KAUNAS UNIVERSITY OF TECHNOLOGY
FACULTY OF CHEMICAL TECHNOLOGY

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ISOLATION AND *IN VITRO* ANTIOXIDANT ACTIVITY ASSESSMENT OF HIGHER-ADDED VALUE COMPONENTS FROM DIATOMS

Master's Final Degree Project

Supervisor
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„Isolation and *in vitro* antioxidant activity assessment of higher-added value components from diatoms"

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CONTENT

Kesminas, Šarūnas. *Aukštesnės pridėtinės vertės komponentų išskyrimas iš titnaginių dumblių ir in vitro antioksidacinio aktyvumo įvertinimas*. Magistro baigiamasis projektas. Vadovas dr. Michail Syrpas; Kauno technologijos universitetas, Cheminės technologijos fakultetas.

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SANTRAUKA

Titnagdumblis, *Phaeodactylum tricornutum,* buvo auginamas su įvairomis vario ir geležies koncentracijomis. Buvo tiriamas žalingas vario ir geležies poveikis *Phaeodactylum tricornutum* augimui, stebimi antioksidacinių savybių, fenolinių jungių kiekio pasikeitimai. Prie labai aukštų vario ir geležies koncentracijų *Phaeodactylum tricornutum* augimas nevyko. Esant mažesnioms, tačiau vistiek aukštoms, vario ir geležies koncentracijoms, titnagdumblio augimas vyko, bet netolygiai, tačiau ištyrus užaugintą biomasę buvo pastebėtas antioksidacinio veikimo ir fenolinių juginių kiekio padidėjimas. Titnagdumbliai, *Phaeodactylum tricornutum* ir *Cylindrotheca closterium,* buvo auginami su skirtingomis pridėtinėmis glicerolio koncentratcijomis. Buvo ištirtas titnagdumblių gebėjimas augti miksotrofinėmis sąlygomis, taip pat, kaip pridėtinis glicerolis įtakojo augimą, antioksidacines savybes, fenolinių junginių kiekį, riebalų rūgščių sudėties pasikeitimus. *Phaeodactylum tricornutum* ir *Cylindrotheca closterium,* gebėjo augti miksotrofinėmis sąlygomis, pridėtinis glicerolis lėmė greitesnį titnagdumblių augimą ir didesnę galutinę biomasės koncentraciją, taip pat padidėjo anitoksidacinis aktyvumas, fenolinių junginių ir riebalų rūgščių kiekis. Buvo atliktas *Phaeodactylum tricornutum* aukšo slėgio skysčių ekstrakcijos parametrų (laiko ir temperatūros) įtakos patikrinimas su didėjančio poliškumo tirpikliais (heksanas, acetonas, etanolis). Ištirta aukšto slėgio skysčių ekstracijos, su heksanu, parametrų (laiko ir temperatūros) įtaka ekstrakto išeiga, DHA ir EPA kiekiams ekstrakte. Ištirta aukšto slėgio skysčių ekstracijos, su acetonu, paramterų (laiko ir temperatūros) įtaka ekstrakto išeigai, TPC ir ABTS dydžiams ekstrakte. Taip pat, ištirta aukšto slėgio skysčių ekstracijos, su etanoliu, paramterų (laiko ir temperatūros) įtaka ekstrakto išeigai, TPC ir ABTS dydžiams ekstrakte. Pateikta *Phaeodactylum tricornutum* ekstrakto, naudojant aukšto slėgio skysčių ekstrakciją, gamybos principinė technologinė schema.

Kesminas, Šarūnas. *Isolation and in vitro Antioxidant Activity Assessment of Higher-added Value Components from Diatoms.* Master's thesis in industrial biotechnology. Supervisor dr. Michail Syrpas. The Faculty of Chemical Technology, Kaunas University of Technology.

Study field and area: Biotechnology, Technology sciences

Key words: *Antioxidant activity, diatoms, fatty acids, mixotrophy, phenolic compounds, PLE*

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SUMMARY

Diatom, *Phaeodactylum tricornutum,* was cultivated with various copper and iron concentrations. Copper and iron stress on *Phaeodactylum tricornutum* growth, changes in antioxidant activity and phenolic content were examined. Very high concentrations of copper and iron inhibited growth of *Phaeodactylum tricornutum,* lower but still relatively high concentrations of copper and iron negatively affected growth, but increased antioxidant activity and phenolic content of cultivated biomass. Diatoms *Phaeodactylum tricornutum* and *Cylindrotheca closterium* were cultivated with different glycerol concentrations. Their ability to grow under mixotrophic conditions were tested, changes in growth, antioxidant activity, phenolic content, fatty acid profile were examined. *Phaeodactylum tricornutum* and *Cylindrotheca closterium* were able to grow under mixotrophic conditions, glycerol addition promoted growth of both diatoms, with that antioxidant activity, phenolic content and fatty acid content were increase in both diatoms also. *Phaeodactylum tricornutum* pressurized liquid extraction (PLE) with three increasing polarity solvents (hexane, acetone, ethanol) at three temperatures (40^oC, 80^oC, 120^oC) and three extraction times (15min, 39min, 60min) were performed. Total yield, EPA and DHA content in lipophilic fractions obtained by pressurized liquid extraction under various experimental conditions were examined. Total yield. total phenolic content (TPC) and *in vitro* antioxidant activity as measured by the ABTS+ assay in polar fractions obtained by pressurized liquid extraction under various experimental conditions were examined. Manufacturing process technology scheme for production of *Phaeodactylum tricornutum* extract using pressurized liquid extraction (PLE) was proposed.

ABBREVIATIONS

Chl a – chlorophyll a;

Chl c – chlorophyll c

Chl – total chlorophyll

TPC – total phenolic content;

ABTS+ – ABTS+ scavenging assay

FRAP – Ferric reducing antioxidant power assay

GC-FID – Gas Chromatography with Flame Ionization Detector

EPA – Eicosapentaenoic acid (20:5n3)

DHA – Docosahexaenoic acid (C22:6n3)

PLE – Pressurized Liquid Extraction

INTRODUCTION

Microalgae are a diverse group of microorganisms with high photosynthetic biomass production efficiency[1], due to their unicellular or small multicellular structure they can live in harsh conditions[2]. Diatoms are one of the most important groups of marine algae, they constitute the base in many marine ecosystems food-chains and are key players in many biogeochemical cycles [3]. Diatoms are abundant and widely spread, which indicates their efficient growth strategies and survival mechanisms[3]. Because of their capacity to produce various bioactive and high added value compounds diatoms have attracted increasing attention for their potential industrial application. Regarding the production of bioactive compounds diatoms have numerous advantages over plant sources including rapid growth rates, minimal requirements for clean water or farmable land, thus not competing for resources with food crops [4]. Diatom ability to produce lipophilic products (fatty acids, steroids, triglycerides) and pigments (chlorophylls, carotenoids) [4] in high contents makes them ideal feedstocks for production of high-added value compounds with applications in nanotechnology, biotechnology, food and pharmaceutical industries[5]. Among diatoms, *Phaeodactylum tricornutum,* has attracted scientific attention as a rich source of essential fatty acids (especially EPA) and carotenoids with high antioxidant activity valuable for human nutrition such as ß-carotene, fucoxanthin, zeaxanthin [6]. *Phaeodactylum tricornutum* can also grow under mixotrophic conditions which could potentially increase the essential fatty acid and bioactive compound content.

Thesis aim: Isolation and in vitro antioxidant activity assessment of higher-added value components from diatoms with the specific focus on *Phaeodactylum tricornutum.*

To achieve this aim following objectives were raised:

- To examine *Phaeodactylum tricornutum's* growth, phenolic content and antioxidant activity responses to copper and iron stress at different metal concentrations.
- To examine the ability of diatoms, *Phaeodactylum tricornutum* and *Cylindrotheca closterium,* to grow under mixotrophic conditions
- To examine total phenolic content and *in vitro* antioxidant activity of diatoms, *Phaeodactylum tricornutum* and *Cylindrotheca closterium,* grown under mixotrophic conditions.
- To determine fatty acid composition of diatoms, *Phaeodactylum tricornutum* and *Cylindrotheca closterium,* grown under mixotrophic conditions by GC-FID analysis
- To determine the influence of pressurized liquid extraction conditions (temperature, extraction time) for increasing polarity fractions (hexane, acetone, ethanol) isolated from *Phaeodactylum tricornutum* biomass.

- To examine total yield, EPA and DHA content in lipophilic fractions obtained by pressurized liquid extraction under various experimental conditions.
- To examine total yield. total phenolic content (TPC) and *in vitro* antioxidant activity as measured by the ABTS+ assay in polar fractions obtained by pressurized liquid extraction under various experimental conditions.
- To suggest technological scheme for production of *Phaeodactylum tricornutum* extract using pressurized liquid extraction (PLE).

1. LITERATURE REVIEW

1.1. Algae

Algae are a diverse group of photosynthetic aquatic organisms[7]. They occur in wide variety of sizes and forms: single microscopic cells, can live in colonies, can be macroscopic or multicellular[7]. Algae can be found in freshwater and saltwater habitats[7]. Vast open seas are dominated by colonial microalgae, known as phytoplankton, almost all primary production in open sea is based on these photosynthetic microorganisms [8]. In shallow water areas, were sunlight penetrates to the bottom, benthic algae dominate. Phytoplankton are the primary producers in aquatic organism food chain [8]. Using nutrients, carbon dioxide, gathering sunlight energy, they create biomass through photosynthesis and are the primary food for other aquatic organisms. One of the major biomass production microorganism are diatoms, responsible for approximately 40% of annual marine biomass production [8].

1.1.1 Diatoms

Diatoms are found as single-cell and colonial, they consist of one of the largest classes of algae. Their cell size varies from 4 to 200 μm and have a light brown or yellowish color with silica exoskeletons, consisting of two overlapping halves [9]. Their skeletons vary in shape, ranging from circular to elliptical, needle-like, sigmoid or polygonal. Skeletons main two parts are referred to as valves, they are routinely used for species identification under a light microscope [10]. Valves differ in shape, size and ornamentation [9]. There are estimated 200 000 species of diatoms worldwide, taxonomy of diatoms is often difficult due to relatively similar morphology of many taxa [9]. The taxonomic process is complicated by dramatic variety of valve shape and size caused by diatom life cycle distinctiveness [10].

1.1.2. General biology of diatoms

The most distinctive characteristic is the possession of siliceous cell walls (frustules), often linked to pill-boxes. In contrast to most algal groups, cell wall morphology has formed the basis of diatom classification, requiring permanent preparations of cleaned frustules to facilitate the identification [11]. Rigid wall surrounds the protoplast of diatom cell contain several components, two valves and a series of linking bands (gridle). Cells are usually seen in girdle or valve view. Valve views present varied shapes, girdle views are usually more or less rectangular or square. Cells may move, shift changing their orientation so that both views are periodically presented. Therefore, both views must be observed and recognized for accurate identification. Valve contour fall into two major categories, circular with radial symmetry and linear with bilateral symmetry.

Cells with valvar markings that can be described as radial around a central point are called centric diatoms and those with bilaterally symmetrical – pennate diatoms (Figure 1) [11].

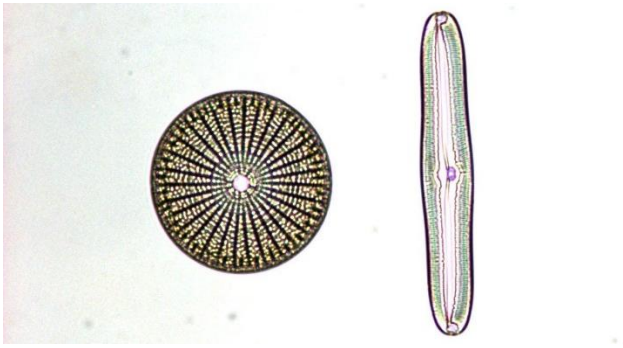


Figure 1. Centric diatom in the left, pennate diatom in the right.

Fundamental differences between centric and pennate diatoms in structure and symmetry relate to differences in features like motility, size and number of plastids as well as ecology. Centric diatoms are mainly planktonic algae. So, existence of oogamy, with production of larger number of motile sperm, can be attributed to a strategy for increasing efficiency of fertilization in open water environments. Pennate diatoms are mostly isogamous, with equal sized non-flagellate gametes. In these algae, ability of fertilization is improved by pairing of gamete parental cells before gamete formation [12].

1.1.3. Diatom growth physiology

Diatoms are one the main contributors to global carbon fixation, responsible for up to 40% of the primary production in the Ocean [13]. Diatoms are dominating in biogeochemical cycles of carbon, nitrogen, phosphorus, silicon and iron. They have a relatively low surface to volume ratio which need nutrient rich conditions comparing to smaller phytoplankton with higher surface to volume ratio which is more efficient in low nutrient conditions [13]. Therefore, diatoms dominate smaller phytoplankton in nutrient-rich areas and vice versa [13]. Specific growth rate values differ by more than one order of magnitude, but generally can be related to cell size. Smaller cells tend to have higher growth rates than bigger cells [14]. Growth rate changes implies smaller cells having a distinct catalytic advantage over the large ones [14]. Due to thickening of the diffusion boundary layer, increase of the diatom cell size decreases the influx or efflux of the nutrient solution [13]. Smaller are of membrane lipid and lower number of transporters. Additionally, increasing cell size, reduces the efficiency of pigmentation in harvesting light, alleged ‘package effect’ [15]. The pigment-protein complexes take longer to repay the energy used for synthesis, because chlorophyll molecules have lower probability of absorbing light photons in a given field in a large cell than in a small cell. Influence of package effect in algae of different sizes and shapes,

play a big role in rate changes of growth and photosynthesis in natural conditions [13]. Algae are an important natural surface water component, they produce and release phenolic compounds, so regulating the bioavailability of trace metals [16].Presence of metals, like copper (Cu) or iron (Fe), above certain concentrations can cause metallic stress. In response to metallic stress, the phenolic metabolism increases for recovery and protection from toxic metal injury [17].Copper and iron do not affect the diatom growth and phenolic contents in the same way [18]. Iron majorly affects the photosynthetic characteristics, it is essential for major biogeochemical processes, including photosynthesis. Major part of iron content in phytoplankton is required for electron transfer during photosynthesis. Fe limitation causes the reduction of maximum photosynthesis rate and the increase of photosynthesis efficiency, by reducing the biosynthesis of light-harvesting pigments [13].In high-nutrient areas iron can act as a fertilizer. Copper at low concentrations acts as nutrient, but in higher concentrations it becomes toxic [18]. At the onset of growth seasons, diatoms shouldn't experience nutrient limitations, thus allowing them to rapidly develop in growing phytoplankton and after the depletion of nutrients, other phytoplankton species may take over. But diatoms can adapt to lowering of the nutrients by reducing in size, increasing their surface to volume ratio [13].

Limitation of nutrients and energy increases cell sinking rates. Fe deficiency stresses the energy production pathways, which are needed to maintain cells buoyancy, also the higher silicification of diatoms thickens the siliceous frustules, resulting in increasing sinking rates. So, with the increase of Fe availability to diatoms may result in decrease in sinking rates by increase in energy production and thinner frustules. But some larger diatoms may decrease their buoyancy, for the ability of vertical migration to access the nutrient-enriched waters below the nutricline [13].Besides the phototrophic conditions, where the sunlight is the sole energy source, some diatoms can utilize mixotrophic conditions [19]. Mixotrophy is when algae grow in presence of sunlight (phototrophic growth) and organic substrates as carbon source (heterotrophic growth) [20]. In phototrophic conditions, when algae are dependent on sunlight, cellular self-shading reduces light availability, by that limiting biomass production and decreasing cell harvesting efficiency [20]. Commercial applications of microalgae are phototrophic, typically in large ponds or long photobioreactors, that occupy extensive amounts of space [21]. Introduction of mixotrophic growth, could result in improved use of light and so increased biomass concentration and reduced cost of biomass production [20] Different carbon sources have been explored and one of the most easily utilized by diatoms is glucose. But due to relatively high price of glucose and usage in food production, it makes it not so desirable for commercial applications [22]. One of the potential carbon sources, that could be used, is glycerol [22]. Because of it being one of the main by-products of biodiesel it is commercially available and has a reasonable market value [23].

Although most microalgae are only capable of phototrophic growth and are unable to use organic carbon sources [20]. It is crucial to search for species with this capability, *Phaeodactylum tricornutum* is one of the diatoms that can grow in mixotrophic conditions[19]. Experiments on *Phaeodactylum tricornutum* were conducted in search of carbon sources that would be fit for mixotrophic growth. In 2005 group of researchers performed an experiment using glycerol, fructose, glucose, lactose, mannose as potential carbon sources for mixotrophic growth[24]. Experiment showed that only glycerol significantly increased, 3-fold compared to untreated samples, the growth profile of *Phaeodactylum tricornutum*. Whereas, other carbon sources only showed minor differences [24]. Other experiment was conducted with *Phaeodactylum tricornutum growth* under mixotrophic conditions with fed-batch cultures. Fed-batch culture grown with glycerol reached 3 times higher biomass concentration compared to untreated culture [25]. Another experiment with *Phaeodactylum tricornutum* mixotrophic growth using glycerol as carbon source had different results. Culture was capable of mixotrophic growth using added carbon source, but the increase in growth was smaller, only 31.6 %, when previous experiments resulted in 300% increase in biomass concentration [26]. Capability of mixotrophic growth show potential for biotechnological applications by increasing the biomass productivity [19]. Further investigation and characterization of glycerol uptake mechanism could inspire biotechnological developments, engineer strains with improved biomass productivity [19].

1.2. Bioactive and high-added value compounds in diatoms

Human population growth increases demand for sustainable compound production. Diatoms have attracted increasing attention for their potential industrial application in producing various bioactive and high added value compounds which have a relatively high market value (Table1). They have numerous advantages over usage of plants for bioactive compounds, microalgae have rapid growth rates, doesn't compete for resources with food crops, don't need fresh water or farmable land. Diatoms are rich in lipids (fatty acids, steroids, triglycerides), in pigments (chlorophylls, carotenoids) which are widely applied in pharmaceutical products, food supplements [4]. Diatoms still are underexploited from an engineering perspective [27].

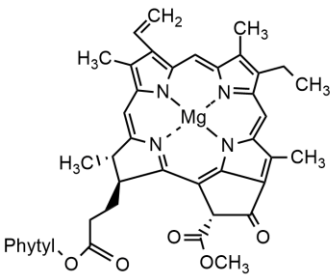
Table 1. High-added value compounds produced from microalgae estimate wholesale market price

High-added value compound	Wholesale market price (US $/kg)	Reference
β-carotene	300-1500	[28]
Astaxanthin	200-7000	[28]
Omega-3 fatty acid oil	80-160	[28]

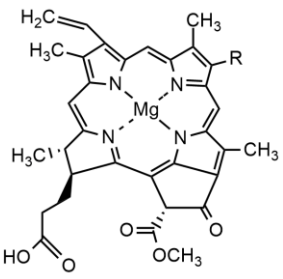
1.2.1. Photosynthetic pigments in diatoms.

Light harvesting or photoprotection in diatoms is done by two types of pigments: chlorophylls and carotenoids. Chlorophylls trap light energy and use it in photosynthesis. More specifically, red and blue portions of the electromagnetic spectrum. Generally, they are magnesium complex of cyclic tetrapyrroles containing a fifth isocyclic ring with a long-chain isoprenoid alcohol ester group (Figure 2). Carotenoids are mainly engaged in photoprotection, but some of them also participates in light harvesting. They consist of carotenes and their oxygenated derivatives, xanthophylls [29].

Photosynthetic organisms contain several types of chlorophylls, but diatoms contain two forms of chlorophylls, it's chlorophyll a and chlorophyll c (Figure 2) [29]. Chl a is the predominant pigment in photochemical energy conversion and Chl c plays a supportive role, maximizing the photosynthetic efficiency. Maximum photosynthetic efficiency is usually obtained with red light. Diatoms are often greatly dispersed in water, so they relatively receive more yellow (500-600 nm) than red (600-690 nm) light, due to absorption by water. Chlorophyll c has a bigger absorption in the 500-600 nm region than chlorophyll a. Because of that chlorophyll c must effectively participate in photosynthesis as an accessory pigment similarly as chlorophyll b in higher plants [30]. Chlorophyll is one of the bioactive compounds that can be extracted from diatoms. It is used as a natural food coloring agent [31]. Chlorophyll is widely used in pharmaceutical products, it increases wound healing by stimulating tissue growth and has antibacterial properties, also used in cancer prevention for its high antioxidant and antimutagenic activity [31].



Chlorophyll a

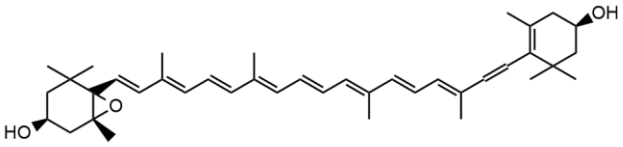


Chrolophyll c_1 R: CH_2CH_3
Chlorophyll c_2 R: $CHCH_2$

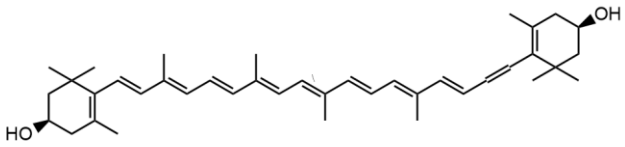
Figure 2. Structural formulas of photosynthetic pigments in diatoms.

Carotenoids are a group of natural pigments whose central structural feature is a linear chain of alternating C-C and C=C bonds. There are about a thousand naturally occurring carotenoids, but only about 50 of them play a role in photosynthesis. In diatoms, they have two main functions: protect against photooxidative damage and serve as light harvesting pigments in photosynthesis. As a part of light-harvesting receivers, carotenoids can absorb light photons and transfer the energy to chlorophyll, in that way widening the harvesting of light in the range of 450-570 nm. As they play an important part in the light-harvesting receiver role, they are also important components of the photosynthetic reaction centers. Additionally, carotenoids protect against photooxidation processes in the photosynthetic apparatus that are caused by the chlorophyll excited triplet state. Carotenoid molecules can absorb the triplet-state energy from chlorophyll, preventing the formation of harmful single-state oxygen radicals[32].

