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RESEARCH PAPER

Fucoxanthin Content of *Cylindrotheca closterium* and Its Oxidative Stress Mediated Enhancement

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Abstract

Production of fucoxanthin by diatoms has become an alternative research area due to their low cost, convenience and diversity. The fucoxanthin content of *Cylindrotheca closterium* and its enhancement by altering the cultivation conditions via oxidative stress were investigated in this study. For this purpose, the extraction parameters were optimized and the highest fucoxanthin concentration (6.58 mg g^{-1}) was achieved within 15.0 minutes at 40 ^{o}C. Then, this yield reached to 10.15 mg g^{-1} in the presence of NaOCl and Fe^{2+}. It is worth noting that, this is the first time that the effect of oxidative stress on fucoxanthin production in diatom has been studied according to our knowledge. Therefore, the results of this study and the discussion about the mechanisms can be a reference for the enrichment of fucoxanthin from other diatoms.

Keywords: Fucoxanthin; *Cylindrotheca closterium*, oxidative stress; diatom; extraction.

Cylindrotheca closterium'un Fukoksantin İçeriği ve Oksidatif Stres ile Arttırılması

Özet

Fukoksantinin diatomlar tarafından üretimi düşük maliyet, kolaylık ve çeşitlilik nedeniyle alternatif bir araştırma alanı haline gelmiştir. Bu çalışmada, *Cylindrotheca closterium'*da bulunan fukoksantin miktarı ve kültür koşullarına uygulanan oksidatif stresin, bu içeriği nasıl arttırdığı araştırılmıştır. Bu amaçla,ekstraksiyon parametreleri optimize edilmiş ve en yüksek fukoksantin derişimi (6,58 mg g^{-1}) 40 ^{o}C''de ve 15,0 dakika içinde elde edilmiştir. Daha sonra, fukoksantin miktarı, NaOCl ve Fe^{2+} varlığında 10,15 mg g^{-1} a kadar ulaşmıştır. Bu çalışma ile ilk kez bir diatomun kültür koşullarına uygulanan oksitafis stres ile fukoksantin miktarındaki değişim gözlenmiştir. Bu nedenle; söz konusu çalışma, sonuçları ve mekanizmalar hakkında yapılan değerlendirmeler ile fukoksantinin diğer diatomlarda da zenginleştirilmesi açısından bir referans olabilir.

Anahtar Kelimeler: Fukoksantin, Cylindrotheca closterium, oksidatif stres, diatom, ekstraksiyon.

Introduction

The use of microalgae in biotechnology has gained a significant attention since they produce various metabolites that are essential to human health. For many years, carotenoids are among these bioactive compounds. Biosynthesis of carotenoids from microalgae have been more preferred instead of using synthetic routes since the reaction efficiency is very low. Moreover, production of carotenoids from algae has several advantages when compared with the other sources due to their cheap production and there is no limited seasonal variation (Christaki *et al*., 2013).

Although many microalgae are known to produce various levels of carotenoids, at present, only *Haematococcus pluvialis* and *Dunaliella* are grown commercially due to their large production of astaxanthin and β-carotene, respectively. For this reason, there is growing commercial interest in the production and use of other carotenoids; such as lutein, cantaxanthin and fucoxanthin, due to their anti-oxidative properties. In order to be a commercial microalga for carotenoid production, it must accumulate large amounts of carotenoids during the cultivation. Nowadays, interest is also being focused on fucoxanthin which is a type of carotenoid and powerful antioxidant mostly produced by brown algae that also gives their color. Recently, several studies have been demonstrated the anti-inflammatory, antioxidant and anti-cancer activity of fucoxanthin (Yan *et al*., 1999; Sangeetha *et al*., 2010; Peng *et al*.,

2011; Fung *et al*., 2013). On account of the high fucoxanthin content, diatoms have become the most important and promising natural resources. Production of fucoxanthin by diatoms has been preferred due to their low cost, convenience and diversity. Therefore, it has been important to find out new species which highly accumulates fucoxanthin. In addition, many microalgae alter their biosynthetic pathways for the formation and accumulation of carotenoids due to changes in environmental conditions, sometimes so-called stress factors as they exhibit a great metabolic plasticity (Jin *et al*., 2003). These conditions may be light, temperature, salinity or presence of oxidizing agents such as H_2O_2 (hydrogen peroxide) or NaOCl (sodium hypochlorite) (El-Baky *et al*., 2009). Mojaat *et al*., (2008) have succeeded to stimultae β-carotene synthesis in Dunaliella salina, by inducing an oxidative stress. However, there has been still no considerable research on the effect of oxidative stress on fucoxanthin. Despite their roles and great abundance, information on the biosynthesis of carotenoids (or a specific carotenoid) is also still incomplete. Therefore, new studies must be directed towards their determination and response to oxidative stress conditions (Sachindra *et al*., 2007; Jaswir *et al*., 2011; Mise *et al*., 2011).

