferase (Boyle *et al*., 2012). These genes have crucial roles in conducting the flux of carbon into triacylglycerol formation; hence, they are considered great targets for engineering oil in microalgae cells (Patil *et al*., 2005; Yoon *et al*., 2012; Xu *et al*., 2018).

Variation in PUFA levels was another consequence that occurred in *T.weissflogii* under ND conditions. A glance at the fatty acids profile in both nitrogen regimes shows that the amount of C20:5 in NN were higher than ND condition, and it was decreased in continue by almost half in nitrogen deficiency conditions. In contrast, this amount for C22:6 has been drastically increased from 3.5 (NN) to 12.63 (ND) (TFA %) (Table 4).

As mentioned, fish and shellfish farming have been caught in the spotlight due to increase in healthy food demand. In general, the highest cost of aquaculture production is the provision of high-quality feed, so today, improving feed efficiency in industrial systems has become a priority. Application of high-nutritious microalgae strains with a proper level in LC-PUFA, protein, and carbohydrates make microalgae as available biomolecules for the fish food chains (Patil *et al*., 2005). However, other parameters such as size, shape,

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digestibility and biochemical composition are required to consider one microalgae strain considered as an appropriate feed. All studies carried out on *T.weissflogii* approved that this strain has an appropriate size (6-20µm x 8-15µm) and it contains the suitable nutritional value (PUFA n-3) for feeding shrimp, and bivalve larviculture industries. Consequently, many aquafeed products used today in the early to end post larval (PL) stages include this diatom as instant algae feed for shellfish and shrimp hatchery.

This study attempts to better understand the impact of nitrogen deficiency condition on PUFA content of *T.weissflogii* during the prolonged culturing. Due to the results, this strain can be considered potential natural PUFA n-3 source for aquafeed industries; however, further studies are required to optimize the commercial process like reduction in time culturing and improving C20:5 and C22:6 levels. Also, it is highly recommended that the long-term nutritional effects of this strain on the growth and fatty acid composition of shrimp and shellfish be investigated.

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