Diadinoxanthin pigment which is a product of a xanthophyll cycle in diatoms may help them to dominate in turbulent waters. Where the amount of light in unicellular organisms is highly unpredictable, constant vertical mixing exposes cells to a short-term high intensity light. During light exposure, the diadinoxanthin cycle converts mono-epoxide diadinoxanthin (Figure 3) into de-epoxide diatoxanthin (Figure 3) and when the light condition changes into lower intensity or into darkness, it converts diatoxanthin back into diadinoxanthin[33]. Diadinoxanthin and diatoxanthin present, similar to other carotenoids, bioactive properties, such as antioxidant, anti-inflammatory, anticancer activity[34]. Diadinoxanthin is one of the major carotenoids produced in *Phaeodactylum tricornutum*[35]. Because of its beneficial activities and successful diatom cultivation in bioreactors, diadinoxanthin and diatoxanthin can potentially be produced from *Phaeodactylum tricornutum*[34]. Efficient extraction of diadinoxanthin and diatoxanthin in an experiment was achieved by extraction medium which consisted of methanol, ammonium acetate and ethyl acetate, because of its low compatibility with chlorophyll a and β-carotene[34].



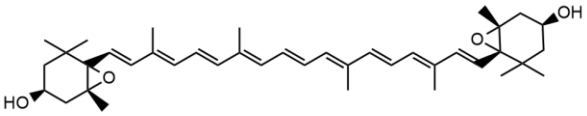
diadinoxanthin



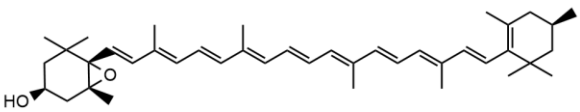
diatoxanthin

Figure 3. Structural formula of diadinoxanthin and diatoxanthin.

Violaxanthin cycle is a similar protective mechanism as diadinoxanthin cycle, in higher plants and green algae it's the man photoprotective mechanism, but in diatoms it's secondary compared to the diadinoxanthin cycle. The violaxanthin cycle is a forward reaction where di-epoxy violaxanthin (Figure 4) is transformed into epoxy free zeaxanthin (Figure 5), while the intermediate product of this reaction is antheraxanthin (Figure 4)., containing one epoxy group[36]. Zeaxanthin most notable biological activity is antioxidant and anti-inflammatory activity, violaxanthin also has anti-inflammatory activity but also present anticancer properties[37].



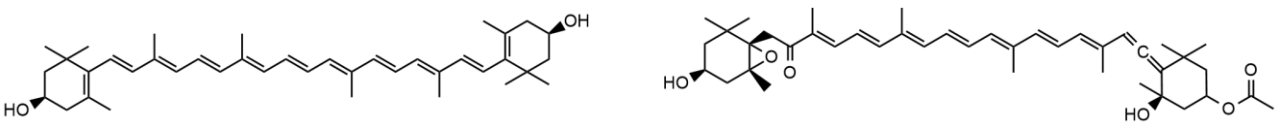
violaxanthin



antheraxanthin

Figure 4. Structural formula of violaxanthin and antheraxanthin.

Fucoxanthin (Figure 5) is one of the most abundant carotenoids, an orange-colored pigment, found long with β-carotene, chlorophylls a and c in diatoms. Diatoms exhibit golden-brown color due to high amounts of fucoxanthin. Because of its many bioactive properties and health benefits it is widely used in pharmaceutical and cosmetic products[38]. Fucoxanthin is shown to have antioxidant, anticancer, anti-inflammatory, anti-obesity, neuroprotective activities, prevents osteoporosis[38]. Fucoxanthin has a unique molecular structure which is similar to peridinin, dinoxanthin and neoxanthin, but different from other carotenoids[39]. Unusual allenoic bond with some oxygenic functional groups contributes to its unique structure[40]. Diatom *Phaeodactylum tricornutum* was identified as a potential fucoxanthin source[41]. Different extraction methods using ethanol shown a consistent fucoxanthin content in produced extracts (15.42-16.51 mg/g freeze dried biomass) as described by Sang Min Kim[41].



zeaxanthin **fuxoxanthin**

Figure 5. Structural formula of zeaxanthin and fucoxanthin.

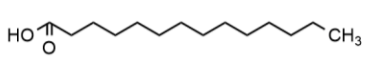
1.2.2. Lipids and fatty acids in diatoms

Diatoms are a great natural source of bioactive compounds with a large variety of lipids (Table 2). On average, lipid content in diatoms can be around 25% - 45% of dry weight, but it can vary depending on the culture and growth conditions.[27]. As marine animals can't produce omega-3 fatty acids by themselves, diatom algae are one of the main polyunsaturated fatty acids source in the marine food chain. Because of diatom lipids significance as edible marine organism (such as mollusk or fish) feed, some microalgae lipid composition investigation started in the early 1960s[42].

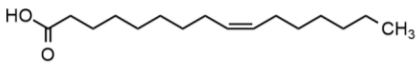
Table 2. Valuable lipids in diatoms

Lipids class	Composition	Representation	Bioactive property	Reference
Fatty acids	Palmitoleic acid	16:3n-3	Anti-bacterial	
	EPA	20:5n-3	Anticancer, antibacterial, anti-inflammatory	
	DHA	22:6n-3	Anti-inflammatory	
	Myristic acid	14:0	Antimicrobial	[27]
TAG	TAG		Biofuels	[43]
Polar lipids	Glycolipids		Antibacterial, anti-inflammatory	
	Phospholipids		Functional foods, cosmetics ingredient	[44]

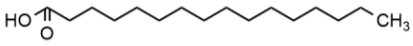
Fatty acids in diatoms vary from C14:0 to C22:6, the most common fatty acids are eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), myristic acid, palmitic acid and palmitoleic acid (Figure 6). Fatty acid profile is similar in many know microalgae, but the fatty acid content varies depending on the strains and culture conditions[43].



Myristic acid (14:0)



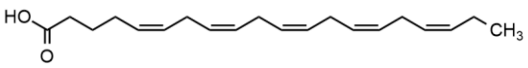
Palmitoleic acid (16:1n-7)



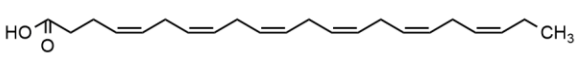
Palmetic acid (16:0)

Figure 6. Common fatty acids in diatoms.

EPA and DHA are the most valuable fatty acids found in diatoms in relatively high concentrations. They belong to the omega-3 fatty acids (Figure 7), alongside linolenic acid. EPA and DHA has been shown to play an important role in prevention or treatment of cardiovascular, inflammatory diseases and been proposed in treatment of various cancers[44]. Myristic acid (Figure 7) exhibits antimicrobial activity[45]. Humans and mammals can synthesize EPA and DHA from linolenic acid, however the conversion efficiency is low, so it necessary to obtain omega-3 acids from dietary sources[27]. Also, linolenic acid can be produced from plant sources, like linseeds contain up to 58% of linolenic acid, however EPA and DHA traditionally is obtained from marine fish oils[44]. Diatoms naturally produce omega-3 polyunsaturated eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), so they could present a sustainable alternative to fish oil for EPA and DHA production[46]. One of the most intensively studied and potential diatom is *Phaeodactylum tricornutum*, because of its relatively easy cultivation and high EPA content, approximately 30% of total fatty acid content[46]. Grown under normal conditions, *Phaeodactylum triconutum* total lipid content can be around 32% of total dry biomass weight[47]. Different growth conditions can increase the fatty acid content in diatoms. Using mixotrophic growth condition, glycerol as a carbon source, for P. *triconutum* increased the fatty acid content, from 7.24% under normal conditions to 16.98% with 0.1M glycerol concentration in the growth medium[48]. Although the eicosapentaenoic acid (EPA) content was only slightly affected by the mixotrophic conditions, increased from 1.90% (Control) to 2.04% (0.1M glycerol concentration)[48].



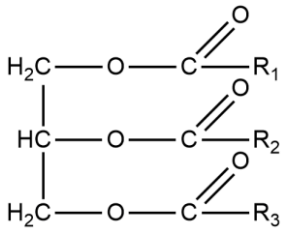
Eicosapentaenoic acid (20:5n-3)



Doxosahexaenoic acid (22:6n-3)

Figure 7. Omega-3 fatty acids.

Microalgae are considered to a potential source of biofuels and biomaterials, especially diatoms, because of their effective accumulation of triacylglycerols(TAGs) (Figure 8)[49]. Comparing to other phytoplanktonic microalgae, diatoms dominate under nutrient-limiting conditions and also because of their greater carbon fixing ability, they grow faster in low light conditions[50]. TAGs vary in diatoms depending on the strain, but myristic acid and palmitoleic acid were found to be the main fatty acids in diatoms[27]. Usage of various environmental stressors were shown to enhance TAG contents[51]. It was shown that under nutrient stress conditions, like silicon limitation or nitrogen deficiency, diatoms start to accumulate TAGs[51]. However, nitrogen deficiency leads to reduced growth rates, but alternatively silicon starvation leads to higher total lipid content in diatoms without the physiological damage[52]. *Phaeodactylum tricornutum* is one of the most widely cultivated diatoms, because of its potential applications. *P. tricornutum* can produce TAG up to 30% of weight[53]. In an experiment were *P. tricornutum* was cultivated under nitrogen starvation conditions, TAG concentration increased from 5% of dry weight to 25% of dry weight[53], in comparison green algae *Scenedesmus obliquus* accumulated 35% of its dry weight as TAGs under nitrogen starvation[54].



TAG

Figure 8. Structural formula of triacylglycerol(TAG), R_1-R_3 represent fatty acid residues

1.3. Extraction and purification of high-value metabolites from diatoms

Microalgae are very interesting for their rich biochemical composition[55], especially diatoms for their high flexibility and easy cultivation[56]. They are recognized as rich raw

materials because they are composed wide selection of bioactive compounds, carotenoids, chlorophylls, polysaccharides, proteins, fatty acids, which over the years were applied in numerous industries, like cosmetics, energy, human food, animal food, pharmaceuticals[55]. Diatom production requires just temperature, nutrients and light to produce larger spectra of high-value bioactive compounds and they can be cultivated all year long[21]. Some of the microalgae cells compounds have a high economical value, but their commercialization is lacking[55]. This is because the extraction and purification processes that are available today have a high cost, are complex, use large amounts of organic solvents, are environmentally harmful or use costly complex equipment which makes it unprofitable[55].

1.3.1. Conventional extraction methods

Organic solvent extraction is a one of the conventional extraction techniques. It is based on a chemical principle "like dissolving like", which is polar solvents dissolve polar solutes and non-polar solvents dissolve non-polar solutes. The organic solvent for the extraction is chosen depending on the target compound that is being extracted[57]. Nonpolar and polar organic solvents are combined for more efficient extraction, as nonpolar solvent interacts with the compounds that are found in cytoplasm and polar organic solvents helps with membrane-associated complexes extraction[57]. For lipid extraction from microalgae solvent mixture chloroform:methanol 1:2 (v/v) is most frequently used, but chloroform is highly toxic, so alternatively hexane: isopropanol 3:2 (v/v) mixture is proposed because of its lower toxicity. Experiment compared chloroform: methanol and hexane:isopropanol mixtures extraction efficiency on marine diatom, *Phaeodactylum tricornutum* [58]. Chloroform:methanol 1:2 (v/v) mixture lipid extraction yield was the highest with 18 g/100g of dry weigh, while extraction with hexane: isopropanol 3:2 (v/v) mixture had 13.8 g/100g of dry weigh yield [58]. For extraction of antioxidant-rich extracts different solvents are used, mainly acetone, ethanol, methanol[59]. In an experiment using tropical marine diatom, *Chaetoceros calcitrans,* most efficient solvents for antioxidant rich extracts were methanol with 22,71 g/100g of dry weight yield and ethanol with 18.06 g/100g of dry weigh yield, in comparison acetone yield was 10.66 g/100g dry weight[59]. Experiment was conducted investigating the best solvent for fucoxanthin extraction from *Phaeodactylum tricornutum*[41]. Five solvents (acetone, ethanol, hexane, water, ethyl acetate) was used at room temperature for 30 minutes, best fucoxanthin extraction was achieved with ethanol (15.71 mg/g dw), where water and hexane were proven ineffective for extracting fucoxanthin [41]. Solvent mixtures can be made for more specific compound extraction, methanol, 0.2 M ammonium acetate and ethyl acetate (81:9:10, v/v) mixture is used for efficient extraction of diadinoxanthin and diatoxanthin from *Phaeodactylum tricornutum* [34].

1.3.2. Sustainable extraction methods

Conventional extraction methods consume significant amounts of energy and uses toxic solvents. Also, conventional solvents can lower the quality of the product by dissolving other compounds during the extraction. Alternatively using sustainable extraction technologies may reduce energy costs, improve eco-friendliness, non-toxicity and efficiency[60].

Pressurized liquid extraction (PLE), also known as accelerated solvent extraction (ASE), is a technique that uses liquid solvents at elevated pressure and temperature[61]. The basic apparatus schematic design is shown in Figure 9 [62]. PLE enhances the extraction yield and decreases time and solvent consumption compared with extractions which are carried out in room temperature and atmospheric pressure. Wide range of temperatures can be used during the extraction, usually from room temperature to 200ºC.[61]

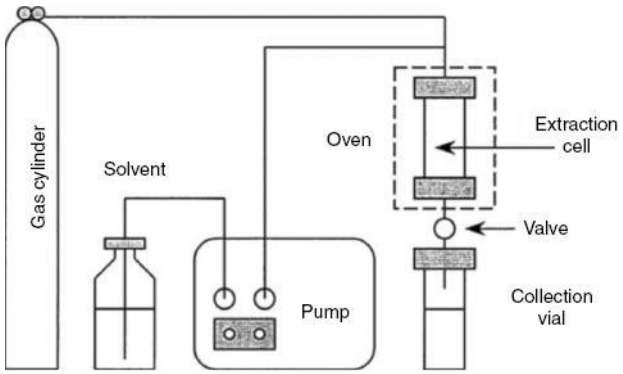


Figure 9. Basic schematic diagram of a pressurized liquid extraction apparatus[62].

When water is used as solvent, PLE is usually referred to as subcritical water extraction (SWE) or pressurized hot water extraction (PHWE)[63]. Due to the elevated temperature and pressure water's physico-chemical properties, dielectric constant changes drastically[63]. At ambient pressure and temperature water is a polar solvent with high dielectric constant but increasing the temperature under moderate pressure water polarity and dielectric constant reduces and the solvent becomes like ethanol or acetone[63]. Depending on the analytes of interest polarity the appropriate solvent is chosen for extraction[61]. PLE can be an alternative for extraction of bioactive compounds from diatoms. For example, Fucoxanthin extraction from diatom *Phaeodactylum tricornutum,* using a conventional solvent extraction method, Soxhlet extraction, ethanol as a solvent for 16h at 40ºC, the extraction yield of fucoxanthin was 0.8%[64] and when using PLE, ethanol as a solvent for 30 min at room temperature, the extraction yield was 1.6%[65]. Optimizing extraction conditions can substantially increase the extraction efficiency. Optimization of bioactive compound, such as phenolic compounds and carotenoids, extraction from *Phaeodactylum tricornutum* showed temperature influence on yield. Using low temperature, 50ºC for 20 min with ethanol, PLE, extract yield was 23.95% and using high temperature conditions,

170°C for 20min with ethanol, extract yield increased significantly, 38.78%[66]. Pressurized liquid extraction can be used for lipid extraction from microalgae using alternative solvents which are food and pharmaceutical industry grade (n-hexane, ethanol). Conducted with microalga *Nannochloropsis oculate* experiment compared extraction yields and total fatty acid profiles using n-hexane and ethanol with PLE. With the same parameters, 60°C and 60 min extraction, extraction yield with ethanol was 36% while with n-hexane only 6.1%. Total fatty acid profile showed that fatty acid content based on extract mass was higher in n-hexane extracts – 70.4%, while in ethanol extracts – 46.9%., however, fatty acid yield based on input mass was higher using ethanol – 16.7%, when using hexane fatty acid yield was 4.3% [67].

Another promising technology in pharmaceutical and food industries is super-critical fluid (SCF) extraction[60]. Compared to organic solvents it has lower extraction time, higher selectivity and non-toxicity[68]. Additionally, it doesn't require separation due to CO_2 evaporation in ambient pressure. CO_2 can be recycled to avoid the greenhouse effect[68]. SFL technology has a high equipment cost, but because of the processes simplicity, the scale up is easy and has gained interest in microalgae extraction[68]. SFC combined with non-toxic polar solvents, like ethanol, produced enhanced extraction yields[69]. Microwave assisted extraction (MAE) is a unique extraction method. It generates heat through molecular interactions by directly delivering microwave energy to the materials[60] which ruptures the cell membrane and extracts intercellular metabolites. Using microwave assisted extraction bypasses microalgae biomass drying reduces extraction and recovery times, reduces solvent usage, increases yield and quality [70], is environmentally friendly, but must be filtrated or centrifuged to remove solid residue[60]. Potentially easy so scale up[60].

1.4. Diatom industrial applications and prospects in biotechnology

Relevance of biotechnological application of diatoms is growing. Diatoms have a high degree of flexibility, can grow virtually in all climate zones, only water, nutrients and light are needed for cultivation[56]. Because of the growing flexibility diatom easily compete with other biological solar-powered factories that are used in biotechnology[56]. Additionally, diatoms can use wastes as nutrients, carbon dioxide from gaseous exhaust, without using fertile soil that can be used for food or crops[56]. Diatoms have been proven to have potential in commercial and industrial applications for pharmaceuticals, fluid fuel production, health foods, biomolecules, contaminated water treatment. Diatom biomass contain components which allow fuel production capabilities: natural oils for biodiesel production, carbohydrates for ethanol production using fermentation, proteins for methane production. In pharmaceutical industry diatoms are used to produce secondary metabolites, which have great antioxidant, anticancer, anti-inflammatory, anti-

obesity, neuroprotective activities, compounds like fucoxanthin, astaxanthin[4]. In food supplement industry omega-3 fatty acids are very important for normal human body health, but traditionally they are obtained from fish oil. Diatoms are alternatively used to produce omega-3 fatty acids, because of their high EPA and DHA content[46], easy cultivation and omega-3 acids obtained from diatoms are vegan[71]. Diatoms can be used for heavy metal detoxification by internal or external metal chelation using phytochelatins[56]. Studies showed that *p. tricornutum* exposed Cd, Pb or Zn start to synthesize phytochelatins as a response[56]. Diatoms in polluted waters could potentially bind and collect metal contaminants, by so reducing the cost of wastewater treatment and even reacquiring raw materials[56]. Diatoms have silica-based exo- and endo-skeletons which makes up majority if their body mass. Diatoms dominate biogenic silica production on earth. Bioactive glasses gather more and more interest for their application as scaffold in bone regeneration[72]. One of the most successful bio glasses is silicate-based, it possesses excellent bioactivity, biodegradability and biocompatibility[72]. Silica for bioactive glass production can be obtain industrially, but in most cases, it uses harsh conditions which alter physiological properties[72]. Potentially diatoms could be biogenic silica source for bioactive glass production, because of the ambient production conditions silica present far superior quality, comparing with other industrial methods[72]. Diatoms can be used for recombinant protein production[73]. They can be cultivated in low cost, have high growth rates, are easy to handle, have high scale up capacity [73]. As genome sequences of microalgae, including diatom *p. tricornutum,* became available, basic genetic tools were established to alter microalgae to secret target proteins into culture medium or express recombinant proteins intracellularly[73]. As many diatoms have no harmful components, target compounds can be extracted from the whole cell[73]. *Phaeodactylum tricornutum* was successfully altered and used to express antibody surface protein[74]. So, diatoms can be a novel low-cost expression form for production of feed supplements, therapeutics[73].

2. MATERIALS AND METHODS

2.1. Materials

2.1.1 Chemicals

2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS•+, Sigma-Aldrich, Steinheim, Germany), 3,4,5-trihydroxybenzoic acid (gallic acid, 99%, Sigma-Aldrich, Steinheim, Germany), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, 97%, Sigma-Aldrich, Steinheim, Germany), Folin & Ciocalteu's phenol reagent ((2M), Fluka Analytical, Bornem, Belgium), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ, Sigma-Aldrich, Steinheim, Germany), NaCl, KCl, KH_2PO_4, $K_2S_2O_8$ (Lach-Ner, Brno, Czech Republic), Na_2HPO_4 (Merck KGaA, Darmstadt, Germany), Na_2CO_3 (Sigma-Aldrich), H_2SO_4, NaOH, H_3PO_4, (Sigma-Aldrich), HCl (35-38%, Chempur, Piekary Slaskie, Poland), acetonitrile, methanol, hexane (HPLC grade, Sigma-Aldrich Chemie, Steinheim, Germany), microcrystalline cellulose (20 μm, Sigma-Aldrich, St. Louis, MO, USA), ASE filters (Glass Fiber_(X)_Cellulose, Dionex Corporation, Sunnyvale, CA,USA), diatomaceous earth (100 % SiO_2, Dionex Corporation, Sunnyvale, CA, USA),), ethanol (96.3%, food grade, Stumbras, Kaunas, Lithuania), nitrogen liquid (AGA SIA, Riga, Latvia), nitrogen gas (99.9%, Gaschema, Jonava region, Lithuania), BIO-ACTIF sea salt (Tropic Marin, Wartenberg, Germany).

2.2. Methods

2.2.1. Diatom growing

Diatom cultures were cultivated in 25 cm^2 or 75 cm^2 culture flasks with plug seal cap. Sterilized culture growth medium was inoculated with a desired diatom culture to reach an initial chlorophyll content of 0.125 mg/mL. Cultures were grown on a growing shelve, temperature at the room was kept at 19 ^oC. Light, adjusted to 1000 lx, was supplied by LED lamps. Photoperiod was set at 16 h of light and 8 h of dark simulating natural light cycle. Cultures were maintained for 7 – 10 days, depending on the experiment conducted.