The aim of this work is to discuss the mechanism behind the oxidative stress applied to *Cylindrotheca closterium* in order to increase the amount of fucoxanthin. The defense mechanisms for the diatoms were evaluated including how conditions stress diatoms and how diatoms respond to oxidative stress by managing reactive oxygen species.

Although this study will require new optimization parameters for fucoxanthin production under oxidative stress in both open and closed systems, it might have important contributions to the literature.

Materials and Methods

Cultivation of *Cylindrotheca closterium*

Cylindrotheca closterium was obtained from Ege University Microalgae Culture Collection, Ege-MACC (codded with EGEMACC44 http://www.egemacc.com/cultures.php) whose NCBI (National Center for Biotechnology Information) access number is JQ809712. It was isolated from Lake Bafa, Muğla-TURKEY. The stock cultures were cultivated in F/2 Medium (Guillard and Ryther 1962). For the preparation of the inoculum, the cells from the stock culture were collected and concentrated by centrifugation and the supernatant was then removed. The collected cells were transferred and incubated aseptically under continuous illumination using cool white fluorescent lamps (Philips, 18 W/54) at 24.0±1ºC for 4 days. Air was supplied to the culture and the ventilation rate was 1.25 vvm. 4-day old culture (at vegetative cell growth phase) was used as inoculum at 10% volume for all experiments. The cells in 2.0 L bottles were incubated 17 days at constant temperature and light. Optical microscope images of *C. closterium* were presented in Figure 1. All glassware and the medium were sterilized in autoclave. Growth rate for *C. closterium* was calculated according to Becker's formula (Equation 1) using the data obtained by the absorbance values at 450 nm.

$$\mu = \frac{\ln x_2 - \ln x_1}{\Delta t} \quad (1)$$

μ : specific growth rate
x_2 : cell concentration at time t_2
x_1 : cell concentration at time t_1
Δt : t_2-t_1

Doubling time was also calculated as Doubling Time = ln 2/μ.

Preparation of Biomass

Cylindrotheca closterium was cultivated as outlined in the previous section. When the culture was ready to be harvested, it was first centrifuged for 5 minutes at 6000 rpm and then filtered through 0.45 μm cellulose acetate filter paper (Whatman). The filtered biomass was washed with deionized water to remove the excess salts that might be left from the culture medium. The obtained biomass was lyophilized, reduced to a size using a mortar and then kept at −20 ºC until the extraction of carotenoids.

Extraction of Fucoxanthin from *Cylindrotheca closterium*

The extraction procedure was adapted from Erdoğan *et al*., (2015). The main difference was that the saponification procedure was not performed in this study since the saponification causes fucoxanthin into fucoxanthinol under severe basic conditions (Yonekura *et al*., 2010; Haugan *et al*., 1992).

In the present study, the effect of extraction parameters on fucoxanthin content were also examined. For this purpose, the effect of contact time, extraction number and temperature were optimized. In addition, the effect of oxidative stress conditions on fucoxanthin content were investigated.

First, the biomass (0.25 g) was added $CaCO_3$ (0.25 g) and extracted with 10.0 mL of THF:DCM (1:1) (v/v) containing 0.010 % (w/v) pyrogallol. Then the mixture was placed in an ultrasonic bath for extraction. After the ultrasonic extraction, the solution was centrifuged at 6000 rpm for 10 minutes and the supernatant was collected. The residue was repeatedly extracted with fresh volume of 10.0 mL extraction solvent until it was colorless (three times). The supernatant solutions were combined, evaporated by the rotary evaporator at 35.0 ºC under vacuum. Then the residue was dissolved in 10.0 mL chloroform and

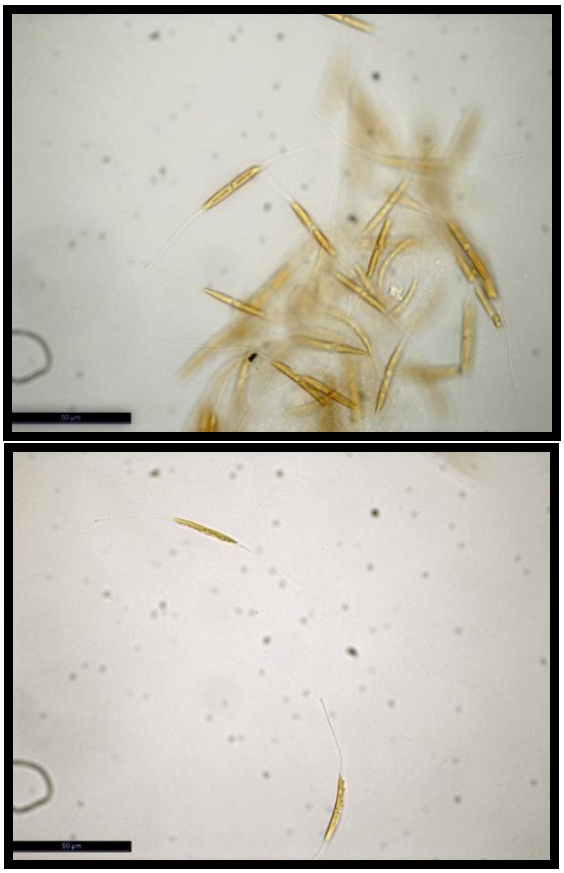

Figure 1. Optical microscope images for *Cylindrotheca closterium* (x60).

diluted with mobile phase prior to HPLC injection.

Usually, saponification is applied in routine carotenoid analysis to eliminate the unwanted lipids and chlorophylls. It also hydrolyzes carotenoid esters, thus simplifies the chromatographic separation, identification and quantification in most of the samples. However, in this study saponification procedure was avoided since fucoxanthin is alkali-labile and is converted to fucoxanthinol in the presence of strong alkali (Britton *et al*., 2004).

Enhancement of Fucoxanthin Content under Oxidative Stress Conditions

As a model for environmental stress, it was also examined whether the use of oxidative stress growth conditions could increase the amount of fucoxanthin in *Cylindrotheca closterium* cultivated in F/2 medium containing H_2O_2, NaOCl and iron. To evaluate the effects of H_2O_2, NaOCl and iron on *C. closterium*, the cultured was exposed to H_2O_2/Fe^{2+}, NaOCl/ Fe^{2+} and H_2O_2 /NaOCl concentrations (0.1 mM) for 17 days, separately. The •OH and $^1O_2$ are among the most powerful oxidative species in nature which are generated based on the Eqs. (2-5) (Ip and Chen 2005; Strati and Oreopoulou 2011):

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \bullet OH \quad (2)$$

$$NaOCl + H_2O \rightarrow HOCl + Na^+ + OH^- \quad (3)$$

$$HOCl + Fe^{2+} \rightarrow Fe^{3+} + Cl^- + \bullet OH \quad (4)$$

$$H_2O_2 + NaOCl \rightarrow H_2O + NaCl + {}^1O_2 \quad (5)$$

Instrumentation and Analyses

The extraction and work-up procedures were conducted under yellow light to prevent photo-isomerization and degradation of carotenoids as they are sensitive to light (Hii *et al*., 2010). Chromatographic determination of fucoxanthin was performed by Agilent 1200 Series HPLC-DAD using a reversed-phase C_{30} column because of its improved separation efficiency. Prior to use, all HPLC-grade mobile phase solutions were degassed using an ultrasonic bath (Elmasonic S80H).The reversed phase column was a YMC Carotenoid C_{30} column, 250 mm x 4.6 mm, 5µm (Waters**)**. The column temperature was set at 25.0 ºC. The mobile phase consisted of 70 % (v/v) methanol and 30 % (v/v) acetonitrile. (Both solutions already contained 0.1 % (v/v) TEA and 0.01 % (w/v) pyrogallol).

The separation was achieved by isocratic elution at a flow rate of 1.0 mL min^{-1}. The injection volume was 20.0 µL and detection was made by diode array detector with quantitation at 450 nm. The full spectrum from 300 to 600 nm was also recorded.