2.2.1.1. Growth medium preparation

For 1 L of artificial sea water growth medium preparation, 33.5g of *Topic Marine BIO-ACTIF* sea salt was added to 980 ml of distilled water in an autoclavable media bottle and dissolved. After the salt was dissolved, media bottle was autoclaved for 15 20 minutes at a 121^oC. After autoclaving, the media was cooled down to room temperature, then 20 ml of Guillard's (F/2)

Marine Water Enrichment Solution and 1 ml of Na2O3Si*9H2O (30 g/L concentration) was added to the autoclaved artificial sea water, solution was mixed and ready for use.

2.2.1.2. Chlorophyll content determination

2 ml of the homogenized culture was taken, centrifugated for 2 minutes at 1300 rpm, after the centrifuged the supernatant (medium) was removed. To the precipitate (diatom biomass) 100µL of methanol was added and vortexed for 30 seconds, then 900 µL of acetone was added and vortexed for 30 seconds. Solution was centrifuged for 2 minutes at 1300 rpm and samples were collected from the supernatant. In a cuvette, 100 µL of methanol and 900 µL of acetone were added and used as a blanc for the spectrophotometer. Sample absorbance measured at 664nm and 630nm with *Shimadzu UV-1280* spectrophotometer. Chlorophyll content was expressed as mg/L, calculated using following formulas:

$$Chl\ a = \frac{11.47 \cdot A_{664} - 0.4 \cdot A_{630}}{n} \quad (1)$$

$$Chl\ c = \frac{24.36 \cdot A_{630} - 3.7 \cdot A_{664}}{n} \quad (2)$$

$$Total\ Chl = Chl\ a + Chl\ c \quad (3)$$

here: Chl a – chlorophyll a content, mg/L, Chl c – chlorophyll c content, mg/L, A_{664} – absorbance measured at 664nm, A_{630} – absorbance measured at 630nm, Total Chl – chlorophyll content, mg/L.

2.2.1.3. Cell density calculation

Glass hemocytometer and the coverslip was cleaned with alcohol and dried before each use. Culture flask was swirled to ensure even cell distribution. Using a pipette, 100 µL of cell suspension was taken and applied to the hemocytometer to fill the chamber underneath the coverslip. Using a microscope with a 10x objective cells were counted in a 1 corner set of 16 corner squares, this was done to all 4 sets of 16 corners. To calculate the number of cells/ml, an average cell count from each set of 16 corner squares were taken, multiplied by 10^4 and if needed multiplied by the dilution for correction (if the dilution was made).

2.2.1.4. *Phaeodactylum tricornutum* correlation between chlorophyll concentration and cell count.

High cell density culture of *Phaeodactylum tricornutum* was grown. Chlorophyll concentration was measured. 9 different dilution samples were made, to have 9 different chlorophyll concentration cultures of *Phaeodactylum tricornutum,* from 0.125 mg/mL to 5.18 mg/mL. All diluted samples were measured with hemocytometer to determine cell density. Values were calculated and added to figure to see the correlation between chlorophyll concentration and

cell density calculated with hemocytometer. Coefficient of determination R^2 from the correlation curve (Figure 10) indicates that there is a linear correlation between chlorophyll concentration and cell density.

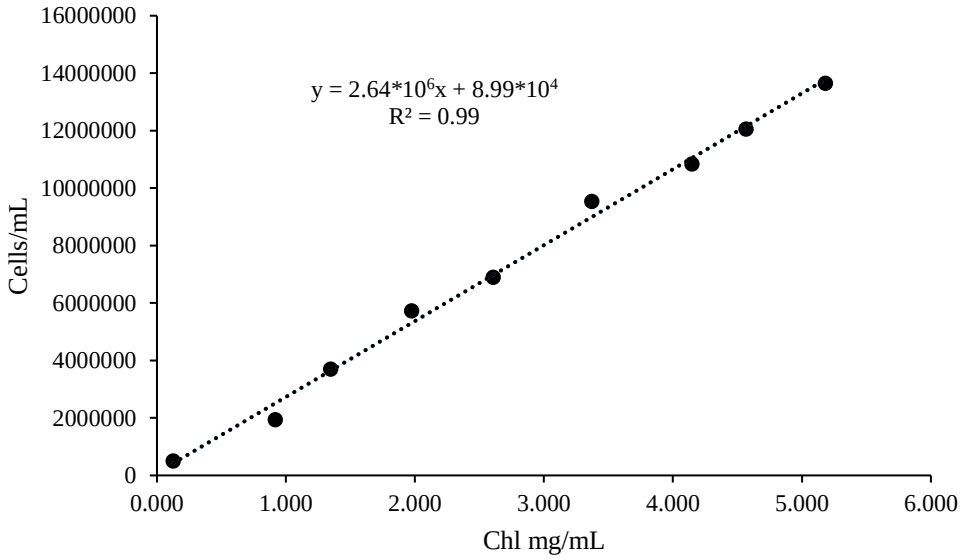


Figure 10. *Phaeodactylum tricornutum* 1052/1A curve that indicates linear correlation between chlorophyll concentration and cell count.

2.2.1.5. Biomass freeze-drying

Culture medium from culture flask was added to a centrifuge tube and centrifugated with *Hettich Universal 320 R* centrifuge at 9000 rpm for 9 minutes, then the supernatant (medium) was removed. Collected culture was frozen using liquid nitrogen. After the culture was frozen it was freeze-dried using a *VirTis BenchTop 2K Benchtop Freeze Dryer* for 24-36 hours at 4 Pa pressure. Dry biomass was collected and stored in a freezer for further experiments.

2.2.2. Antioxidant activity measurements of biomass and extracts

Antioxidant activity of dry biomass was evaluated by Folin-Ciocalteu's. ABTS+, FRAP assays. Solid dilutions for analysis were prepared with dry biomass and microcrystalline at concentrations of 50µg/mg to 400 µg/mg.

Antioxidant activity of extracts was evaluated by Folin-Ciocalteu's, ABTS+ assays. Various extracts were dissolved in acetone=methanol mixture (1:9, v/v) and further diluted with methanol to final concertation from 0.5mg/ml to 5mg/ml.

2.2.2.1. Total phenolic content (TPC) by Folin-Ciocalteu's assay

For biomass antioxidant activity assessment, 10mg of sample (400 µg/mg.) or cellulose (blank) were mixed with 150 µL of distilled H_2O, 750 µL of Folin-Ciocalteu's reagent (diluted with distilled water (1:9, v/v)) and 600 µL of Na_2CO_3 solution (75 g/L), vortexed for 15s, shaken in the dark for 2 hours, centrifugated at 4500 rpm for 5 min and the absorbance of optically clear supernatant was measured at 760 nm with *Shimadzu UV-1280* spectrophotometer. When using extracts instead of biomass, 150 µL of extract sample (0.5mg/ml) were mixed with 750 µL of Folin-Ciocalteu's reagent (diluted with distilled water (1:9, v/v)) and 600 µL of Na_2CO_3 solution (75 g/L), vortexed for 15s, shaken in the dark for 2 hours, centrifugated at 4500 rpm for 5 min and the absorbance of optically clear supernatant was measured at 760 nm with *Shimadzu UV-1280* spectrophotometer. For calibration, gallic acid solutions (150 µL) at various concentrations (0-80 µL) were used. Total phenolic content of dry biomass and extracts was express as gallic acid equivalents (mg GAE/g dry biomass, mg GAE/g extract) by means of dose-response curves for gallic acid. Calibration curve:

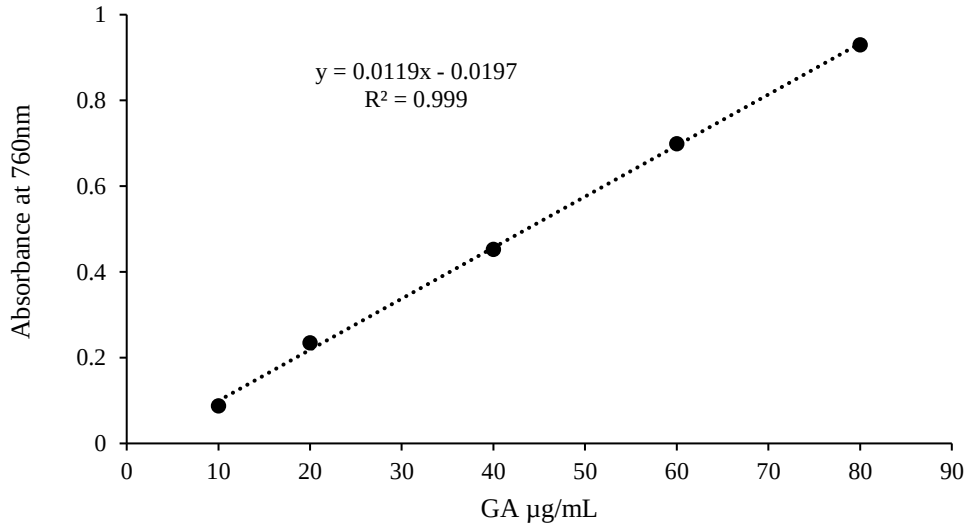


Figure 11. Total phenolic content (TPC) assay calibration curve with gallic acid (GA).

2.2.2.2. The $ABTS^+$ scavenging assay.

Phosphate buffered saline (PBS) solution (75mmol/L; pH 7.4) was prepared by dissolving 8.18g NaCl, 0.27g KH_2PO_4, 1.42g Na_2HPO_4 and 0.15g KCl in 1 L of distilled water. The ABTS+ solution was prepared by mixing 50 ml of ABTS (2mmol/L in PBS) with 200 µL $K_2S_2O_8$ and letting the mixture to stay in the dark at room temperature for 15-16 before use. Working solution was prepared by diluting the $ABTS^+$ solution with PBS to obtain absorbance of 0.700 ±0.01 at 734 nm. Using the dry biomass, 10mg of sample (40 µg/mg) or cellulose (blank) were mixed with 25

µL of MeOH and 1500 µL of ABTS+ solution, vortexed for 15s, shaken in the dark for 2 hours, centrifugated at 4500 rpm for 5 min and the absorbance of optically clear supernatant was measured at 734 nm *Shimadzu UV-1280* spectrophotometer. For the extracts, 25 µL of sample (5 mg/ml) was mixed with 1500 µL of ABTS+ solution, vortexed for 15s, shaken in the dark for 2 hours, centrifugated at 4500 rpm for 5 min and the absorbance of optically clear supernatant was measured at 734 nm *Shimadzu UV-1280* spectrophotometer. Trolox solutions (25 µL) at various concentrations (0-1500 µmol/L in MeOH) were used for calibration. $TEAC_{ABTS}$ of dry biomass and extracts were calculated by means of dose-response curves for Trolox. Calibration curve:

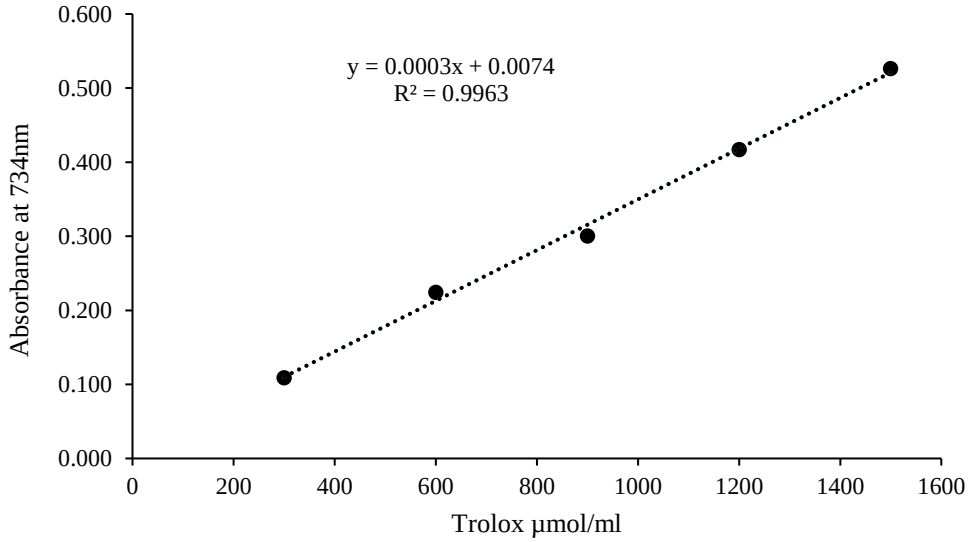


Figure 12. ABTS+ scavenging assay calibration curve with Trolox.

2.2.2.3. Ferric reducing antioxidant power (FRAP) assay

FRAP reagent was prepared by mixing 10 mmol/L 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) (in 40 mmol/L HCL solution), 20 mmol/L $FeCl_3*_6H_2O$ and 300 mmol/L sodium acetate buffer solution (pH 3.6), in ratio 1:1:10. 10 mg of sample (40 µg/mg) or cellulose (blank) were mixed with 50 µL of MeOH, 150 µL of distilled water and 150 µL of fresh FRAP reagent, vortexed for 15s, shaken in the dark for 2 hours, centrifugated at 4500 rpm for 5 min and the absorbance of optically clear supernatant was measured at 734 nm *Shimadzu UV-1280* spectrophotometer. Trolox solutions (50 µL) at various concentrations (0-1500 µmol/L in MeOH) were used for calibration. $TEAC_{ABTS}$ of dry biomass and extracts were calculated by means of dose-response curves for Trolox. Calibration curve:

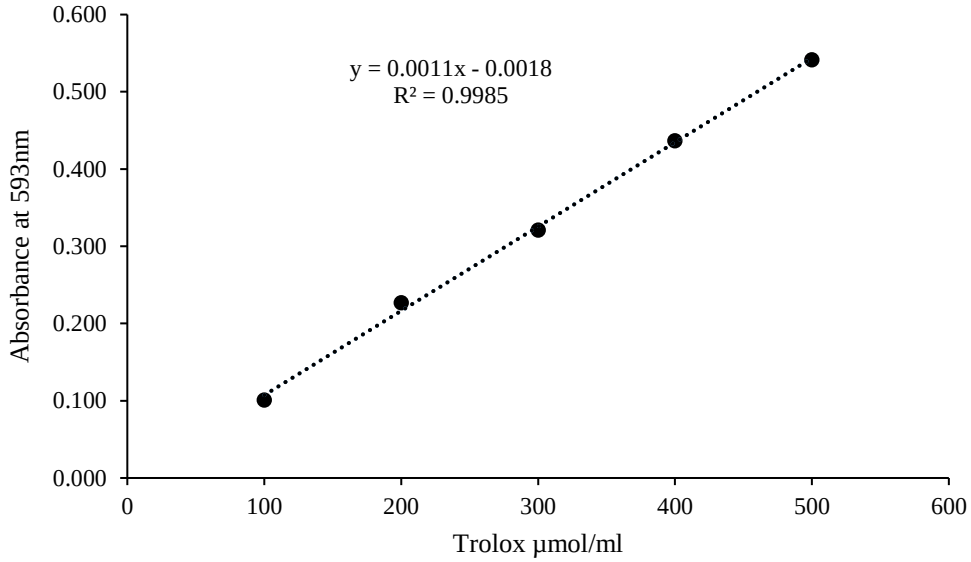


Figure 13. Ferric reducing antioxidant power (FRAP) assay calibration curve with Trolox.

2.2.3. Pressurized-liquid extraction (PLE)

Response surface methodology (RSM) and central composite design (CCD) were utilized for the experimental design setup of PLE. Two independent variables and their variation were chosen: for hexane, acetone and ethanol temperature was 40-120 ^{o}C and 15-60 minutes of extraction time, for water temperature was 120-140 ^{o}C and 15-60 minutes of extraction time. The response factors (RF) were total yield of PLE extract, TPC and $ABTS^{+}$ assays for acetone and ethanol, total yield of PLE extract, EPA and DHA content for hexane. The number of experiments was defined, based on the equation:

$$N = 2^{f} + 2f + c \qquad (4)$$

here: f - the number of factors; c – the number of center points.

Complete design consisted of 13 experimental runs for each solvent with 4 factorial points, 4 axial points and 4 center points (Table 3) was established using the software *Stat–Ease Inc Design-Expert 7.0.0.* The multiple regression equation was used to fit the second-order polynomial equation, expressing predicted response as a function of independent variables:

$$Y = \beta 0 + \sum_{i=1}^{3} \beta iXi + \sum_{i=1}^{3} \beta iiXi^{2} + \sum_{i=1}^{3} \sum_{j>1}^{3} \beta ijXiXj \qquad (5)$$

here: Y – the predicted response; β_0 – a constant; β_i, β_{ii} and β_{ij} – the coefficients for linearity; X_i and X_j – independent variables.

All extractions were performed in duplicate and in random order. Results were analyzed using *Stat–Ease Inc Design-Expert 7.0.0.* Statistical significance of the model and each variable was determined using the Student test (*p*-value) at 5% probability level ($p<0.05$). The adequacy of the model was determined by evaluating the '*lack of fit*' coefficient and the Fisher test value (*F*-value) obtained from analysis of variance.

Table 3. Levels of independent variables for PLE parameter optimization

Solvent	**Experimental factors**	**Variable levels**		
		-1	0	+1
Hexane	Extraction temperature (T,^{o}C)	40	80	120
	Extraction time (τ, min)	15	39	60
		(5min x 3 cycles)	(13min x 3 cycles)	(20min x 3 cycles)
Acetone	Extraction temperature (T,^{o}C)	40	80	120
	Extraction time (τ, min)	15	39	60
		(5min x 3 cycles)	(13min x 3 cycles)	(20min x 3 cycles)
Ethanol	Extraction temperature (T,^{o}C)	40	80	120
	Extraction time (τ, min)	15	39	60
		(5min x 3 cycles)	(13min x 3 cycles)	(20min x 3 cycles)

PLE was performed in *Thermo Scientific Dionex ASE 350 Accelerated Solvent Extractor* apparatus. 2±0.001 g of dry *Phaeodactylum tricornutum* biomass was mixed with 2 g of diatomaceous earth (1/1, w/w) and placed to 10 ml stainless steel extraction cells with two cellulose filters in both ends. Extractions were conducted with hexane, acetone and multistep extractions were conducted with ethanol, water. The system pressure (103 bar or 10.3 MPa), pre-heating time (5 min), cell flush volume (100%) and purge time (120 s) with nitrogen to collect the extracts in the vials was kept constant for all PLE experiments. Organic solvents were evaporated with rotary evaporator at different pressure at 45°C by *BUCHI Labotechnic Rotavapor R-210*. H_2O extracts were additionally freeze-dried to remove residual water. The yields of extracts were determined gravimetrically (±0.001 g), extracts were kept in brown glass bottles in the freezer prior to the analysis. The solid residues were collected, dried (50°C) in and kept in a dry, well-ventilated place prior to the analysis. All extractions were performed in duplicates.

2.2.4. Fatty acid methyl ester (FAME) analysis.

One step derivatization-extraction was done. For dry biomass, in tubes 10mg of dry biomass and 700 µL of 2.5% H_2SO_4 solution (in MeOH) was added. Tubes were sealed, vortexed for 15s, kept in a water bath at 80 ^oC for 90 minutes., then removed and left to cool down to room temperature. 300 µL of hexane and 300 µL of 0.25% NaCl aqueous solution was added. Samples were vortexed for 15 s and then centrifuged at 4500 rpm for 5 min. 200 µL of the hexane fraction was removed, dried with Na_2SO_4, stored and then analyzed with GC-FID.

For extracts, 10mg of extract and 700 µL of 2.5% H_2SO_4 solution (in MeOH) was added. Tubes were sealed, vortexed for 15s, kept in a water bath at 80 ^oC for 90 minutes., then removed and left to cool down to room temperature. 300 µL of hexane and 300 µL of 0.25% NaCl aqueous solution was added. Samples were vortexed for 15 s and then centrifuged at 4500 rpm for 5 min. 200 µL of the hexane fraction was removed, dried with Na_2SO_4, stored and then analyzed with GC-FID, for analysis, extract samples were diluted, 20 µL of hexane phase was diluted with 180 µL of GC-grade hexane.

Analysis was carried out with gas chromatograph *Carlo Erba HRGC 5300 Mega Series* using a flame ionization detector with a pole *SPTM-2560* column (100 m long, 0,25 mm internal diameter the adsorbent layer of 0,20 µm (*Supelco, Bellefonte, PA, USA*) Oven temperature was programmed from 80°C to 240°C and increasing every 4°C/min. Injector temperature – 220°C and detector – 240°C. Injected amount of sample – 1µl. For compounds identification, a *Supelco*^{TM} mixture of 37 fatty acids, were used as standards. Fatty acid methyl esters were identified by the retention time and the percentage of fatty acid composition was calculated comparing peak areas to the corresponding compounds from standard.

3. RESULTS AND DISCUSSION

3.1. *Phaeodactylum tricornutum* under metallic stress

Heavy metals are frequent pollutants found in aquatic environments. Heavy metals can be divided into essential and non-essential for diatom growth. Essential metals can be toxic at high concentrations, having influence on physiological and biochemical processes. Copper and iron are essential nutrients for diatoms, but in high concentrations they are toxic. When diatoms are growing under copper or iron stress they tend to produce more phenolic compounds to protect themselves against heavy metal toxicity.

Cultures of *Phaeodactylum tricornutum* 1052/1A were produced with 5 different metal concentrations. As a reference growth, 1052/1A was grown in seawater with f/2 medium with no added metals (Figure 14). At reference culture conditions the cell density increased from $2.38*10^5$ cells/mL to $4.26*10^5 \pm 1.19*10^4$ cells/mL, culture reached maximum concentration of $4.89*10^5 \pm 1.03*10^4$ cells/mL at day 4, decline phase started from day 4. Growth of *Phaeodactylum tricornutum* was also carried out in the presence of 30mM of Cu (II) (Figure 4.1). Cell density increased only very slightly, from $2.38*10^5$ cells/mL to $2.66*10^5 \pm 1.66*10^4$ cells/ml, lag phase of the cells increased heavily compared to the control sample, lasted for 5 days and after that the growth rate was very small compared to the control sample. Another concentration of Cu was also tested, 60mM of Cu (II) (Figure 14). Growth of *Phaeodactylum tricornutum* was very inconsistent under this concentration of Cu, dropped below the starting cell density from $2.38*10^5$ cells/mL to $1.89*10^5 \pm 1.36*10^4$ cells/mL at day 2, then reached the starting cell density at day 4, then dropped again. In the end, culture, under 60mM of Cu (II), cell density dropped to $2.13*10^5 \pm 2.21*10^4$ cell/mL.