Extraction was realized by using ultrasonic bath (Elmasonic S80H) and the solvents were removed with the use of rotary evaporator (Heidolph Hei-VAP

Advantage). Optical image was obtained under a trinocular light microscope (Olympus CH40). The elemental composition of the microalga was determined with LECO-932 elemental analyzer. Elemental analysis has indicated that *Cylindrotheca closterium* is composed of 23.2 % C, 3.5% N, 3.5% H, and 0.6% S by mass. The remaining part is mainly composed of silicon and oxygen since it is a diatom.

The stock standard solutions of carotenoids were prepared under yellow light (Philips lamp TLD 36W/16 yellow, light transmission at 500-750 nm) at room temperature within the shortest possible time. For the preparation of fucoxanthin stock standard, 5.0 mg of all-trans fucoxanthin, and for internal standard 5.0 mg of all-trans-neoxanthin were accurately weighed and dissolved in chloroform in separate volumetric flasks (50.0 mL). Calibration standards (0.010-5.0 mgL^{-1}) were prepared from the stock solution and contained a fixed concentration of internal standard (2.0 mgL^{-1}). All standard and sample solutions were kept in amber colored volumetric flasks wrapped with aluminum foil. Calibration curve was obtained by plotting the ratio of absorbances of the analyte and the internal standard as a function of analyte concentration. Absorbance values were measured in terms of peak area. It is a great problem that there is no available certified reference standard for each carotenoid. For this reason, in order to check the accuracy of calibration curve, certain concentration of a fucoxanthin solution (within the limit of linearity) was injected after each sample introduction. Tolerance limit for concentration was under 5%.

Magnitude of analytical background was obtained by measuring the blank solution 10 times. The detection and quantification limits (LOD based on 3s and LOQ based on 10s, respectively) were determined using the standard deviation of the blank sample.

Chemicals

HPLC and analytical grade solvents were used in all of the experiments. The analytical standards (all-trans-fucoxanthin and all-trans-neoxanthin) were purchased form CaroteNature GmbH.

Results and Discussion

HPLC-DAD Analysis of Fucoxanthin from *Cylindrotheca closterium*

A simple isocratic elution procedure was developed for the determination of fucoxanthin. The system suitability tests were performed using three replicate injections of the standard solutions. Neoxanthin was used as internal standard due to similarity of its structure to fucoxanthin. Theoretical plates (N), asymmetry factor, LOD and LOQ values, linearity range, regression equation and other parameters are given in Table 1. The developed LC-DAD method described is fast, specific and precise for the determination of fucoxanthin. The multidimensional nature of diode array spectra makes it an extremely significant tool for the study of complex products such as carotenoids and chlorophylls. According to the data obtained by HPLC-DAD throughout the analysis, fucoxanthin showed the maximum absorbance at 450.0 nm. Therefore all the HPLC-DAD analyses were performed at this wavelength. In the present study, the employed chromatographic conditions (Figure 2) can separate fucoxanthin and neoxanthin (internal standard). In this chromatogram, peaks eluting in the first five minutes are due to chlorophyll content of *Cylindrotheca closterium*.

Effect of Contact Time on Fucoxanthin Extraction

The effect of contact time on fucoxanthin extraction from the microalga was investigated by conducting the experiments in THF:DCM (1:1) at 25.0 °C. It has been reported that the following steps occur in a liquid-solid extraction process*: (i) solvent transfer from the bulk of the solution to the surface of*

Table 1. Summarized system suitability and validation parameters with proposed LC method (isocratic elution with 70:30-MeOH:ACN at 450 nm, flow rate:1.0 mL min^{-1})

System suitability and validation parameters	
Theoretical plate (N)	13322.5
Asymmetry factor	1.04
*Range (µg mL^{-1})	0.010-5.0
LOD (µg mL^{-1})	0.0017
LOQ (µg mL^{-1})	0.0185
r^2	0.9994
Regression equation	y = 0.435 x + 0.00371
Peak purity (%)	97.99
Injection precision (RSD)	<1%
Column resolution (Rs)	>2
Capacity factor (k')	2.75
Selectivity factor (α)	2.65

* (n=3)

the solid; (i) penetration or diffusion of the solvents into the pores of the solid; (iii) dissolution of the solute in the solvent; (iv) solute diffusion to the surface of the particle; and (v) solute transfer to the bulk of the solution. Any of the five basic steps may be responsible for limiting the extraction rate because of the solubility of the solute or its miscibility with the solvent. Therefore, the rate of the extraction is most likely to be controlled by the diffusion phenomena (Strati and Oreopoulou 2011). As it is shown in Figure 3, fucoxanthin extraction depended on extraction time, showing a high initial rate of extraction that decreased with time until almost an equilibrium was reached. Therefore, 15 min appeared to be sufficient for the maximum extraction of fucoxanthin from *Cylindrotheca closterium*.