Two different Fe(II) concentration culture mediums were prepared, 50mM and 100mM of Fe(II) (Figure 14). With both concentrations of Fe(II) *Phaeodactylum tricornutum* cell density dropped down for 3 days, from $2.38*10^5$ cells/mL to $1.59*10^5 \pm 1.31*10^4$ cell/mL with 50mM of Fe(II) and $9.58*10^4 \pm 2.26*10^4$ cells/mL with 100mM of Fe(II). After 3 days of decreasing cell density, cultures started to recover and started to grow, but the growth rate was minimal and in the end cultures with both Fe(II) concentrations didn't reach the starting cell density, *Phaeodactylum tricornutum* with 50mM of Fe(II) had $1.95*10^5 \pm 6.12*10^3$ cells/mL and $1.54*10^5 \pm 1.18*10^3$ cells/mL with 100mM of Fe(II).

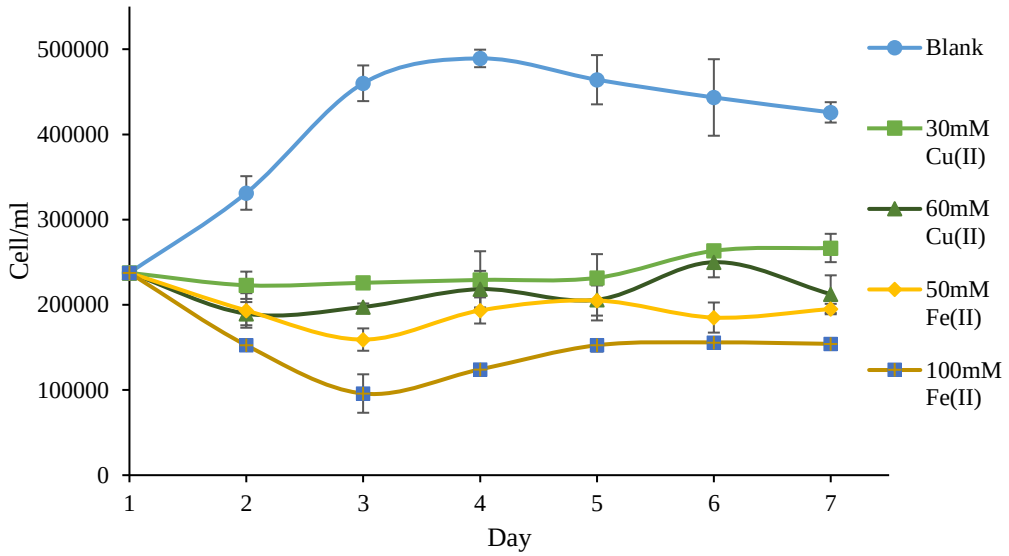


Figure 14. Diatom *Phaeodactylum tricornutum* 1052/1A growth with high concentrations of Cu and Fe metals. Metal concentrations: 1 – blank control, 2 - 30 mM Cu(II), 3 - 60 mM Cu(II), 4 - 50 mM Fe(II), 5 - 100 mM Fe(II).

Very high concentrations of copper and iron had a severe effect on growth of *Phaeodactylum tricornutum.* Heavy metal toxic effect stopped the growth of the cultures and the concentrations of heavy metals were too high for *Phaeodactylum tricornutum* to produce enough antioxidant to cope with the toxicity. Only the culture with the lowest concentration of copper didn't decrease in cell density, the lag phase was extended to 5days and after that the slow growth began. Chosen copper and iron concentrations were too high, because of that cultures growth were too low to produce enough biomass to test the effects of high concentrations of heavy metals on *Phaeodactylum tricornutum.*

Previously chosen heavy metal concentrations were too high for *Phaeodactylum tricornutum* to grow, another experiment with significantly lower, but still relatively high, concentrations of copper and iron was conducted. Cultures of *Phaeodactylum tricornutum* 1052/1A were produced with 3 different metal concentrations, all cultures were produced in biological triplets.. To compare the effects of heavy metals, *Phaeodactylum tricornutum* was produced in seawater with f/2 as a reference growth (Figure 15). At these conditions cell density increased from $4{,}99*10^5$ cells/mL to $2.65*10^6 \pm 0.2*10^6$ cells/mL after 7 days of cultivation. Maximum cell density was reached at day 6, $2.85*10^6 \pm 0.19*10^6$ cells/mL, after 6 days the decline phase started.

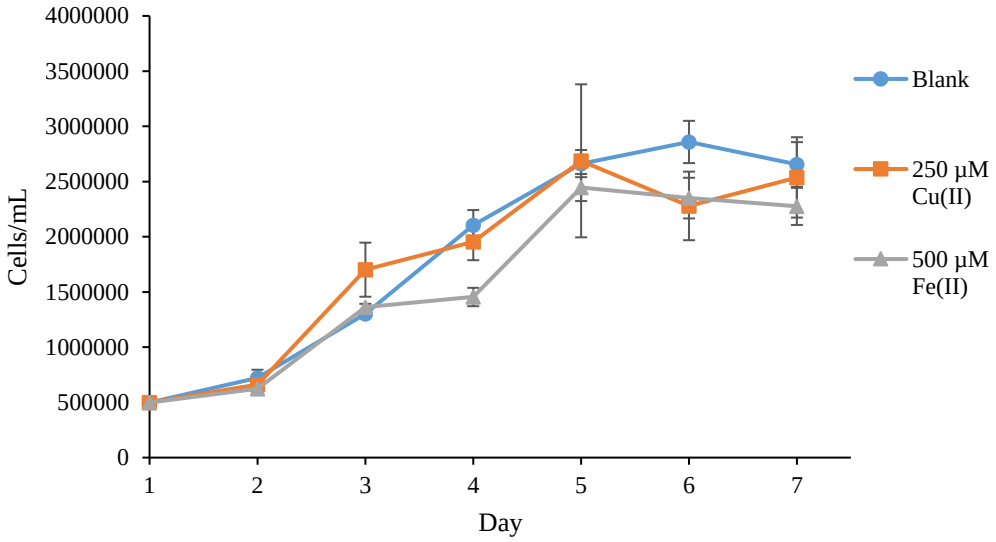


Figure 15. Diatom *Phaeodactylum tricornutum* 1052/1A growth exposed to copper and iron. Metal concentrations: 1 – blank control, 2 - 250 µM Cu(II), 3 - 500 µM Fe(II).

Other culture of *Phaeodactylum tricornutum* 1052/1A was carried out under conditions of 250 µM of Cu(II) (Figure 15). Cell density increased from $4{,}99*10^5$ cells/mL to $2.53*10^6 \pm 0.36*10^6$ cells/mL, cell density was very similar to the reference culture, counting in the standard deviation. Although the lag phase was the same compared to the reference culture, but the exponential growth from day 2 to day 3, was significantly higher with copper. *Phaeodactylum tricornutum* reference culture from day 2 to day 3 grew by $0.58*10^6$ cells/mL and culture with copper by $1.04*10^6$ cells/mL, almost 80% higher growth for the culture with copper. During the 7-day cultivation, culture with Cu(II) grew inconsistently with increases and decreases in growth rate, also from 5th cultivation day cell density decreased from $2.68*10^6 \pm 0.69*10^6$ cells/mL to $2.28*10^6 \pm 0.31*10^6$ cells/mL but then recovered during the next day.

Third culture of *Phaeodactylum tricornutum* 1052/1A was cultivated with 500µM of Fe(II) (Figure 15). Under these conditions, cell density after 7 days of cultivation reached $2.28*10^6 \pm 0.17*10^6$ cells/mL, lowest concentration compared to reference and copper cultures. Just like with the copper culture, *Phaeodactylum tricornutum* culture with iron growth turned static from day 3 to day 4 and then jumped back to exponential growth for 1 day from day 4 to day 5. Maximum cell density was reached after 5 days, $2.45*10^6 \pm 0.12*10^6$ cells/mL, and then slow decline phase started till the end of cultivation.

Copper and iron affected growth of *Phaeodactylum tricornutum*, but the effect wasn't the same. *Phaeodactylum tricornutum* under copper stress grew inconsistently comparing to the reference culture but reached almost the same cell density. Looking at the result it might be that culture was capable of producing exudates to decrease the copper toxic effect. Rico et al. (2013) found that *Phaeodactylum tricornutum* cell density with 315nM Cu(II) concentration decreased

by 20% and with 790 nM (Cu(II) concentration decreased by 47.5% compared to the blank culture [75]. When in this study *Phaeodactylum tricornutum* cell density with 250 µM Cu(II) concentration only decreased by 5 % compared to the blank culture. In another experiment *Phaeodactylum tricornutum* cell density with 1.57 µM Cu(II) concentration was 50% lower than control culture and with 0.78 µM Cu(II) cell density didn't decreased significantly compared to the control culture [76]. *Phaeodactylum tricornutum* under iron stress had the lowest cell density and grew inconsistently compared to the reference culture. Cell density with 500µM Fe(II) concentration decreased by 16 % compared to the reference culture. Rico et al. (2013) found that *Phaeodactylum tricornutum* cell density increased very significantly when cultivated with 900 nM Fe(III) added to the culture medium, cell density increased by 471% compared to the reference culture. This concentration didn't have a toxic effect on *Phaeodactylum tricornutum* [75]. When in this study chosen Fe(II) concentration of 500µM, exhibited Fe-stress toxic effect on culture growth. Culture was able to cope with toxicity of high concentration of iron, but not as well as with the high concentration of copper. To test if *Phaeodactylum tricornutum* produced phenolic compounds in response to heavy metal toxicity, cultures biomass was collected, freeze-dried and different antioxidant assays were performed.

To test the differences in *Phaeodactylum tricornutum* phenolic compound production under heavy metal stress three different antioxidant assays were performed. Ferric reducing antioxidant power, ABTS+ scavenging and total phenolic content assays were chosen (Figure 16). Ferric reducing antioxidant power assay is based on ferric- tripyridyltriazine complex reduction to ferrous form which produces blue color with absorption at 593 nm, color development indicate that antioxidant is present. Trolox is used as a standard for FRAP assay and results are presented as Trolox equivalent. Testing *Phaeodactylum tricornutum* that was grown with heavy metals (Figure 16) FRAP assay showed differences in antioxidant activity between the reference culture and cultures with copper and iron (Figure 16). Culture exposed to copper had the highest antioxidant activity - 7.395 ± 0.518 mM Trolox/g biomass, which was more than 10 times higher than the reference culture 0.675 ± 0.066 mM Trolox/g biomass. *Phaeodactylum tricornutum* exposed to iron had also a big increase in antioxidant activity, 5.646 ± 0.511 mM Trolox/g biomass, more than 8 times higher than the reference culture.

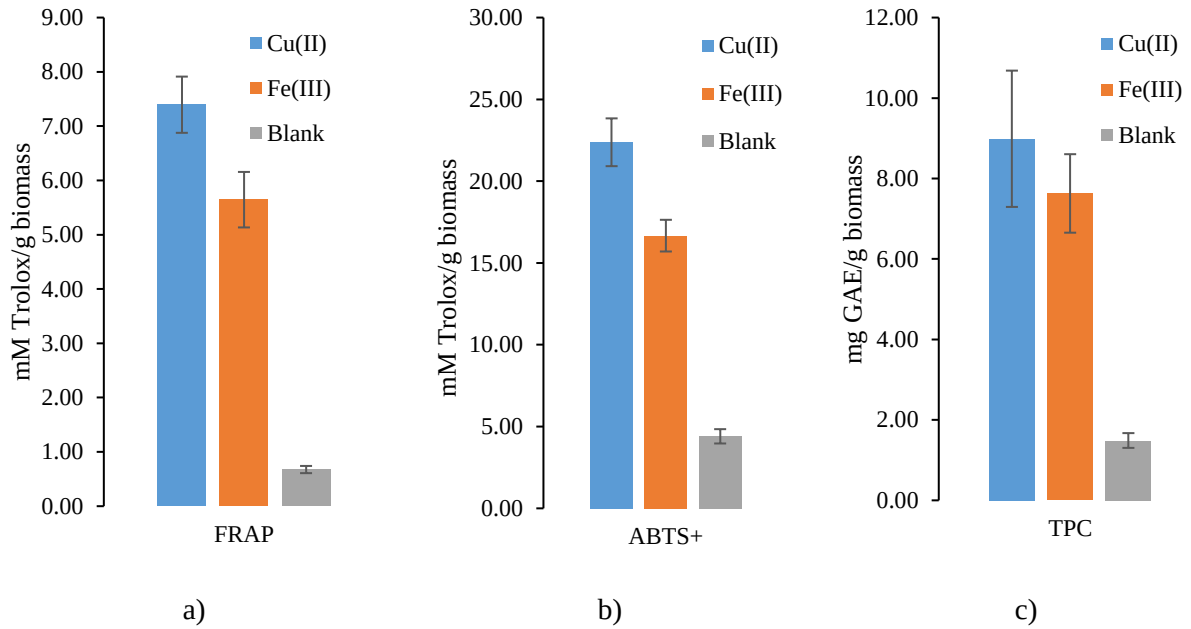


Figure 16. Diatom *Phaeodactylum tricornutum* 1052/1A biomass grown with low concentrations of Cu and Fe metals ferric reducing antioxidant power (a), ABTS+ scavenging (b), total phenolic content (c) assays. Metal concentrations analyzed: 250 µM Cu(II), 500 µM Fe(II).

ABTS scavenging assay is based on ABTS radical conversion from blue color radical, which absorbs light at 734 nm, to its colorless form. Trolox is used as a standard for ABTS assay, antioxidant activity is expressed as Trolox equivalent antioxidant capacity. ABTS assay was used to determine the antioxidant capacity changes in *Phaeodactylum tricornutum* that was grown with high concentrations of heavy metals (Figure 16). Highest antioxidant capacity was observed with copper culture, it reached 22.378 ± 1.462 mM Trolox/g biomass, which was more than 5 times higher than the blank reference culture (4.403 ± 0.437 mM Trolox/g biomass). *P tricornutum* cultivated with iron antioxidant activity was 16.669 ± 0.986 mM Trolox/g biomass, smaller than the cultures with copper but almost 4 times higher than the reference culture. Like FRAP assay, ABTS assay showed antioxidant activity increases in the same samples.

Total phenolic content is based on Folin-Ciocalteu's method, Folin-Ciocalteu's reagent reacts with phenolic compounds. Total phenolic content of *Phaeodactylum tricornutum* grown with heavy metals were measured using the Folin-Ciocalteu's method (Figure 16). Culture grown with Cu(II) showed the highest phenolic content 8.987 ± 1.694 mg GA/g biomass which was 6 times higher than the phenolic content of the blank reference culture 1.488 ± 0.183 mg GA/g biomass. *P tricornutum* cultivated with Fe(II) phenolic content was 7.629 ± 0.976 mg GA/g biomass, which increased 5 times compared to the blank reference culture.

All the assays showed an increase in antioxidant activity with cultures exposed to heavy metals. Copper had biggest impact, in all three assays culture grown with 250 µM Cu(II) had the highest antioxidant activity 4-10 times increase compared to the reference culture. *Phaeodactylum tricornutum* has defensive mechanism when exposed to high concentrations of copper, increases

phenolic compound production to slow down copper toxicity. Similar experiment with *Phaeodactylum tricornutum* and copper stress was conducted by a group of researchers, they cultivated *Phaeodactylum tricornutum* with 790 nM Cu(II), phenolic compound concentration increased 2.4 times compared to the control culture [75].When in this study, *Phaeodactylum tricornutum* cultivated with 250 μM Cu(II), phenolic content increased 6 times compared to the control culture. During the experiment the defense mechanism showed to be effective because cell density of culture grown with copper was almost the same as the cell density of the reference culture (Figure 15). *Phaeodactylum tricornutum* response to high concentrations of Fe(II) was similar. High concentrations of iron affected the growth of diatom (Figure 15), in the end of cultivation cell density of the iron culture was about 15% lower compared to the reference and copper cultures. Also, in all the assays antioxidant activity of the iron culture was lower than the copper culture. *Phaeodactylum tricornutum* increased the phenolic compound production as a response to iron to lower the toxicity, but the cell mechanism wasn't as effective as with treating copper toxicity.

3.2. Mixotrophic conditions

To test the ability of *Phaeodactylum tricornutum* 1052/1A to grow under mixotrophic conditions, culture mediums with different glycerol concentrations were prepared. For a reference medium without glycerol were prepared and two different glycerol concentrations mediums were prepared with 0.01M and 0.1M glycerol concentration. Also, to see if *Phaeodactylum tricornutum* 1052/1A is capable of autotrophic growth, 0.02M glycerol concentration medium was prepared. All cultures were grown in biological triplets for 10 days. *Phaeodactylum tricornutum* culture using reference medium cell density increased from $4.2*10^5$ cells/mL to $2.94*10^6 \pm 0.29*10^6$ cells/mL, maximum cell density was reached during 7 day of growth, $3.13*10^6 \pm 0.35*10^6$ cells/mL, after 7 days of cultivation decline phase started due to nutrient limitation (Figure 17).

Other culture of *Phaeodactylum tricornutum* was cultivated using 0.01M glycerol concentration medium. Cell density increased from $4.2*10^5$ cells/mL to $5.9*10^6 \pm 0.74*10^6$ cells/mL, 2 times higher cell density than the reference culture (Figure 17). During the first 4 days the culture growth with glycerol enricher medium was the same as the culture with no added glycerol. From day 5 cell growth increased comparing to the reference culture, cell growth continued and lasted for all 10 days, there was no decline phase and the maximum cell density was reached during the last day of cultivation.

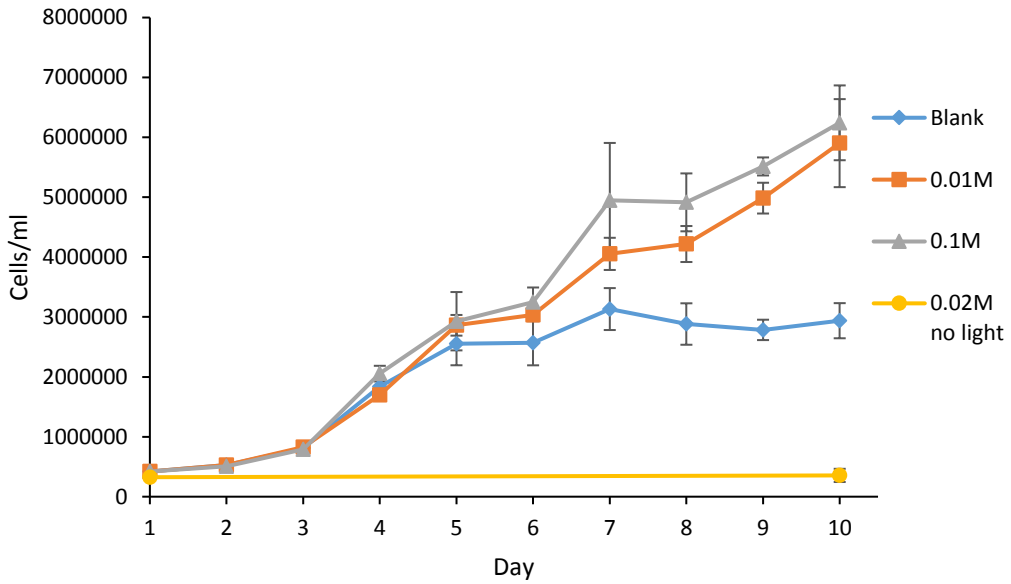


Figure 17. Diatom *Phaeodactylum tricornutum* 1052/1A growth under mixotrophic conditions using different glycerol concentrations (1, 2, 3) and growth under heterotrophic conditions (4). Glycerol concentrations used: 0.01M glycerol, 0.1M glycerol and 0.02M glycerol with no light.

Third culture of *Phaeodactylum tricornutum* was cultivated using 0.1M glycerol concentration medium. Cell density increased from $4.2*10^5$ cells/mL to $6.24*10^6 \pm 0.63*10^6$ cells/mL, 2.12 times higher cell density than the reference culture and 12% higher cell density than culture grown with 0.01M glycerol concentration (Figure 17). Cell growth was similar with 0.01M

glycerol concentration culture, first 6 days culture growth was the same, but starting day 7 cell growth slightly increased compared to the 0.01M. There was no decline phase and the maximum cell density was reached during the last day of cultivation.

The last culture of *Phaeodactylum tricornutum* was cultivated with 0.02M glycerol concentration enriched medium and with no light conditions to test heterotrophic growth ability. After 10 days of cultivation with no light source there was no significant increase in cell density (Figure 17).

Growth experiment with added glycerol to the medium showed that diatom *Phaeodactylum tricornutum* 1052/1A is capable to growth under mixotrophic conditions effectively consuming glycerol as a carbon source. Ending cell density with both glycerol concentrations was more than 2 times higher than the reference culture, but the difference between 0.1M and 0.01M was 11%, 0.1M glycerol concertation culturing having the higher cell density (Figure 17). Similar experiment was conducted by a group of researchers using *Phaeodactylum tricornutum* UTEX-640 culture [48]. They also used 0.01M and 0.1M glycerol concertation enriched medium and cultivation was carried out for 11 days. Similar results were obtained, there as an increase biomass concentration in cultures with added glycerol, culture with 0.01M glycerol concentration had a 1.4 times increased biomass concentration and culture with 0.1M glycerol concentration had a 1.7 times increased biomass concentration compared to the blank reference culture[48]. Another experiment conducted by a different group of researches using *Phaeodactylum tricornutum* and 0.1M glycerol concentration also achieved similar results, culture biomass concentration with added glycerol increased 1.6 times compared to the reference culture [77]. Other group of researchers also reached similar results, *Phaeodactylum tricornutum* was cultivated with 0.05M glycerol concentration medium, after 10 days of cultivation culture with added glycerol had a 2-fold cell density increase compared to the blank control culture[19].

For another experiment a different diatom strain was used to test mixotrophic growth to compare to *Phaeodactylum tricornutum,* diatom *Cylindrotheca closterium* IIDO2 was chosen. Experimental setup was identical to the previous experiment, reference medium with no added glycerol and two different glycerol concentration (0.01M, 0.1M) mediums were prepared, cultivation was carried out in biological triples for 10 days under the same conditions. Growth was expressed as chlorophyll concentration mg/mL increase because the correlational curve wasn't prepared for *Cylindrotheca closterium. Cylindrotheca closterium* IIDO2 reference culture chlorophyll concentration increased from 0.125 mg/ml to 0.732 ± 0.051 mg/ml, lag phase lasted for two days and exponential growth stared from day 3 until day 5, from day 5 until the end of cultivation growth was in static phase (Figure 18).

Other culture of *Cylindrotheca closterium* was cultivated using 0.01M glycerol concentration enriched medium. Culture chlorophyll concentration increased from 0.125 mg/mL to 1.122 ± 0.034 mg/mL, chlorophyll concertation increased 1.5 times compared to blank reference culture. First 5 days the culture growth was the same as the reference culture, but the glycerol culture exponential growth lasted 1 day longer (until day 6). After 6 days of cultivation growth became static and concentration declined at day 8, but then again started to grow from day 8 until the end of cultivation. Highest chlorophyll concentration was recorded during the last day of cultivation.

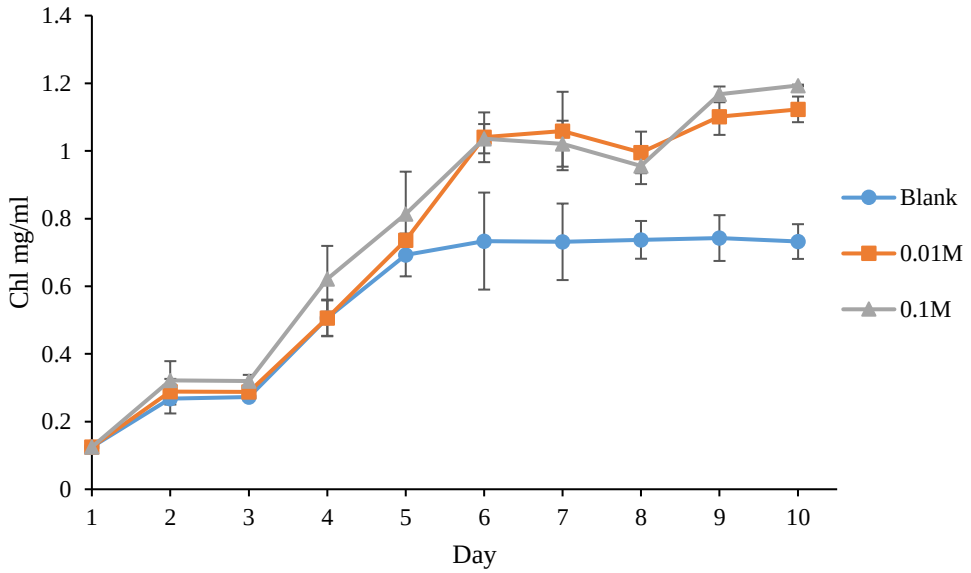


Figure 18. Diatom *Cylindrotheca closterium* IIDO2 growth under mixotrophic conditions using different glycerol concentrations. Glycerol concentrations used: 0.01M glycerol and 0.1M glycerol.

Third culture of *Cylindrotheca closterium* was cultivated using 0.1M glycerol concentration enriched medium. Culture chlorophyll concentration increased from 0.125 mg/mL to 1.193 ± 0.003 mg/mL, chlorophyll concentration increased 1.6 times compared to the reference culture, there was only 6% difference between the two cultures grown with glycerol, culture with 0.1M glycerol concentration medium having the higher chlorophyll concentration (Figure 18). Growth behavior was identical to the culture with 0.01M glycerol concentration, exponential growth lasted until day 6, then chlorophyll concentration declined until day 8 and then chlorophyll concentration again increased until the end of cultivation. Highest chlorophyll concentration was during the last day of cultivation.

From gathered data it can be said that *Cylindrotheca closterium* IIDO2 can utilize glycerol as a carbon source and grow under mixotrophic conditions. *Cylindrotheca closterium* growth increased with glycerol added to the medium, 1.6 times with 0.1M glycerol and 1.4 with 0.01M glycerol, but not as much as *Phaeodactylum tricornutum* growth increased with glycerol, 2.31 times with 0.1M glycerol and 2.12 times with 0.01M glycerol. But there was an experiment where

group of researchers tested two *Cylindrotheca* species (*Cylindrotheca closterium* and *Cylindrotheca fusiformis)* with different glycerol concentrations (from 0.1M to 0.4M) but their results were different, in their case addition of carbon source didn't improve growth of neither of the diatom strains [78]. Other research group tested three strains of *Cylindrotheca closterium* (VD05, VD06 and VD18) with galactose as an added carbon source, in their experiment two strains (VD05, VD06) were incapable of using galactose and their growth didn't increase, but *Cylindrotheca closterium* VD18 growth improved with galactose added to the medium which showed mixotrophic growth capability [79].

After *Phaeodactylum tricornutum* 1052/1A and *Cylindrotheca closterium* IIDO2 cultivation, biomass from samples were collected, freeze-dried and used for further experiments. To test effects of mixotrophic growth with different glycerol concentrations to phenolic content and antioxidant activity for different diatom species, phenolic content (TPC) and antioxidant activity (ABTS+, FRAP) assays were performed.

Total phenolic content (TPC) assay was performed using Folin-Ciocalteu's method to see the effect of mixotrophic growth to phenolic compound production in *Phaeodactylum tricornutum* 1052/1A and *Cylindrotheca closterium* IIDO2 (Figure 19).

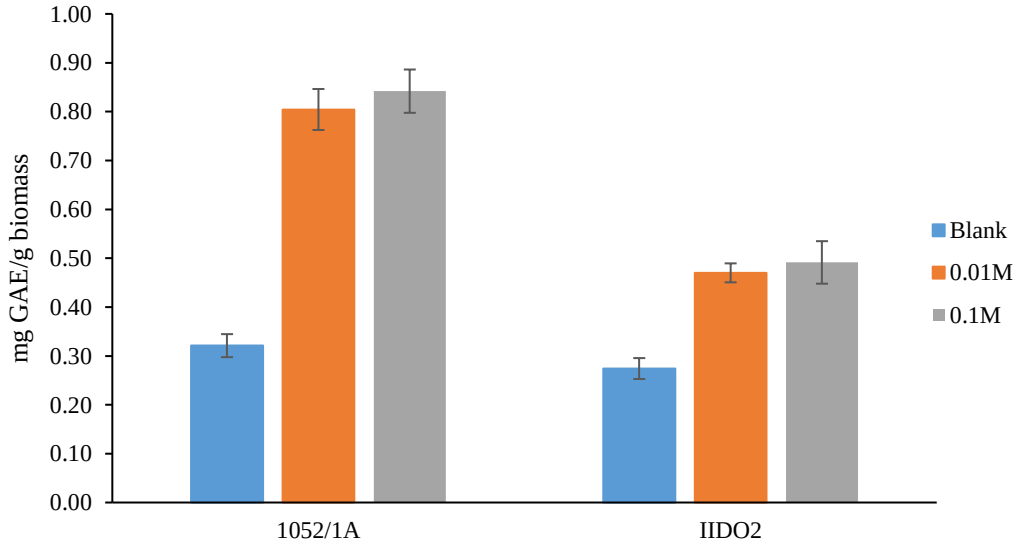


Figure 19. *Phaeodactylum tricornutum* 1052/1A and *Cylindrotheca closterium* IIDO2 collected biomass total phenolic content (TPC) assay.

Phaeodactylum tricornutum and *Cylindrotheca closterium* blank culture phenolic compound content was very similar, respectively 0.321 ± 0.024 mg GAE/g biomass and 0.274 ± 0.021 mg GAE/g biomass. There was a big increase in phenolic content for *Phaeodactylum tricornutum* grown under mixotrophic conditions. Culture that was grown with 0.01M glycerol concentration phenolic content was 0.804 ± 0.042 mg GAE/g biomass, which was 2.5 times higher than the blank reference culture, but the difference between two glycerol concentrations was very small, 0.1M glycerol concentration phenolic content was 0.842 ± 0.044 mg GAE/g biomass, 2.6 times higher

compared to the reference culture and only 5% higher than 0.01M culture. *Cylindrotheca closterium* cultures increase in phenolic content was smaller than *Phaeodactylum tricornutum,* 0.01M glycerol concentration culture phenolic content was 0.47 ± 0.019 mg GAE/g biomass, 1.7 times higher than the blank reference culture. And like for *Phaeodactylum tricornutum,* difference between *Cylindrotheca closterium* different glycerol concentrations were small, 0.1M glycerol concertation culture phenolic content was 0.491 ± 0.044 mg GAE/g biomass, only 5% increase compared to 0.01M.

For antioxidant activity changes two antioxidant activity assays were performed, ferric reducing antioxidant power (FRAP) and ABTS+ scavenging assay (ABTS+) assays. First ferric reducing antioxidant power assay (FRAP) was performed with *Phaeodactylum tricornutum* 1052/1A and *Cylindrotheca closterium* IIDO2 biomasses. Both diatom species (1052/1A and IIDO2) blank reference culture antioxidant activity was similar, *Phaeodactylum tricornutum* antioxidant activity was 0.933 ± 0.088 mM Trolox/g biomass and *Cylindrotheca closterium* – 1.023 ± 0.091 mM Trolox/g biomass (Figure 20).

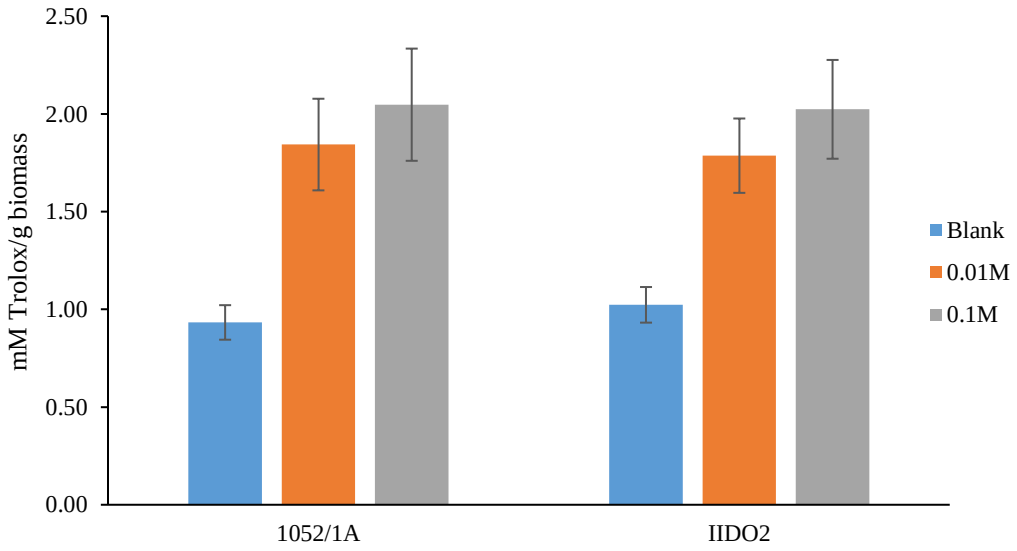


Figure 20. *Phaeodactylum tricornutum* 1052/1A and *Cylindrotheca closterium* IIDO2 collected biomass ferric reducing antioxidant power (FRAP) assay.

Phaeodactylum tricornutum cultures with both glycerol concentrations shown an increase in antioxidant activity (Figure 20). Culture grown with 0.01M glycerol concentration antioxidant activity was 1.843 ± 0.234 mM Trolox/g biomass, which increased 1.97 times compared to the blank reference culture, and culture grown with 0.1M glycerol concertation antioxidant activity reached 2.047 ± 0.287 mM Trolox/g biomass, which was 2.2 times higher than the reference culture and only 11% increase compared to 0.01M culture. Results for *Cylindrotheca closterium* were very similar. Culture grown with 0.01M glycerol concentration antioxidant activity increased to 1.786 ± 0.19 mM Trolox/g biomass, which was 1.75 time higher than the blank reference culture. Culture grown with 0.1M glycerol concentration antioxidant activity was 2.023 ± 0.253

mM Trolox/g biomass, 1.98 times higher than the reference culture and 13% increase compared to 0.01M culture.

ABTS+ scavenging assay was also performed for both diatom species to see changes in antioxidant activity (Figure 21). *Phaeodactylum tricornutum* 1052/1A blank reference culture antioxidant activity was 3.968 ± 0.526 mM Trolox/g biomass (Figure 21). Cultures that was grown with added glycerol exhibited antioxidant activity increase with both concentrations. *Phaeodactylum tricornutum* culture grown with 0.01M glycerol concentrations antioxidant activity was 5.993 ± 0.492 mM Trolox/g biomass, 1.5 increase compared to the blank reference culture (Figure 21). Culture grown with 0.1M glycerol concentration antioxidant activity was 6.949 ± 0.571 mM Trolox/g biomass, 1.75 higher than blank reference culture and 16% increase compared to 0.01M culture.

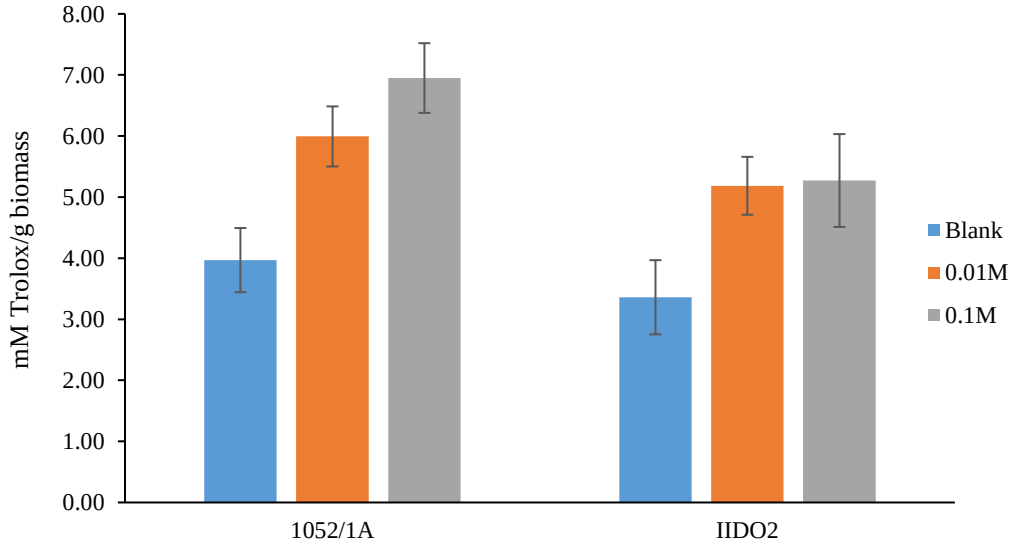


Figure 21. *Phaeodactylum tricornutum* 1052/1A and *Cylindrotheca closterium* IIDO2 collected biomass ABTS+ scavenging assay.

Cylindrotheca closterium IIDO2 blank reference culture antioxidant activity was 3.360 ± 0.608 mM Trolox/g biomass (Figure 21). Cultures grown with added glycerol had an increase in antioxidant activity (Figure 21). Culture grown with 0.01M glycerol concentration antioxidant activity was 5.185 ± 0.474 mM Trolox/g biomass, almost the same as the culture with 0.1M glycerol concentration, 5.272 ± 0.760 mM Trolox/g biomass, cultures with both glycerol concentration antioxidant activity increased 1.55 times compared to the blank reference culture.

Introduction of mixotrophic growth to *Phaeodactylum tricornutum* 1052/1A and *Cylindrotheca closterium* IIDO2 cultures changed their phenolic content and antioxidant activity. Both species had an increase in phenolic content and antioxidant activity. Differences between the blank reference cultures and cultures with glycerol were significant in all performed assays, from 1.5 to 2.6 times increase, but between different glycerol concentrations, counting in standard deviations, changes were very small. *Phaeodactylum tricornutum* phenolic content increased

significantly more compared to *Cylindrotheca closterium,* almost 2 times higher increase (Figure 19). That could be the result of *Phaeodactylum tricornutum* the superior ability to use glycerol as carbon source for mixotrophic growth. Because of the utilization of both inorganic and organic carbon sources during mixotrophic growth, cultures capable of mixotrophic growth result in increased growth rate and metabolite rate. *Phaeodactylum tricornutum* and *Cylindrotheca closterium* resulted in increased total phenolic content (Figure 19). As mixotrophic growth increases the yield of metabolites, *Phaeodactylum tricornutum* and *Cylindrotheca closterium* resulted in increased antioxidant activity compared to the blank reference culture. Ferric reducing antioxidant power assay showed an increase in antioxidant activity for both diatom species, counting the standard deviations, increase was the same for both species with both glycerol concentrations, minimal differences (Figure 20). ABTS+ scavenging assay also showed an increase in antioxidant activity for *Phaeodactylum tricornutum* and *Cylindrotheca closterium. Phaeodactylum tricornutum* had a higher antioxidant activity in all the samples (Blank, 0.01M and 0.1) than *Cylindrotheca closterium,* but both species, counting in the standard deviation, had almost the same increase in antioxidant activity under mixotrophic growth with both concentrations of glycerol compared to the blank reference culture (Figure 21). All cultures had a 1.5 time increase in antioxidant activity compared to the blank reference culture, except *Phaeodactylum tricornutum* with 0.1M glycerol concentration which had a slightly higher, 1.75 times increase compared to the blank reference culture. Experiment conducted by a group of researchers tested relationship between growth conditions and antioxidant properties. *Chlorella vulgaris* and *Scenedesmus obliquu* were grown under autotrophic, heterotrophic and mixotrophic conditions, highest phenolic content and antioxidant activity was recorded in cultures grown under mixotrophic conditions [80]. The total phenolic content in *Chlorella vulgaris* grown under mixotrophic condition increased from 0.191 mg GAE/g to 0.397 mg GAE/g and for *Scenedesmus obliquu* increased from 0.111 mg GAE/g to 0.55 mg GAE/g. Moreover, the antioxidant activity of the same strains was evaluated by the phosphomolybdate method, mixotrophic growth significantly influence both microalgae antioxidant activity, *Chlorella vulgaris* increased from 1836.48 mg AEAE/g for control cultures to 4322.58 mg AEAE/g for treated, accordingly *Scenedesmus obliquu* increased from 1815.6 mg AEAE/g to 5096.76 mg AEAE/g [80].

Fatty acid profile of *Phaeodactylum tricornutum* grown under mixotrophic conditions is shown in Table 4. There are 11 fatty acids shown in the table. There were found four saturated fatty acids (Myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0) and lignoceric acid (C24:0)) and seven unsaturated fatty acid from which one monounsaturated fatty acid (palmitoleic acid (C16:1) and six polyunsaturated fatty acids (oleic acid (C18:1n9c), linoleic acid (C18:2n6c), linolenic acid (C18:3n3), eicosadienoic acid (C20:2), eicosapentaenoic acid (C20:5n3),

docosahexaenoic acid (C22:6n3)). The was an increase in almost all fatty acid content with *Phaeodactylum tricornutum* grown with glycerol, only stearic acid decreased when glycerol was introduced, decreased from 0.228 ± 0.063 mg/g biomass in blank culture to 0.174 ± 0.007 mg/g biomass in 0.0M glycerol concentration culture and 0.143 ± 0.025 mg/g biomass in 0.1M glycerol concentration culture. Myristic acid had the biggest increase, from 0.163 ± 0.039 mg/g biomass in blank culture to 1.362 ± 0.048 mg/g biomass in 0.01M glycerol concentration culture and 1.172 ± 0.037 mg/g biomass in 0.1M glycerol concentration culture. Highest content of fatty acids was in culture grown with 0.01M glycerol concentration with all fatty acids, except myristic acid (blank culture highest concentration) and linoleic acid (0.1M highest concentration). Eicosadienoic acid wasn't present in blank culture of *Phaeodactylum tricornutum* but could be found in cultures grown with 0.01M and 01M glycerol concentrations.

Table 4. *Phaeodactylum tricornutum* 1052/1A grown under mixotrophic conditions with glycerol, dry biomass fatty acid composition using GC-FID, expressed as mg/g biomass.

Fatty acid	***Phaeodactylum tricornutum* 1052/1A**		
	Blank	**0.01M**	**0.1M**
Myristic acid (C14:0)	0.163 ± 0.039	1.362 ± 0.048	1.172 ± 0.037
Palmitic acid (C16:0)	0.649 ± 0.142	0.927 ± 0.117	0.882 ± 0.036
Palmitoleic acid (C16:1)	0.517 ± 0.024	1.063 ± 0.052	1.147 ± 0.063
Stearic acid (C18:0)	0.228 ± 0.063	0.174 ± 0.007	0.143 ± 0.025
Oleic acid (C18:1n9c)	0.199 ± 0.021	0.631 ± 0.039	0.59 ± 0.032
Linoleic acid (C18:2n6c)	0.112 ± 0.024	0.244 ± 0.015	0.205 ± 0.012
Linolenic acid (C18:3n3)	0.019 ± 0.004	0.062 ± 0.006	0.059 ± 0.005
Eicosadienoic acid (C20:2)	-	0.084 ± 0.008	0.065 ± 0.005
Lignoceric acid (C24:0)	0.042 ± 0.012	0.13 ± 0.013	0.086 ± 0.022
Eicosapentaenoic acid (C20:5n3)	0.547 ± 0.044	1.664 ± 0.179	1.328 ± 0.129
Docosahexaenoic acid (C22:6n3)	0.028 ± 0.003	0.083 ± 0.01	0.067 ± 0.017
Total saturated fatty acids:	1.27 ± 0.455	2.592 ± 0.174	2.284 ± 0.052
Total unsaturated fatty acids:	0.517 ± 0.024	1.063 ± 0.052	1.147 ± 0.063
Total polyunsaturated fatty acids:	0.906 ± 0.065	2.768 ± 0.242	2.315 ± 0.198

Fatty acid profile of *Cylindrotheca closterium* grown under mixotrophic conditions is shown in Table 5. Fatty acid profile is similar to *Phaeodactylum tricornutum.* There are 11 fatty acids shown in the table. There were found four saturated fatty acids (Myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0) and lignoceric acid (C24:0)) and seven unsaturated fatty acid

from which one monounsaturated fatty acid (palmitoleic acid (C16:1) and six polyunsaturated fatty acids (oleic acid (C18:1n9c), linoleic acid (C18:2n6c), linolenic acid (C18:3n3), eicosadienoic acid (C20:2), eicosapentaenoic acid (C20:5n3), docosahexaenoic acid (C22:6n3)). There was an increase in all fatty acids in cultures grown with glycerol compared to blank culture. Just like with *Phaeodactylum tricornutum* Eicosadienoic acid wasn't present in blank culture of *Cylindrotheca closterium* but could be found in cultures grown with 0.01M and 0.1M glycerol concentrations. While all fatty acid concentrations increased compared to the blank culture, but some fatty acid concentrations were higher produced with 0.01M glycerol concentration and other were higher produced with 0.1M glycerol concentration. Myristic acid, linoleic acid, eicosapentaenoic acid concentrations were higher when *Cylindrotheca closterium* was cultivated with 0.01M glycerol concentration. Palmitic acid, palmitoleic acid, stearic acid concentrations were higher when cultivated with 0.1M glycerol concentration. Oleic acid, linolenic acid, lignoceric acid, docosahexaenoic acid and eicosadienoic acid concentrations counting in standard deviation were the same with both glycerol concentrations.