Effect of Number of Extractions on Fucoxanthin Extraction

To increase the amount of fucoxanthin extracted, successive experiments were performed until no color was observed in the extracts. As illustrated in Figure 4, 95% of fucoxanthin is extracted in the first run. It has been observed that the cells of *Cylindrotheca closterium* was sturdy and significant decoloration was observed within its cells when compared with the non-extracted cells (data not shown) after the first extraction.

Effect of Temperature on Fucoxanthin Extraction

Generally, temperature has a positive effect on both extraction efficiency and extraction rate provided that it is not too high to cause the degradation of some of the active components of the biomaterial. In order to study the effect of temperature on the extraction of fucoxanthin from *Cylindrotheca closterium*, 25, 40 and 55 ^{o}C were applied and the results are summarized in Figure 5.

The results indicated that an increase in the extraction efficiency was observed when the temperature was risen from 25 to 40 ^{o}C. Higher temperature probably allows the liberation of pigments due to increased kinetic energy and the contact between solvent molecules and the biomass. However, upon a further increase of temperature to 55 ^{o}C, the extraction of fucoxanthin decreases which might be stemming from undesirable reactions such as isomerization and/or oxidation of fucoxanthin.

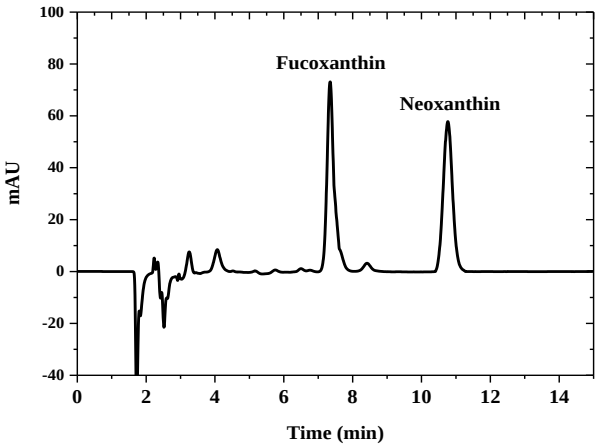

Figure 2. HPLC chromatogram for *Cylindrotheca closterium* extract (isocratic elution, 70:30-MeOH:ACN, column temperature: 25 ^{o}C, flow rate: 1.0 mL min^{-1}, absorbance at 450 nm).

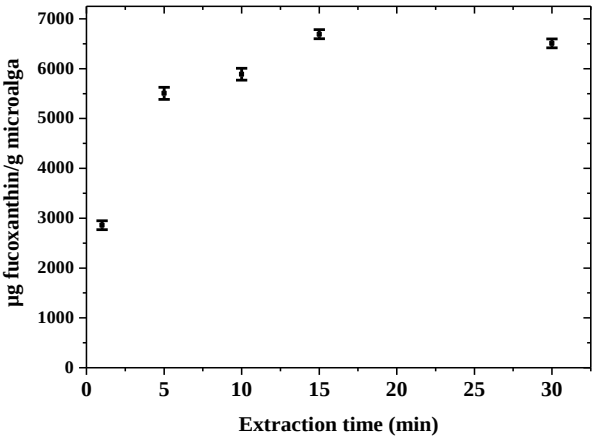

Figure 3. Effect of contact time on the extraction of fucoxanthin from *Cylindrotheca closterium* (Experimental conditions: 0.25 g microalga, 10.0 mL THF/DCM, 25 ^{o}C, n=3).

Effect of Oxidative Stress on Fucoxanthin Production During Cultivation of *Cylindrotheca Closterium*

In microalgae, reactive oxygen species (ROS) are continuously produced in chloroplasts, mitochondria and peroxisomes. To avaoid damage to cell components, production and scavenging of ROS must be strictly balanced; hence, the antioxidant protective mechanisms must take place (Goiris *et al*., 2015).