Table 5. *Cylindrotheca closterium* IIDO2 grown under mixotrophic conditions with glycerol, dry biomass fatty acid composition using GC-FID, expressed as mg/g biomass.

Fatty acid	***Cylindrotheca closterium* IIDO2**		
	Blank	**0.01M**	**0.1M**
Myristic acid (C14:0)	0.088±0.017	0.833±0.039	0.704±0.071
Palmitic acid (C16:0)	0.414 ± 0.02	0.523 ± 0.019	0.622 ± 0.036
Palmitoleic acid (C16:1)	0.418 ± 0.019	0.600 ± 0.035	0.687 ± 0.043
Stearic acid (C18:0)	0.138 ± 0.005	0.144 ± 0.005	0.162 ± 0.015
Oleic acid (C18:1n9c)	0.152 ± 0.037	0.367 ± 0.024	0.375 ± 0.026
Linoleic acid (C18:2n6c)	0.055 ± 0.015	0.194 ± 0.018	0.178 ± 0.009
Linolenic acid (C18:3n3)	0.008 ± 0.004	0.052 ± 0.007	0.055 ± 0.005
Eicosadienoic acid (C20:2)	-	0.038 ± 0.005	0.029 ± 0.004
Lignoceric acid (C24:0)	0.034 ± 0.006	0.049 ± 0.009	0.077 ± 0.018
Eicosapentaenoic acid (C20:5n3)	0.436 ± 0.041	0.956 ± 0.072	0.901 ± 0.071
Docosahexaenoic acid (C22:6n3)	0.022 ± 0.002	0.043 ± 0.008	0.053 ± 0.008
Total saturated fatty acids:	0.702 ± 0.071	1.549 ± 0.072	1.566 ± 0.114
Total unsaturated fatty acids:	0.418 ± 0.019	0.600 ± 0.035	0.687 ± 0.043
Total polyunsaturated fatty acids:	0.674 ± 0.088	1.641 ± 0.130	1.592 ± 0.112

It was found that *Phaeodactylum tricornutum* highest increase in fatty acid concentrations was present in culture grown with 0.01M glycerol concentration and smaller with 0.1M glycerol concentration. Which were different results from most other research papers. Experiment conduction with *Phaeodactylum tricornutum* and different glycerol concentrations (0.01M and 0.1M) found that most fatty acid concentrations increased with higher glycerol concentrations, but unexpectedly eicosapentaenoic acid did not undergo a significant change compared to control culture, only 12% increase with 0.01M and 7% increase with 0.1M [48]. While in this study eicosapentaenoic acid concentration increased by 304% with 0.01M glycerol concentration and by 243% with 0.1M glycerol concentration compared to the blank culture. Other experiment result tendency was more similar, but still quite different, *Phaeodactylum tricornutum* with 0.1M glycerol concentration showed an increase in fatty acids, myristic acid increased by 255%, palmitic acid increased by 227%, palmitoleic acid increased by 362% and eicosapentaenoic acid increased by 150% compared to the blank culture [81]. While in this study *Phaeodactylum tricornutum* cultivated with 0.1M myristic acid increased by 719%, palmitic acid increased by 135%, palmitoleic acid increased by 221% and eicosapentaenoic acid increased by 242% compared to the blank reference culture. It may appear that high concentrations of carbon increase synthesis of fatty acids. It is seen that *Phaeodactylum tricornutum* grown under mixotrophic conditions with glycerol greatly increased fatty acid content.

From results it seems that *Cylindrotheca closterium*, like *Phaeodactylum tricornutum*, under mixotrophic conditions with glycerol increases fatty acid content, but the increases weren't consistent between different concentrations of glycerol (0.01M and 0.1M). Results were different from other researches, in most cases *Cylindrotheca closterium* growth didn't increase under mixotrophic conditions with glycerol or growth even decreased when glycerol was added. This strain of *Cylindrotheca closterium* exhibited ability to consume glycerol to enhance growth and fatty acid content. *Cylindrotheca closterium* eicosapentaenoic acid content increased from 0.436 ± 0.041 mg/g biomass to 0.956 ± 0.072 mg/g biomass with 0.01M glycerol concentration and to 0.901 ± 0.071 mg/g biomass with 0.1M glycerol concentration, showing the ability of mixotrophic growth. Experiment conducted with *Cylindrotheca closterium* and three different glycerol (0.1M, 0.2M, 0.4M) concentrations resulted in decreased culture growth and no significant changes in fatty acid content[82]. In the same experiment *Cylindrotheca fusiformis* also didn't show any capability of mixotrophic growth with glycerol, the cell density didn't change compared the blank control culture and the fatty acid content didn't had any significant differences[82]. Other microalgae species which exhibit the ability of mixotrophic growth, have changes in fatty acid content, *Navicula saprophil* was capable of mixotrophic growth fatty acid content changed also, eicosapentaenoic acid content increased from 17.3 mg/g biomass to 19.2 mg/g biomass when

cultivated under mixotrophic conditions [83]. It can be said from the results that microalgae and diatoms which have the ability and are cultivated under mixotrophic conditions, exhibit increase in fatty acid content.

3.3. Optimization of pressurized liquid extraction (PLE) of *Phaeodactylum tricornutum* using different polarity solvents.

Phaeodactylum tricornutum has a high lipid content and a high production rate of eicosapentaenoic acid (EPA), also docosahexaenoic acid (DHA) is present. EPA and DHA are essential fatty acids for human metabolism. Besides lipids, *Phaeodactylum tricornutum* is also rich in bioactive compounds like pigments and phenolic compounds which have high antioxidant activity. *Phaeodactylum tricornutum* pressurized liquid extraction (PLE) using three solvents of different polarities (hexane, acetone and ethanol) at three temperatures (40 ^{o}C, 80^{o}C and 120 ^{o}C) and three extraction times (15 min, 39 min, 60 min) was investigated and optimized.

3.3.1. Hexane extraction

Experimental setup using non-polar solvent hexane with two independent variables and their impact on extract yield, EPA and DHA content can be seen in Table 6. Because hexane is a non-polar solvent, *Phaeodactylum tricornutum* extracts should contain non-polar compounds – lipids, so as additional responses to extraction yield - EPA and DHA contents were chosen. Experimental models with hexane were evaluated by ANOVA. Not all models for hexane extraction were significant. Experimentally obtained extract yields from *Phaeodactylum tricornutum* varied from 6.92 ± 0.32% to 16.77 ± 0.72%, DHA contents were from 1.735 ± 0.039 mg/g extract to 3.874 ± 0.169 mg/g extract and EPA contents were from 183.088 ± 3.796 mg/g extract to 351.667 ± 40.391 mg/g extract.

Table 6. *Phaeodactylum tricornutum* central composite design matrix of two test variables (static extraction (**τ**) time and temperature (T)) and experimental results from the response variables using hexane as a solvent.

Run	**Parameter**		**Response 1**		**Response 2**		**Response 3**	
Nr.	T	τ	Actual Yield	Predicted Yield	Actual DHA	Predicted DHA	Actual EPA	Predicted EPA
	^{o}C	min.	%	%	mg/g extract	mg/g extract	mg/g extract	mg/g extract
1	80	39	12.97 ± 0.44	12.58	2.99 ± 0.166	3.124	248.96 ± 7.847	279.636
2	120	60	15.1 ± 1.01	15.04	3.087 ± 0.036	3.153	183.088 ± 3.796	183.528
3	80	39	14.57 ± 1.24	12.58	3.135 ± 0.363	3.124	280.793 ± 20.597	279.636
4	80	39	13.17 ± 0.77	12.58	2.997 ± 0.065	3.124	286.589 ± 8.801	279.636
5	40	39	8.28 ± 0.39	9.26	1.768 ± 0.103	1.664	267.449 ± 19.235	252.76
6	80	39	11.97 ± 0.89	12.58	3.758 ± 0.731	3.124	316.155 ± 48.56	279.636
7	120	15	15.37 ± 1.45	16.14	3.874 ± 0.169	3.874	217.408 ± 5.522	234.203
8	80	39	10.5 ± 0.29	12.58	2.57 ± 0.463	3.124	233.757 ± 36.05	279.636
9	120	39	16.77 ± 0.72	16.07	3.371 ± 0.118	3.305	205.248 ± 6.254	188.013
10	80	15	12.18 ± 0.74	11.43	3.354 ± 0.358	3.516	351.667 ± 40.391	319.266
11	80	60	11.59 ± 0.39	12.62	3.278 ± 0.771	3.126	280.414 ± 41.583	280.891
12	40	60	11.33 ± 0.7	10.37	1.735 ± 0.039	1.821	260.674 ± 6.165	259.756
13	40	15	6.92 ± 0.32	6.89	1.861 ± 0.241	1.879	270.224 ± 23.198	285.83

The quadratic regression model analysis for extraction yield from *Phaeodactylum tricornutum* showed that the model was significant ($p<0.05$) and "*Lack of fit*" was not significant ($p>0.1$) relative to the pure error (Table 7). Independent variables, temperature and extraction time had a different effect on extract yield using hexane. Temperature had a significant effect ($p<0.05$), while extraction time was not significant ($p>0.1$). Interaction between temperature and extraction time didn't have a significant effect ($p>0.1$). Both second order model terms T^2 and τ^2 were not significant ($p>0.1$).

Table 7. ANOVA for extraction yield response surface quadric model.

Source	Sum of squares	Df	Mean square	F value	p-value Prob>F
Model	79.71	5	15.94	8.11	0.0079*
Temperature (T)	72.49	1	72.49	36.89	0.0005*
Time (τ)	2.10	1	2.10	1.07	0.3356**
T* τ	5.22	1	5.22	2.66	0.1471**
T^2	0.020	1	0.020	0.010	0.9226**
τ^2	0.073	1	0.73	0.37	0.5608**
Residual	13.76	7	1.97		
Lack of Fit	4.61	3	1.54	0.67	0.6122**
Pure Error	9.14	4	2.29		
Cor Total	93.47	12			

* - significant, ** - not significant

Determination coefficient (R^2) if 0.853 showed the adequacy of the model which indicated the model is decent fit to the experimental data. But the predicted R^2 value of 0.365 was not as close to the adjusted R^2 value of 0.748 as normally expected. This can indicate a possible problem with the model or data.

Relationship between independent variable and the extraction yield is presented in the following equation:

$$Yield\ (\%) = 12.54 + 3.48 \cdot T + 0.59 \cdot \tau - 1.14 \cdot T \cdot \tau + 0.085 \cdot T^2 - 0.52 \cdot \tau^2 \quad (1)$$

Predicted values of extraction yield were calculated using a second order polynomial equation (1) and were compared with actual experimental values in Figure 23.

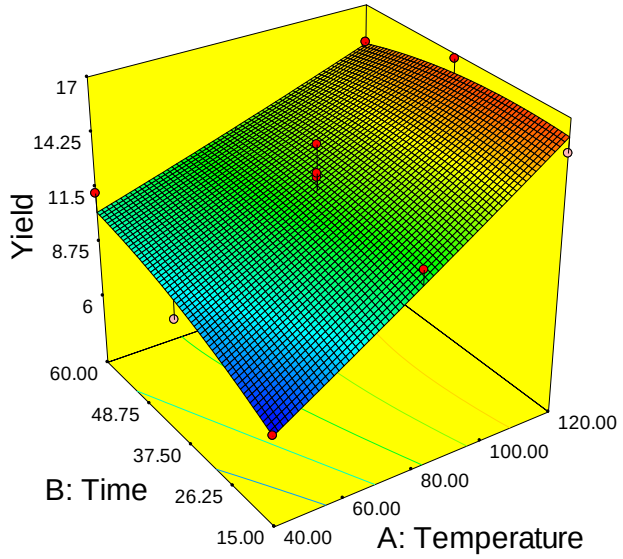


Figure 22. Three-dimensional response surface of influence of extraction temperature and static extraction time on extraction yield.

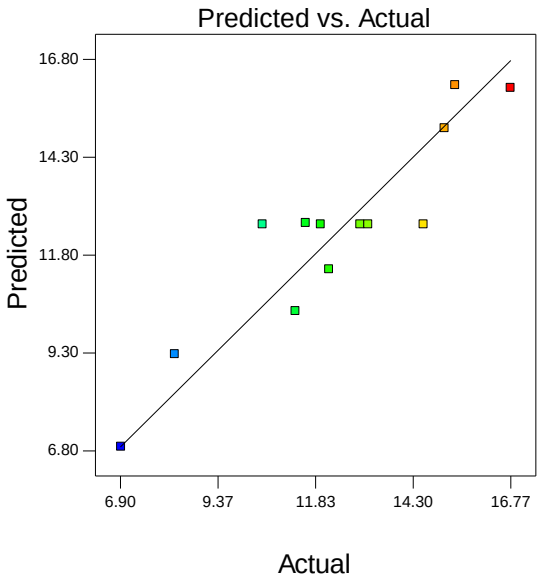


Figure 23. Comparison between actual and predicted extraction yield values.

The three-dimensional response surface plot (Figure 22) show the relationship of the extraction yield and the extraction time, temperature. Model plot reveals interactions between different parameters and evaluate their effect on extraction yield. It can be seen that temperature has the biggest effect on extraction yield. Highest extraction yield (16.77 ± 0.72 %) was reached with 120 ^{o}C temperature and 39 minutes of extraction time. Extraction time didn't have a great impact on extraction yield when temperature was above 60 ^{o}C. Differences between 15 min, 39 min and 60 min extraction time extract yields with 120 ^{o}C temperature were only about 9%. Based on the results obtained, *Phaeodactylum tricornutum* using hexane should be extracted at higher temperatures, preferably at 120 ^{o}C, and extraction time should be in the range of 15 – 39 minutes, because the extraction yield differences were small in that time range. In a recent experiment, extraction from algae *Fucus vesiculosus* with hexane using PLE system was tested, extractions were performed for 10 minutes at 80 ^{o}C, 120 ^{o}C and 160 ^{o}C temperatures, respectively, extraction yields were 2.79 ± 0.12 %, 3.72 ± 0.24 % and 4.49 ± 1.54 %, similarly increasing temperature increases extraction yield with constant extraction time [84]. Pieber *et al*. tested the influence of extraction time influence on extraction yield with PLE using microalgae *Nannochloropsis granulate* [85]. Extraction time varied from 50 minutes to 150 minutes, highest yield was found with 50 minutes, it was shown that longer extraction times reduced the extraction yield [85]. This is in close agreement with this study, where similar extraction time (39 min) resulted in the highest extraction yield (16.77 ± 0.72 %) and longer extraction time resulted in lower extraction yield. In another research the temperature effect on extraction yield of non-polar pressurized liquid extracts of microalgae *Dunaliella salina* showed that an increase in temperature led to an increase in the extraction yield, the highest extraction yield (17.7%) was reached with 160^{o}C temperature[86].

This experiment also confirms findings in this study that increasing extraction temperature increases extraction yield.

The quadratic regression model analysis for DHA content from *Phaeodactylum tricornutum* hexane extract showed that the model was significant ($p<0.05$) and "*Lack of fit*" was not significant ($p>0.1$) relative to the pure error (Table 8). Independent variables, temperature and extraction time had a different effect on DHA content. Temperature had a significant effect ($p<0.05$), while extraction time was not significant ($p>0.1$). Interaction between temperature and extraction time didn't have a significant effect ($p>0.1$). Second order model term T^2 was significant ($p>0.05$), while τ^2 was not significant ($p>0.1$).

Table 8. ANOVA for DHA content response surface quadric model.

Source	Sum of squares	Df	Mean square	F value	p-value Prob>F
Model	5.59	5	1.12	9.86	0.0045*
Temperature (T)	4.15	1	4.15	36.58	0.0005*
Time (τ)	0.23	1	0.23	2.01	0.1993**
T* τ	0.11	1	0.11	0.97	0.3569**
T^2	1.13	1	1.13	9.96	0.0106*
τ^2	0.093	1	0.093	0.82	0.3941**
Residual	0.79	7	0.11		
Lack of Fit	0.056	3	0.019	0.10	0.9548**
Pure Error	0.74	4	0.18		
Cor Total	6.38	12			

* - significant, ** - not significant

Determination coefficient (R^2) if 0.876 showed the adequacy of the model which indicated the model is decent fit to the experimental data. The predicted R^2 value of 0.768 was in agreement with the adjusted R^2 value of 0.787, which indicate that this design could be used for modeling design.

Relationship between independent variable and the extraction yield is presented in the following equation:

$$DHA\ (mg/g\ extract) = 3.14 + 0.83 \cdot T - 0.19 \cdot \tau - 0.17 \cdot T \cdot \tau - 0.64 \cdot T^2 + 0.18 \cdot \tau^2 \quad (2)$$

Predicted values of extraction yield were calculated using a second order polynomial equation (2) and were compared with actual experimental values in Figure 25.

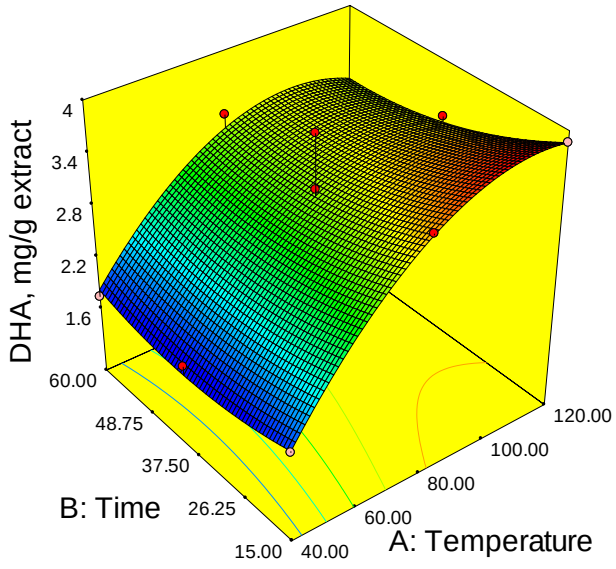


Figure 24. Three-dimensional response surface of influence of extraction temperature and static extraction time on extract DHA content.

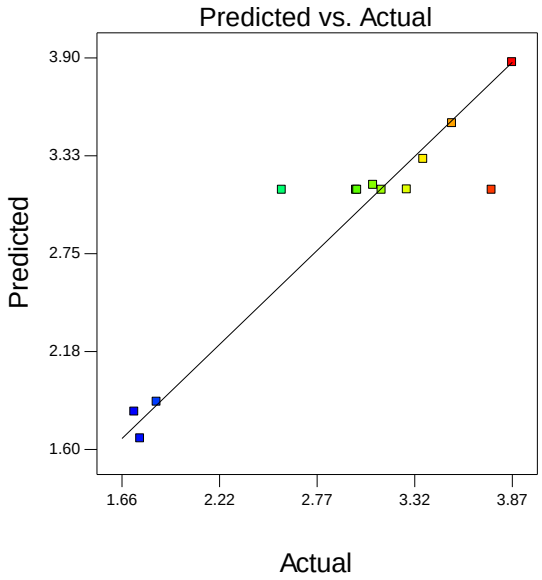


Figure 25. Comparison between actual and predicted DHA content values.

The three-dimensional response surface plot (Figure 24) show the relationship of the DHA content and the extraction time, temperature. Model plot reveals interactions between different parameters and evaluate their effect on DHA content. Just like with the extraction yield, temperature had the biggest effect on DHA content. In most temperatures extraction time didn't have a significant effect on DHA content, but from 80 ^{o}C to 120 ^{o}C temperature, increasing the extraction time resulted in decrease in DHA content. Highest DHA content (3.874 ± 0.169 mg/g extract) was reached when extraction temperature was 120 ^{o}C and extraction time was 15 min. Based on obtained results, it is evident that DHA from *Phaeodactylum tricornutum* using hexane should be obtained at higher temperatures, from 80 ^{o}C to 120 ^{o}C, and at low extraction time (15 minutes) because increasing the extraction times, decreased the DHA content.

The quadratic regression model analysis for EPA content from *Phaeodactylum tricornutum* hexane extract showed that the whole model was not significant ($p>0.05$), but the "*lack of it*" was also no significant. Only temperature was a significant model term, T ($p<0.05$) and T^2 ($p<0.05$). The three-dimensional response surface plot of EPA content showed that highest EPA content (351.667 ± 40.391 mg/g extract) was at 80 ^{o}C temperature and started dropping down when the temperature was increased or decreased, time didn't have a significant influence, when time was increased from 15 min to 39 min with 80^{o}C, EPA content decreased by 11%.

Otero *et al*. reported the fatty acid profile differences of pressurized liquid extracts with 5 solvents, including hexane, with algae *Fucus vesiculosus.* Different temperatures (80^{o}C, 120^{o}C and 160^{o}C) with 10-minute extraction time were tested [84]. It was found that the highest fatty acid content was at 120^{o}C as an extraction temperature, which come in line with the results in this study with DHA model, where highest DHA concentration (3.874 ± 0.169 mg/g extract) were

found in extract obtained with 120^{o}C extraction temperature. However, EPA concentrations didn't follow the same trend, highest EPA concentration (351.667 ± 40.391 mg/g extract) was found in extract obtained with 80^{o}C extraction temperature.

3.3.2. Acetone extraction

Experimental setup using polar solvent acetone with two independent variables and their impact on extract yield, TPC and ABTS+ can be seen in Table 9. Acetone is more polar solvent than hexane, increasing polarity increases the number of compounds that will be extracted, more polar compounds like polyphenols and pigments. Because of increased polarity of the solvent, alongside extract yield two different responses were chosen, total phenolic compound content and antioxidant activity (ABTS+ assay) response. Experimental models with acetone were evaluated by ANOVA. Not all models for acetone extraction were significant. Experimentally obtained extract yields from *Phaeodactylum tricornutum* varied from 11.04 ± 1.69 % to 30.39 ± 1.27 %, compared to hexane extracts, yield increased by 60 – 81%. TPC varied from 15.967 ± 1.526 mg GAE/g extract to 38.667 ± 6.118 mg GAE/g extract and ABTS+ varied from 288.267 ± 10.004 mM Trolox/g extract to 522.133 ± 22.458 mM Trolox/g extract.

Table 9. *Phaeodactylum tricornutum* central composite design matrix of two test variables (static extraction time (τ**)** and temperature (T)) and experimental results from the response variables (Yield, TPC and ABTS+) using acetone as a solvent.

Run	**Parameter**		**Response 1**		**Response 2**		**Response 3**	
Nr.	T	τ	Actual Yield	Predicted Yield	Actual TPC	Predicted TPC	Actual ABTS+	Predicted ABTS+
	^{o}C	min.	%	%	mg GAE/g extract	mg GAE/g extract	mM Trolox/g extract	mM Trolox/g extract
1	80	39	15.89 ± 1.54	15.42	21 ± 1.557	22.346	380.267 ± 2.231	382. 857
2	120	60	30.39 ± 1.27	30.29	36.7 ± 1.647	35.69	522.133 ± 22.458	515.919
3	80	39	15.57 ± 0.37	15.42	23.544 ± 2.237	22.346	394.578 ± 23.42	382. 857
4	80	39	15.32 ± 1.35	15.42	23.544 ± 2.237	22.346	394.578 ± 23.42	382. 857
5	40	39	11.04 ± 1.69	12.34	24.133 ± 1.888	22.231	414.667 ± 3.183	422.123
6	80	39	17.06 ± 1.26	15.42	24.433 ± 1.455	22.346	381.867 ± 3.477	382.857
7	120	15	19.27 ± 0.86	20.17	38.667 ± 6.118	34.585	440.8 ± 14.28	395.865
8	80	39	13.76 ± 1.44	15.42	23.4 ± 1.57	22.346	421.6 ± 12.81	382. 857
9	120	39	24.39 ± 1.82	23.59	28.7 ± 1.145	33.792	394.933 ± 7.978	446.082
10	80	15	15.69 ± 0.37	14.88	15.967 ± 1.526	21.334	288.267 ± 10.004	357.748
11	80	60	18.29 ± 1.31	19.6	27 ± 1.058	25.823	441.6 ± 5.858	430.725
12	40	60	15.22 ± 0.07	14	26.1 ± 0.946	28.287	430.933 ± 10.692	448.022
13	40	15	14.78 ± 1.26	14.69	21.7 ± 0.908	20.415	446.667 ± 58.844	422.122

The quadratic regression model analysis for extraction yield from *Phaeodactylum tricornutum* showed that the model was significant ($p<0.05$) and "*Lack of fit*" was not significant ($p>0.1$) relative to the pure error (Table 10). Independent variables, temperature and extraction time had a similar effect on extract yield using acetone. Temperature had a significant effect ($p<0.0001$), extraction time was also significant ($p<0.05$). Interaction between temperature and extraction time had a significant effect ($p<0.05$). Both second order model terms T^2 ($p<0.05$) and τ^2 ($p<0.05$) were also significant.

Table 10. ANOVA for extraction yield response surface quadric model.

Source	Sum of squares	Df	Mean square	F value	p-value Prob>F
Model	287.46	5	57.49	31.64	0.0001*
Temperature (T)	177.53	1	177.53	97.71	<0.0001*
Time (τ)	33.42	1	33.42	18.39	0.0036*
T* τ	29.31	1	29.31	16.13	0.0051*
T^2	17.92	1	17.92	9.86	0.0164*
τ^2	10.8	1	10.8	5.95	0.0449*
Residual	12.72	7	1.82		
Lack of Fit	7.07	3	2.36	1.67	0.3094**
Pure Error	5.65	4	1.41		
Cor Total	300.18	12			

* - significant, ** - not significant

Determination coefficient (R^2) if 0.958 showed the adequacy of the model which indicated the model is a good fit to the experimental data. The predicted R^2 value of 0.741 was in reasonable agreement with the adjusted R^2 value of 0.927, which indicate that this design could be used for modeling design.

Relationship between independent variable and the extraction yield is presented in the following equation:

$$Yield\ (\%) = 15.25 + 5.44 \cdot T + 2.36 \cdot \tau + 2.71 \cdot T \cdot \tau + 2.55 \cdot T^2 + 1.99 \cdot \tau^2 \quad (3)$$

Predicted values of extraction yield were calculated using a second order polynomial equation (3) and were compared with actual experimental values in Figure 27.

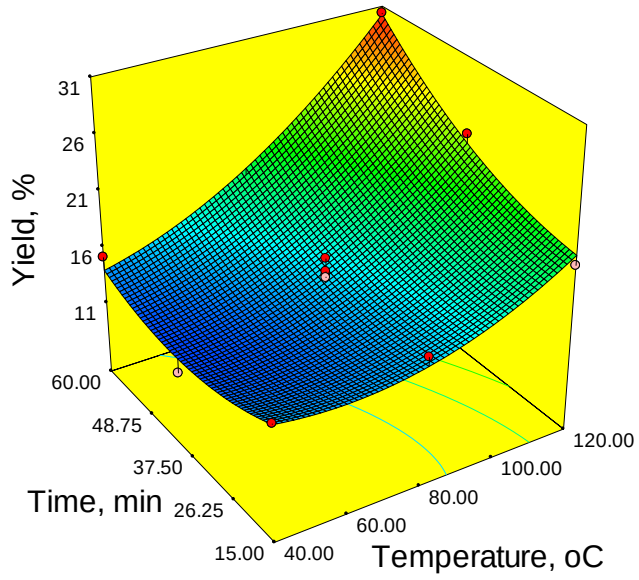


Figure 26. Three-dimensional response surface of influence of extraction temperature and static extraction time on extraction yield.

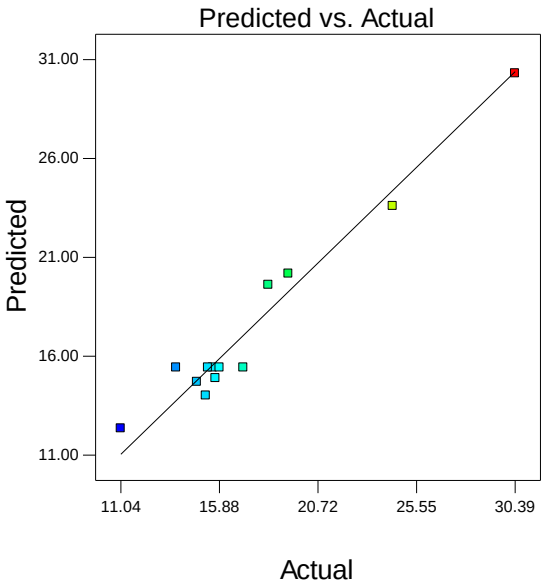


Figure 27. Comparison between actual and predicted yield values.

The three-dimensional response surface plot (Figure 26) show the relationship of the extraction yield and the extraction time, temperature. Model plot reveals interactions between different parameters and evaluate their effect on extraction yield. Both independent variables affected the extraction yield. Increase in extraction time and temperature affected the extraction yield. Highest extraction yield (30.39 ± 1.27 %) was reached when the temperature was 120 ^{o}C and extraction time was 60 minutes. At low temperature, time didn't have a significant effect, but from 80 ^{o}C, increase in extraction time showed an increase in extraction yield, even more when the temperature was 120 ^{o}C. Extraction yield (30.39 ± 1.27 %) of 120 ^{o}C and 60min, compared to extraction yield (19.27 ± 0.86 %) of 120 ^{o}C and 15min, increased by 60%. Based on obtained results, it is evident that with acetone *Phaeodactylum tricornutum* extraction yield increases when temperature and extraction time is increased, preferably extraction at high temperatures (120 ^{o}C) with longer extraction times (60 min) should be used to maximize extraction yield. Plaza *et al.* tested temperature (50^{o}C, 100^{o}C, 150^{o}C and 200^{o}C) effect on extraction yield of acetone extracts obtained by PLE from *Chlorella vulgaris*[87]. It was found that increasing extraction temperature resulted in increased extraction yield, highest extraction yield (31.19%) was reached with the highest temperature 200^{o}C [87], just like in this study, highest yield (30.39 ± 1.27 %) was reached with the highest extraction temperature – 120^{o}C.

The quadratic regression model analysis for TPC from *Phaeodactylum tricornutum* showed that the model was significant ($p<0.05$) and "*Lack of fit*" was also significant ($p<0.05$) relative to the pure error (Table 11).). Independent variables, temperature and extraction time had a different effect on TPC. Temperature had a significant effect ($p<0.05$), while extraction time was not significant ($p>0.1$). Interaction between temperature and extraction time didn't have a significant effect ($p>0.1$). Second order model term T^2 was significant ($p>0.05$), while τ^2 was not significant ($p>0.1$).

Table 11. ANOVA for total phenolic content (TPC) response surface quadric model.

Source	Sum of squares	Df	Mean square	F value	p-value Prob>F
Model	360.33	5	72.07	5.53	0.0222*
Temperature (T)	174.46	1	174.46	13.4	0.0081*
Time (τ)	30.2	1	30.2	2.32	0.1716**
T* τ	11.47	1	11.47	0.88	0.3792**
T^2	104.99	1	104.99	8.06	0.0251*
τ^2	5.29	1	5.29	0.41	0.5443**
Residual	91.16	7	13.02		
Lack of Fit	84.54	3	28.18	17.02	0.0097*
Pure Error	6.62	4	1.66		
Cor Total	451.49	12			

* - significant, ** - not significant

Determination coefficient (R^2) of 0.798 showed the adequacy of the model which indicated the model could be a fit to the experimental data. But The predicted R^2 value of -0.815 implies that overall mean is a better predictor of TPC response than the model (25.76 ± 3.61 mg GAE/g extract).

Relationship between independent variable and the TPC is presented in the following equation:

$$TPC\ (mg\ GAE/g\ extract) = 22.19 + 5.39 \cdot T + 2.24 \cdot \tau - 1.69 \cdot T \cdot \tau + 6.17 \cdot T^2 + 1.39 \cdot \tau^2 \quad (4)$$

Predicted values of extraction yield were calculated using a second order polynomial equation (4) and were compared with actual experimental values in Figure 29.

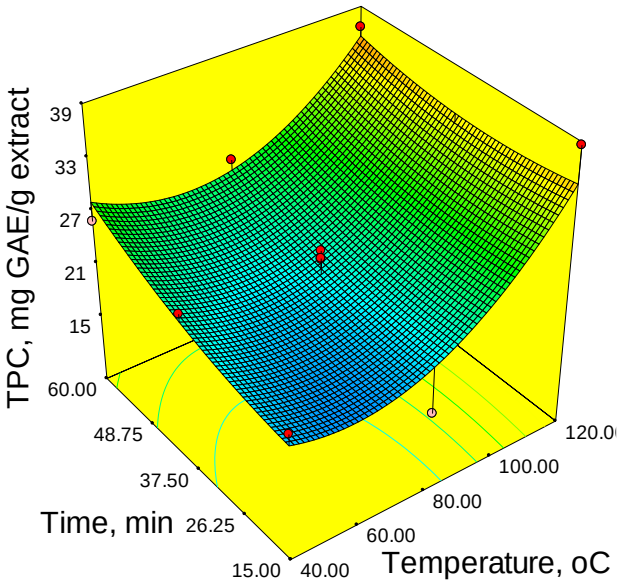


Figure 28. Three-dimensional response surface of influence of extraction temperature and static extraction time on extract total phenolic content (TPC)

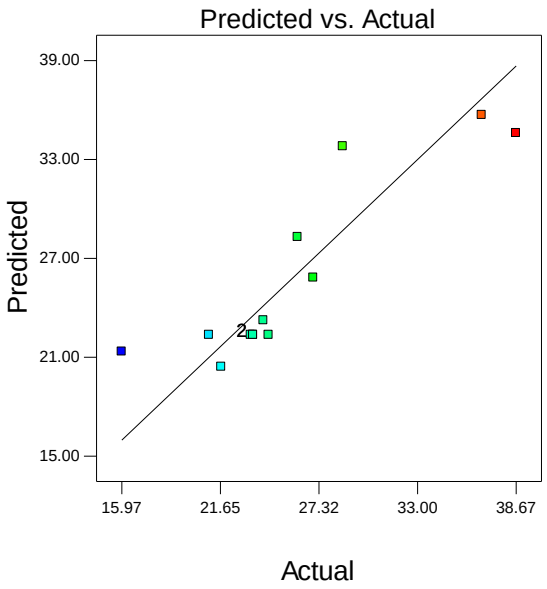


Figure 29. Comparison between actual and predicted extract total phenolic content (TPC) values.

The three-dimensional response surface plot (Figure 28) show the relationship of the TPC and the extraction time, temperature. Model plot reveals interactions between different parameters and evaluate their effect on TPC. It can be seen that temperature has the biggest effect on TPC. Highest total phenolic content (TPC) was reached with 120 ^{o}C temperature and 15 min extraction time (38.667 ± 6.118 mg GAE/g extract), very similar total phenolic content (TPC) was reached with the same temperature, but with 60 min extraction time (36.7 ± 1.647 mg GAE/g extract), only 5% difference between TPC values. Extraction time effect on TPC value is inconsistent in this model. Based on obtained data, temperature has the biggest effect on total phenolic content (TPC) in acetone extracts of *Phaeodactylum tricornutum*, 120 ^{o}C temperature and 15min or 60 min extraction should be used for extract with the highest total phenolic content. As the extraction yield is the highest when extraction time is longer, so for the best results 120 ^{o}C temperature and 60 min extraction time model should be used.

Antioxidant assay (ABTS+) response model was also analyzed, but the was no significance in any of the model terms, model was not significant. But the highest ABTS+ value (522.133 ± 22.458 mM Trolox/g extract) was reached with 120 ^{o}C temperature and 60 min extraction time. For the highest extraction yield, total phenolic content and ABTS+ value acetone extraction of *Phaeodactylum tricornutum* should be performed in high temperature (120 ^{o}C) with long extraction time (60 min).

Plaza *et al.* tested temperature effect on pressurized liquid extraction from *Chlorella vulgaris*, tested the effect of extraction temperature on extraction yield and extract antioxidant activity using acetone as a solvent [87]. It was found that increase in temperature increased the

extract yield, with the highest yield (36.43%) achieved with 200^{o}C as extraction temperature, which is reasonable agreement with this study where increasing temperature increased extraction yield. Plaza *et al.* found that increasing extraction temperature reduces the extract antioxidant activity, highest antioxidant activity was found in extract obtained with 50^{o}C extraction temperature[87]. While in this study with *Phaeodactylum tricornutum*, increasing temperature resulted in increased antioxidant activity and total phenolic content of the extract.

3.3.3. Ethanol extraction

Experimental setup using polar solvent ethanol with two independent variables and their impact on extract yield, TPC and ABTS+ can be seen in Table 12. Ethanol is more polar solvent than acetone, increasing polarity should increases the number of compounds that will be extracted, more polar compounds like polyphenols and pigments. Because of increased polarity of the solvent, alongside extract yield two responses were chosen, total phenolic compound content and antioxidant activity (ABTS+ assay) response. Experimental models with ethanol were evaluated by ANOVA. Not all models for ethanol extraction were significant. Experimentally obtained extract yields from *Phaeodactylum tricornutum* varied from 18.29 ± 0.33 % to 32.49 ± 0.38 %, compared to hexane extracts, yield increased by 93 - 164 %, significant increase. Extraction yields also increased compared to acetone extracts, with lower temperatures and extraction times, yield differences were bigger, ethanol yields were around 60 % higher than acetone. When extraction time and temperature increased, yield differences got smaller, for example when both solvents were used with 120 ^{o}C and 60 min extraction parameters, difference between yields were only 7% ethanol having the higher extraction yield. TPC varied from 28.3 ± 0.553 mg GAE/g extract to 32.667 ± 0.303 mg GAE/g extract, variability of ethanol extracts total phenolic content with different temperatures and extraction times was lower compared to acetone extracts. At lower temperatures and extractions times TPC content of ethanol increased by up to 70%, compared to acetone, but with 120^{o}C and 60 min extraction parameters TPC content in ethanol extract was lower by 16%. In ethanol extracts ABTS+ varied from 340.267 ± 13.984 mM Trolox/g extract to 473.067 ± 5.164 mM Trolox/g extract, the was a similar tendency like with TPC, in ethanol extracts there was an increase of ABTS+ value in lower temperatures and extraction times compared to acetone extracts, variability was also lower in ethanol extracts, but acetone had a higher ABTS+ value with 120 ^{o}C and 60 min extraction parameters, 10 % higher than the ethanol extract.

Table 12. *Phaeodactylum tricornutum* central composite design matrix of two test variables (static extraction time (τ**)** and temperature (T)) and experimental results from the response variables (Yield, TPC and ABTS+) using ethanol as a solvent

Run	**Parameter**		**Response 1**		**Response 2**		**Response 3**	
Nr.	T	τ	Actual Yield	Predicted Yield	Actual TPC	Predicted TPC	Actual ABTS+	Predicted ABTS+
	^{o}C	min.	%	%	mg GAE/g	mg GAE/g	mM Trolox/g	mM Trolox/g
1	80	39	31.23 ± 2.59	29.76	29.567 ± 1.553	29.604	388.267 ± 19.733	384.805
2	120	60	32.14 ± 2.32	31.81	32.667 ± 0.303	32.535	473.067 ± 5.164	476.324
3	80	39	32.08 ± 1.48	29.76	29.1 ± 0.606	29.604	368.533 ± 10.345	384.805
4	80	39	29.25 ± 1.83	29.76	31.3 ± 0.425	29.604	388.267 ± 4.807	384.805
5	40	39	20.19 ± 0.21	21.82	29.7 ± 0.535	29.129	340.267 ± 13.984	348.706
6	80	39	28.09 ± 0.16	29.76	29.567 ± 1.553	29.604	388.267 ± 19.733	384.805
7	120	15	24.39 ± 2.18	23.23	31.033 ± 0.787	30.778	430.267 ± 4.677	418.138
8	80	39	31.29 ± 2	29.76	28.3 ± 0.553	29.604	408 ± 14.404	384.805
9	120	39	26.75 ± 0.32	28.24	31 ± 0.573	31.387	412.8 ± 12.733	421.671
10	80	15	22.47 ± 0.65	24.87	29.233 ± 0.548	29.297	382.133 ± 6.367	406.188
11	80	60	32.49 ± 0.38	33.22	30.733 ± 0.558	30.486	424.4 ± 4.146	417.655
12	40	60	25.58 ± 1.08	25.18	29.367 ± 0.253	29.746	356.267 ± 11.963	359.755
13	40	15	18.29 ± 0.33	17.056	28.933 ± 0.617	29.125	406.933 ± 10.541	395.006

The quadratic regression model analysis for extraction yield from *Phaeodactylum tricornutum* showed that the model was significant ($p<0.05$) and "*Lack of fit*" was not significant ($p>0.1$) relative to the pure error (Table 13). Independent variables, temperature and extraction time had a similar effect on extract yield using ethanol. Temperature had a significant effect ($p<0.05$), extraction time was also significant ($p<0.05$). Interaction between temperature and extraction time was not significant ($p>0.1$). Second order model term T^2 was significant ($p<0.05$), but τ^2 was not significant ($p>0.1$).

Table 13. ANOVA for extraction yield response surface quadric model.

Source	Sum of squares	Df	Mean square	F value	p-value Prob>F
Model	248.55	5	49.71	12.76	0.0021*
Temperature (T)	61.45	1	61.45	15.77	0.0054*
Time (τ)	104.67	1	104.67	26.86	0.0013*
T* τ	0.055	1	0.055	0.014	0.9086**
T^2	61.75	1	61.75	15.85	0.0053*
τ^2	0.53	1	0.53	0.14	0.7237**
Residual	27.28	7	3.90		
Lack of Fit	16.32	3	5.44	1.98	0.2585**
Pure Error	10.96	4	2.74		
Cor Total	275.83	12			

* - significant, ** - not significant

Determination coefficient (R^2) if 0.901 showed the adequacy of the model which indicated the model is a good fit to the experimental data. The predicted R^2 value of 0.489 was not as close to the adjusted R^2 value of 0.831 as one might normally expect, which may indicate a large block effect or a possible problem with the model or data. Model reduction, response transformation maybe be considered.

Relationship between independent variable and the extraction yield is presented in the following equation:

$$Yield\ (\%) = 29.49 + 3.20 \cdot T + 4.18 \cdot \tau + 0.12 \cdot T \cdot \tau - 4.73 \cdot T^2 - 0.44 \cdot \tau^2 \quad (5)$$

Predicted values of extraction yield were calculated using a second order polynomial equation (5) and were compared with actual experimental values in Figure X.