Hydrogen peroxide, a type of ROS, is a product of microalgae via their oxidative metabolism. Nearly all living things decompose low concentrations of hydrogen peroxide to water and oxygen. H_2O_2 can injure cells at high concentrations or lead to acclimation at moderate levels. Particularly in the presence of iron, hydrogen peroxide decomposes and generates the highly reactive hydroxyl radical through Fenton reaction. Moreover, the uncontrolled production of reactive oxygen species (ROS) may destroy proteins, lipids and carotenoids. As with many other organisms, microalgae develop defence mechanisms against high levels of ROS (Wei *et al*., 2008). In the presence of ROS, the antioxidative carotenoids might be produced in order to protect the cells against oxidative damage.

Unfortunately, there is not enough information in literature about the mechanism of the stimulating formation of fucoxanthin by ROS. In the present study, the enhancement in the fucoxanthin accumulation using suitable concentrations of ROS in *Cylindrotheca closterium* was tested. It was found and demonstrated in Table 2 that the fucoxanthin accumulation in *C. closterium* can be said to be remarkable, 10.19 ± 0.22 mg/g (approximately 55 % enhancement compared to the reference value, 6.58 ± 0.17 mg/g), upon the addition of NaOCl in the presence of Fe^{2+} to the growth medium compared to the effects of H_2O_2/Fe^{2+} or H_2O_2/NaOCl. On the other hand, doubling time was increased and biomass productivity decreased only by half.

The results given in Table 2 have shown that NaOCl/Fe^{2+} stimulates the production of fucoxanthin more efficiently than the other oxidative stress conditions. It was observed that the addition of NaOCl in the presence of Fe^{2+} increased the

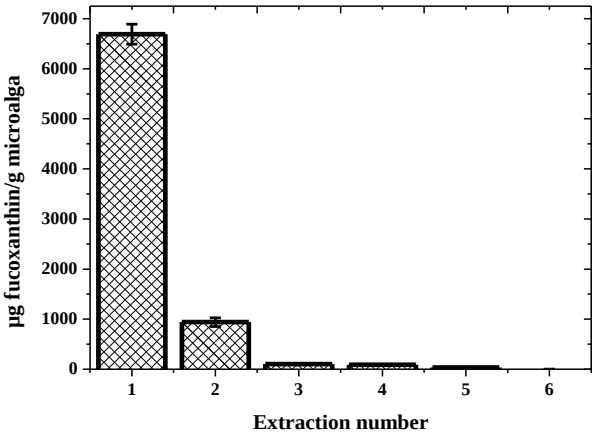

Figure 4. Effect of extraction number on extraction of fucoxanthin from *Cylindrotheca closterium* (Experimental conditions: 0.25g microalga, 10.0 mL THF/DCM, 15 min sonication, 25ºC, n=3).

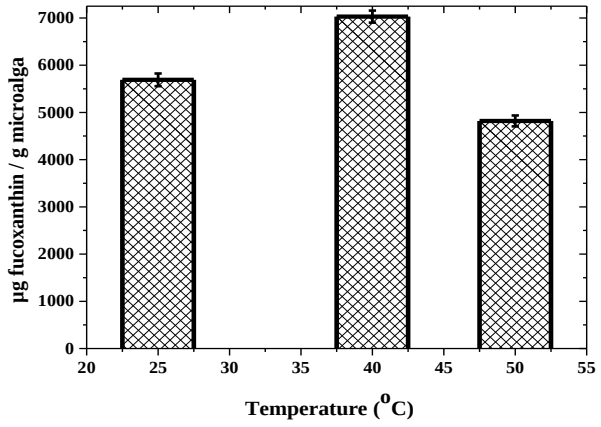

Figure 5. Effect of temperature on the extraction of fucoxanthin from *Cylindrotheca closterium* (Experimental conditions: 0.25 g microalga, 10.0 mL THF/DCM, 15 min sonication, n=3).

accumulation of fucoxanthin. Probably, the microalgae could easily overcome the effect of H_2O_2/Fe^{2+}. However, the case may not be similar in other conditions. As the fucoxanthin is the main carotenoid that is highly accumulated in *C. closterium*, the increase in its amount due to the presence of $NaOCl/Fe^{2+}$ might be regarded as a real stress condition since the specific growth rate decreases and doubling time increases. When the effects of oxidative stress conditions on the growth productivity of *C. closterium* was examined (Figure 6), it could be seen that there occurred a significant difference. It seems that the species adapts itself to the stress conditions. This adaptive response might be considered as an adjustment mechanism for this type of oxidative stress. Probably, microalgae attempts to compensate this effect by increasing its fucoxanthin content. Different types of mechanisms acting alone or in interaction may cause the change in fucoxanthin level by modifiying enzyme activities through transcriptional or post-transcriptional mechanisms that activate signalling or defence mechanisms (Noctor *et al*.,, 2015).

In microalgae, a distinction is usually made between primary and secondary carotenoids. Whereas primary carotenoids are structural and functional components of the photosynthetic apparatus, and thus essential for survival, secondary carotenoids are produced at high levels when cells are exposed to specific environmental stimuli (Jin *et al*., 2003).

As in the case of all living things, microalgae have also several defensive systems , both enzymatic and non-enzymatic for the detoxification of ROS. In literature, it has been reported that three reaction mechanisms describe the reaction of free radicals with carotenoids, that is, electron transfer, hydrogen atom transfer, and radical addition to the carotenoids (Martínez and Barbosa 2008; Martínez *et al*., 2008). In order to scavenge free radicals, carotenoids can either donate or accept unpaired electrons. Usually, antioxidant molecules become oxidized by donating electrons to the free radical. However, carotenoids can also quench free radicals by accepting an unpaired electron, rendering it harmless by translocation over the conjugated side chain. Based on these reasons, it could be possible that the synthesis of fucoxanthin was induced while quenching with ROS.

Conclusions

It could be derived from the results of this study that *Cylindrotheca closterium* contains high amount of fucoxanthin and its content can be increased by applying oxidative stress conditions. In the present work, fucoxanthin as being the major carotenoid in *C. closterium*, has been affected by the variation of cultivation settings and its accumulation has been induced by the presence of reactive oxygen species (ROS). The presence of $NaOCl/Fe^{2+}$ in F/2 medium. stimulates the production of fucoxanthin under oxidative stress conditions.

This study can be interpreted in a way that *C. closterium* might have a great potential for the production of fucoxanthin. Moreover, the optimum

Table 2. Growth rates and fucoxanthin accumulation of *Cylindrotheca closterium* grown control and under oxidative stress conditions

Oxidative stress sources in F/2 medium	μ_{max} (day^{-1})	Doubling time (day)	Fucoxanthin accumulated (mg g^{-1})
Control	0.243	2.85	6.58 ± 0.17
0.1 mM H_2O_2 + 0.1 mM Fe^{2+}	0.121	5.73	6.62 ± 0.12
0.1 mM NaOCl + 0.1 mM Fe^{2+}	0.141	4.92	10.19 ± 0.22
0.1 mM H_2O_2 + 0.1 mM NaOCl	0.136	5.10	7.79 ± 0.19

*Experiments were performed three times.

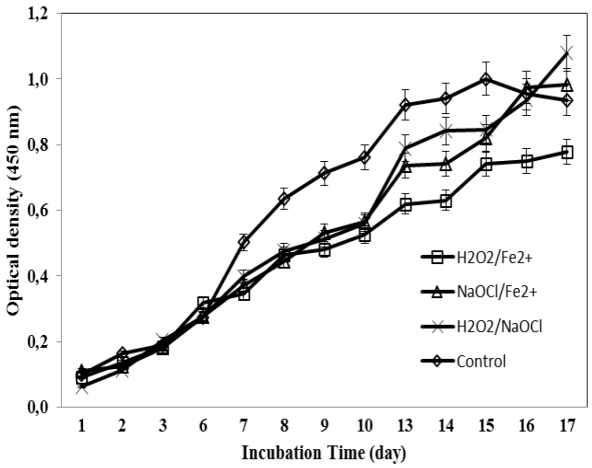

Figure 6. Effects of oxidative stress conditions on the growth productivity of *Cylindrotheca closterium.*

concentration of Fe^{2+} and NaOCl have to be determined to find out a better relation between fucoxanthin synthesis caused by oxidative stress. Yet, the growth rate must be taken into account and it must be sufficient for continuous processes.

Finally, it can be said that microalgae can experience increased levels of oxidative stress and the defence mechanisms may include antioxidant enzymes such as superoxide dismutase, catalase, peroxidases, and glutathione reductase, as well as non-enzymatic antioxidant molecules such as phytochelatins, pigments, polysaccharides, and polyphenols. These findings could be useful updated knowledge and emphasizes the need for future researches.

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