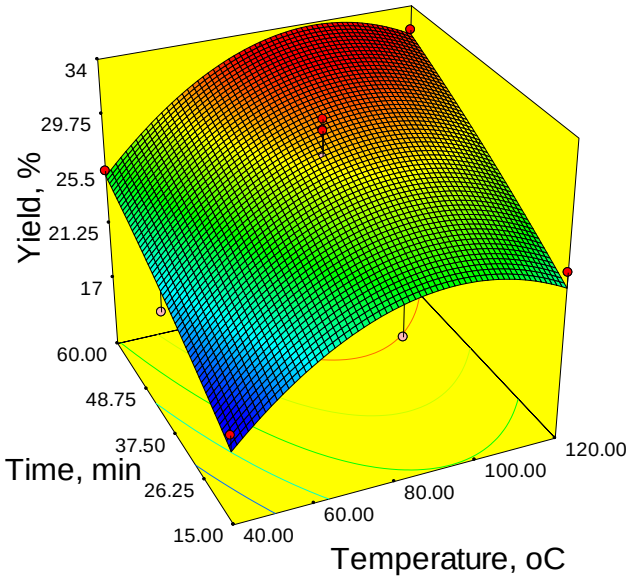


Figure 30. Three-dimensional response surface of influence of extraction temperature and static extraction time on extract yield

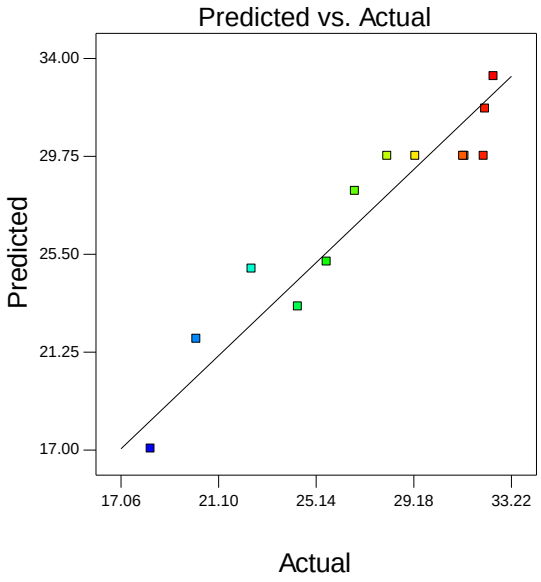


Figure 31. Comparison between actual and predicted extraction yield values.

The three-dimensional response surface plot (Figure 30) show the relationship of the extraction yield and the extraction time, temperature. Model plot reveals interactions between different parameters and evaluate their effect on extraction yield. Both independent variables affected the extraction yield. Highest extraction yield (32.49 ± 0.38%) was reached when the temperature was 80 ^{o}C and extraction time was 60 minutes, virtually the same extraction yield (32.14 ± 2.32 %) was reached with 120 ^{o}C temperature and 60 min extraction time. Extraction time had an effect with all tested temperatures, increasing the extraction time resulted in increased extraction yield. With increased time, temperature increase from 80 ^{o}C to 120 ^{o}C didn't have a significant effect on the extraction yield, counting in the standard deviations extraction yields were almost the same. Based on obtained data it is apparent that extraction from *Phaeodactylum tricornutum* should be done with increased extraction time, preferably 60 minutes and with increased temperature, in the range of 80 – 120 ^{o}C. Cha *et al.* experiment conducted with *Chlorella vulgaris* examined temperature effect on pressurized liquid extract total yield using ethanol as a solvent. Results showed that increasing temperature increased the extraction yield, highest yield (40%) was achieved with the highest extraction temperature (160^{o}C), while with 110^{o}C extraction temperature yield was 30.5% [88]. These results are similar to the findings of this study with *Phaeodactylum tricornutum, where* increasing temperature led to an increase in extraction yield, with 120^{o}C as extraction temperature showed a similar extraction yield of 32.14 ± 2.32 %.

The quadratic regression model analysis for ABTS+ value from *Phaeodactylum tricornutum* showed that the model was significant ($p<0.05$) and "*Lack of fit*" was not significant ($p>0.1$) relative to the pure error (Table 13). Independent variables, temperature and extraction time had a different effect on ABTS+ value using ethanol. Temperature had a significant effect ($p<0.05$), extraction time was not significant ($p>0.1$). Interaction between temperature and extraction time had a significant effect ($p<0.05$). Second order model term T^2 was not significant ($p>0.1$), but τ^2 was significant ($p<0.05$).

Table 14. ANOVA for ABTS+ response surface quadric model.

Source	Sum of squares	Df	Mean square	F value	p-value Prob>F
Model	12322.79	5	2464.56	8.96	0.006*
Temperature (T)	7313.29	1	7313.29	26.59	0.0013*
Time (τ)	197.24	1	197.24	0.72	0.4251**
T* τ	2185.86	1	2185.86	7.95	0.0258*
T^2	0.41	1	0.41	$1.482*10^{-3}$	0.9704**
τ^2	2084.32	1	2084.32	7.58	0.0284*
Residual	1924.94	7	274.99		
Lack of Fit	1146.12	3	382.04	1.96	0.2617**
Pure Error	778.82	4	194.71		
Cor Total	14247.73	12			

* - significant, ** - not significant

Determination coefficient (R^2) if 0.865 showed the adequacy of the model which indicated the model is a decent fit to the experimental data. The predicted R^2 value of 0.0.156 was not as close to the adjusted R^2 value of 0.0.768 as one might normally expect, which may indicate a large block effect or a possible problem with the model or data. Model reduction, response transformation maybe be considered.

Relationship between independent variable and the extraction yield is presented in the following equation:

$$ABTS+ \text{ (mM Trolox/g extract)} = 384.3 + 34.93 \cdot T + 5.73 \cdot \tau + 23.36 \cdot T \cdot \tau + 0.38 \cdot T^2 - 27.62 \cdot \tau^2 \quad (6)$$

Predicted values of extraction yield were calculated using a second order polynomial equation (6) and were compared with actual experimental values in Figure 33.

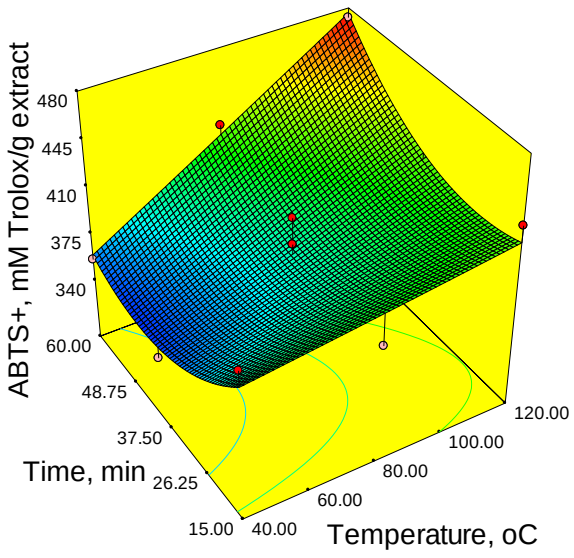


Figure 32. Three-dimensional response surface of influence of extraction temperature and static extraction time on extract ABTS+ value.

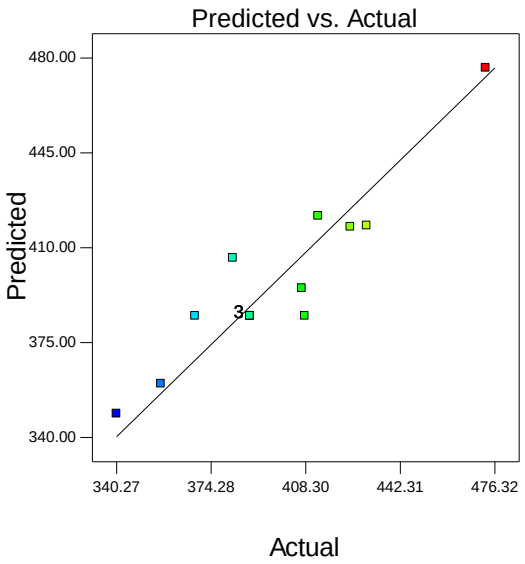


Figure 33. Comparison between actual and predicted extract ABTS+ values.

The three-dimensional response surface plot (Figure 26) show the relationship of the extraction yield and the extraction time, temperature. Model plot reveals interactions between different parameters and evaluate their effect on extraction yield. Temperature had the bigger effect on ABTS+ value. Increasing temperature increased ABTS+ value with all extraction times. Extraction time changes didn't achieve significant differences in ABTS+ value with different temperatures, except for 120 ^{o}C. Highest ABTS+ value (473.067 ± 5.164 mM Trolox/g extract) was reached when temperature was 120 ^{o}C and 60 min extraction time. Based on obtained data it is apparent that extraction from *Phaeodactylum tricornutum* should be done with increased temperature and extraction time to achieve the highest ABTS+ value, preferably 120 ^{o}C and 60 min. The same temperature and extraction time conditions would reach a high extraction yield.

Total phenolic content (TPC) response model was also analyzed, model was not significant, but temperature was a significant model term. The highest TPC value (32.667 ± 0.303 mg GAE/g extract) was reached with 120 ^{o}C temperature and 60 min extraction time. Which was in correlation with the extraction yield model and ABTS+ model results. For the highest extraction yield, total phenolic content and ABTS+ value, ethanol extraction of *Phaeodactylum tricornutum* should be performed in high temperature (120 ^{o}C) with long extraction time (60 min).

Koo *et al.* experiment conducted with microalgae *Chlorella ellipsoidea* optimized pressurized liquid extraction of zeaxanthin using ethanol as a solvent [89]. Different temperatures ,40^{o}C to 160^{o}C, were tested at different extraction times (1min – 24min) and it was found that highest yield (4.23mg/g) of zeaxanthin was with 100^{o}C temperature and 24 minutes of extraction time [89]. Antioxidant activity results of ethanolic zeaxanthin-rich extracts were similar with this study where highest antioxidant activity extracts were obtained with 120^{o}C extraction temperature.

Herrero *et al*. optimized extraction of antioxidants from *Dunaliella salina* by pressurized liquid extraction with ethanol [90]. It was found that temperature had the biggest impact on extraction yield and extracts antioxidant activity. optimum conditions for total yield were 160^{o}C and 30min. and for highest antioxidant activity 160^{o}C and 18min [90]. Similar results were found in this study also, where high yield was found with high extraction temperatures (80-120^{o}C) and the highest antioxidant activity was in extracts obtained with high extraction temperature (120^{o}C. Rodríguez-Meizoso *et al* examined pressurized liquid extraction of bioactive compounds from microalgae *Phormidium* species, reported that highest antioxidant activity extract was achieved with 150^{o}C extraction temperature, whereas100^{o}C temperature extract had very similar but slightly lower antioxidant activity [91] which is in close agreement with this study.

4. RECOMENDATIONS

4.1. Technological scheme for production of *Phaeodactylum tricornutum* extract using pressurized liquid extraction (PLE).

Based on the results from experiments, technological scheme for production of *Phaeodactylum tricornutum* extract using pressurized liquid extraction (PLE) is suggested (Figure 34).

Starting culture inoculate of *Phaeodactylum tricornutum* is prepared *1*. *Phaeodactylum tricornutum* inoculate *1* is added to photobioreactor *5* alongside with culture medium, which can be enriched by glycerol, metallic solution, starting chlorophyll concentration of 0.125 mg/mL is reached in photobioreactor *5*. *Phaeodactylum tricornutum* is cultivated in the photobioreactor *5* till reaches static growth phase. After the end of cultivation, culture is transferred by membrane pump *7* to centrifuge *8* to concentrate biomass. Separated medium is transferred into waste container *9*, concentrated biomass is transferred by membrane pump *10* to a freeze-dryer *11* for drying. Then dry biomass is transferred by screw conveyer *12* to grinder mill *13* to grind dry biomass. Grinded *Phaeodactylum tricornutum* biomass is transferred by screw conveyer *14* to pressurized liquid extraction (PLE) system *17*, desired solvent is supplied to the PLE system *17* from the solvent tank *15* by a membrane pump *16*. After the extraction, extract is transferred by membrane pump *18* to the vacuum evaporator *19* to evaporate the solvent, evaporated solvent is collected in the solvent tank *20*. Evaporated *Phaeodactylum tricornutum* extract is transferred by membrane pump *21* to the nitrogen gas dryer *22* to dry the extract and remove residual water and solvent. Wet nitrogen gas is collected in the gas tank *23*. Dried *Phaeodactylum tricornutum* extract is transferred by screw conveyer *24* to the packaging machine *25* to pack and store the dry *Phaeodactylum tricornutum* extract.

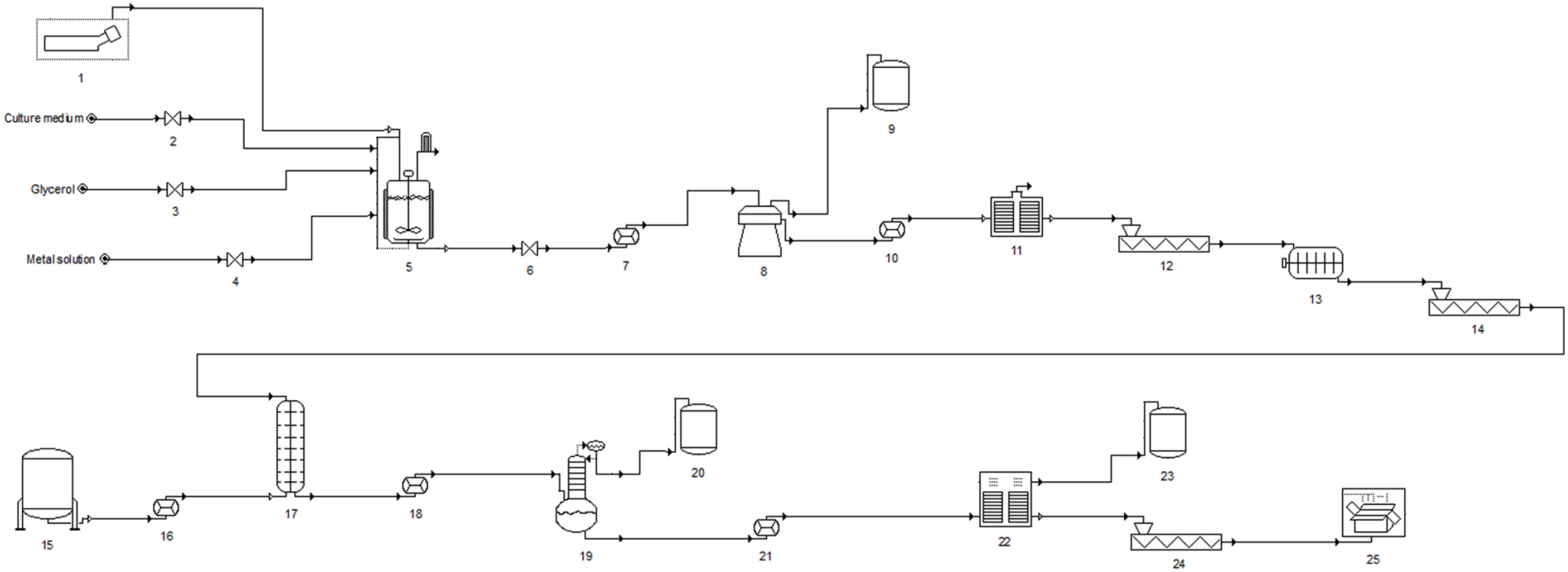


Figure 34. Technological scheme for production of *Phaeodactylum tricornutum* extract using pressurized liquid extraction (PLE): 1 – starting culture of *Phaeodactylum tricornutum*; 2,3,4,6 – gate valve; 5 – photobioreactor; 7,10,16,18,21 – membrane pump; 8 – centrifuge; 9 – waste from centrifuge; 11 – freeze dryer; 12, 14 – transporter; 13 – grind mill; 15 – solvent tank; 17 – pressurized liquid extraction system; 19 – vacuum evaporator; 20 – evaporated solvent collection tank; 22 – nitrogen gas dryer; 23 – wet nitrogen gas collection tank; 24 – transporter; 25 – dry extract packaging.

5. CONCLUSIONS

1. *Phaeodactylum tricornutum* response to copper and iron stress was examined. With high concentrations namely 30mM and 60mM of Cu(II), 50 mM and 100 mM of Fe(II) concentrations the toxic effect was apparent. Under these conditions no growth was observed for *Phaeodactylum tricornutum* culture verifying previous reports where similarly high concentrations of metals led to growth inhibition.
2. *Phaeodactylum tricornutum* cultures grown *w*ith lower concentrations of copper and iron, 250 µM Cu(II) and 500 µM Fe(II), were able to grow, but in smaller cell counts as compared to the control culture. In cultures supplemented with 250 µM of Cu(II) and 500 µM of Fe(II) the cell density decreased by 5% and 16% respectively. The cell count at the final day of experiment (7th day) for control culture was $2.65*10^6 \pm 0.2*10^6$ cells/mL, for cultures supplemented with 250 µM of Cu(II) was $2.53*10^6 \pm 0.36*10^6$ cells/mL, whereas culture grown with 500 µM of Fe(II) concentration showed the smallest cell density of $2.28*10^6 \pm 0.17*10^6$ cells/mL. The highest total phenolic content (8.987 ± 1.694 mg GA/g biomass) was shown with 250 µM of Cu(II) culture, followed by cultures supplemented with Fe(II) (7.629 ± 0.976 mg GA/g biomass) and then control sample (1.488 ± 0.183 mg GA/g biomass). FRAP values ranged from 7.395 ± 0.518 mM Trolox/g biomass to 5.646 ± 0.511 mM Trolox/g biomass to 0.675 ± 0.066 mM Trolox/g biomass. ABTS+ values ranges from increased 5 times, from 4.403 ± 0.437 mM Trolox/g biomass to 22.378 ± 1.462 mM Trolox/g biomass compared to the reference culture.
3. Results showed that *Phaeodactylum tricornutum* was able to utilize glycerol as a carbon source, thus this diatom is capable for cultivation under mixotrophic conditions. Utilization of added carbon source (glycerol) resulted in increased cell density with two glycerol concentrations (0.01M and 0.1M). Cell density increased by 2 times and 2.12 times as compared to the control for cultures grown with 0.01M and 0.1M respectively. Mixotrophic growth also increased total phenolic content and antioxidant activity of the cultivated mixotrophic cultures. The highest increase in all of the assays was observed with 0.1M glycerol treatment. Under these conditions *Phaeodactylum tricornutum's* FRAP value increased by 2.2 times (2.047 ± 0.287 mM Trolox/g biomass), ABTS+ value increased by 1.75 times (6.949 ± 0.571 mM Trolox/g biomass) and TPC value increased by 2.6 times (0.842 ± 0.044 mg GAE/g biomass) as compared to the reference culture. More over mixotrophic growth altered the fatty acid content of *Phaeodactylum tricornutum*, with both glycerol concentrations (0.01M and 0.1M). Total fatty acid content was higher for 0.01M glycerol treated culture, followed by 0.1M glycerol treated culture and then the control.

4. *Cylindrotheca closterium* was also capable of mixotrophic growth. Added glycerol increased and prolonged cell growth, resulting in increased cell density, but not as effective as with *Phaeodactylum tricornutum.* Both concentrations of glycerol (0.01M and 0.1M) had the same effect on *Cylindrotheca closterium,* cell density increased by 1.6 times compared to the reference culture. Mixotrophic growth also increased phenolic content and antioxidant activity of the cultivated mixotrophic cultures. Total phenolic content increased with both glycerol concentrations, there wasn't a significant difference between two concentrations, TPC increased by 1.7 times (0.491 ± 0.044 mg GAE/g biomass) compared to the blank culture. Phenolic content was significantly lower than *Phaeodactylum tricornutum* phenolic content, almost 2 times lower. *Cylindrotheca closterium* FRAP value increased by 2 times (2.023 ± 0.253 mM Trolox/g biomass), ABTS+ value increased by 1.55 times (5.272 ± 0.760 mM Trolox/g biomass) compared to the reference culture. *Cylindrotheca closterium* fatty acid content increased with both glycerol concentrations, but inconsistently, some fatty acids had a higher concentration in culture treated with 0.1M glycerol concentration and others with 0.01M glycerol treated cultures. Looking at the fatty acids totals of both glycerol concentration cultures, there was no significant difference between the two glycerol treated cultures.
5. The lipophilic fraction yield of hexane extracts obtained by pressurized liquid extraction ranged from 6.92 % to 16.77 %, whereas the EPA and DHA content ranged from 183.088 mg/g extract and 1.735 mg/g extract to 351.667 mg/g extract and 3.874 mg/g extract, respectively. Temperature showed the biggest effect on extraction yield and DHA content. Highest extraction yield (16.77 %) was obtained with 120^{o}C and 39 minutes of extraction temperature and time. Highest DHA content (3.874 mg/g extract) was obtained with the maximal temperature (120^{o}C and lowest extraction time (15 minutes). Highest EPA content (351.667 mg/g extract) was obtained with 80^{o}C and 15 minutes of extraction temperature and time.
6. The polar fraction yield of acetone extracts obtained by pressurized liquid extraction ranged from 11.04% to 30.39%, while the TPC and ABTS+ values ranged from 15.967 mg GAE/g extract and 288.267 mM Trolox/g extract to 38.667 mg GAE/g extract and 522.133 mM Trolox/g extract. Extraction temperature had the bigger influence than extraction time. Highest extraction yield (30.39 %) was obtained with 120^{o}C and 60 minutes extraction temperature and time. Highest total phenolic content (TPC) (38.667 mg GAE/g extract) was obtained with 120^{o}C and 15 min extraction temperature and time. Highest ABTS+ value (522.133 mM Trolox/g extract) was obtained with maximal temperature (120^{o}C) and highest extraction time (60 minutes).

7. The polar fraction yield of ethanol extracts obtained by pressurized liquid extraction ranged from 18.29% to 32.49%, while the TPC and ABTS+ values ranged from 28.3 mg GAE/g extract and 340.267 mM Trolox/g extract to 32.667 mg GAE/g extract and 473.067 mM Trolox/g extract. Extraction temperature and extraction time had similar effects. Highest extraction yield (32.49%) was obtained with 80^{o}C and 60 minutes extraction temperature and time. Highest total phenolic content (TPC) (32.667 mg GAE/g extract) was obtained with 120^{o}C and 60 min extraction temperature and time. Highest ABTS+ value (473.067 mM Trolox/g extract) was obtained with maximal temperature (120^{o}C) and highest extraction time (60 minutes).
8. From obtained experimental results *Phaeodactylum tricornutum* extract production with pressurized liquid extraction (PLE) principal technological scheme was suggested

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