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RESEARCH PAPER

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# Bioactive Compounds Produced by *Dunaliella* species, Antimicrobial Effects and Optimization of the Efficiency

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## Abstract

In the present work, production of bioactive compounds by four different *Dunaliella* species (*Dunaliella* sp. 1-4) and antimicrobial activities of them was investigated against different bacteria. The effects of different nitrogen concentrations (0.5 g/L-2.0 g/L), illumination intensities (1200lx-4800lx), NaCl concentrations [10-25%(w/v)], and incubation periods (7-28 d) on the content of the bioactive compounds and their antimicrobial activities were studied. The highest antimicrobial activity of bioactive compounds was observed in *Dunaliella* sp. 2; optimum conditions for the highest antimicrobial activity were found extracts obtained from biomasses grown in media with 1.0 g/L nitrogen and 20% (w/v) NaCl, under 4800 lx light intensity, after 14 d of incubation period. Different solvent types [ethanol, methanol, hexane, chloroform, Tris-HCl (pH:8; 0.5 M), and water] were also investigated to find more efficient antimicrobial activity. Chloroform-extracts had the highest bioactive character within the solvents tested. The chloroform-extracts were chemically characterized by GC–MS and HPLC-DAD. Stearic acid having the highest peak area as fatty acids found in *Dunaliella* sp. 2 was detected. However, pigments such as lutein followed by α-carotene and zeaxanthin with antimicrobial activity were identified; simple phenols like ferulic acid responsible for the antimicrobial activity was analysed.

## Introduction

Microalgae are one of the cheapest organisms that can be used in different biotechnological applications (Demirel, Yılmaz, Ozdemir, & Dalay, 2018). Microalgae can produce several compounds like chlorophyll, carotene, phenolics, proteins, and fatty acids (Rao, Reddy, & Aradhya, 2010). These natural compounds are bioactive substances having usage area as sources of new pharmaceuticals and other biotechnological products. Investigation of bioactive compounds having antibacterial, antiviral, antifungal, antioxidant, anticancer, anti-inflammatory, antimutagen, antidiabetic properties in microalgae is among the most popular topics (Gamal, 2010). The usage of extremophile microalgae in biotechnological applications like production of bioactive compounds has much greater potential. Such microorganisms maintain their vitality by producing more stable materials in adapting to the changing environmental conditions. Among extremophile microalgae, *Dunaliella* species are capable of maintain their survival under high salt concentrations up to 35% and known as the only eukaryotic photosynthetic organism that has been found in some concentrated saline lakes (Ben-Amotz & Avron, 1990). Under stress conditions, extremophile

microalgae might produce unique substances to adapt the changing condition. Thus, with changing environmental requirements, the content of the bioactive compounds might also be changed. It is known that algae had a chemical defence system being synthesized to survive in a competitive environment (Barros, Pinto, Sigaud-Kutner, Cardozo, & Colepicolo, 2005)

Extracts of *Dunaliella* species have been investigated having antimicrobial activity against several microorganisms. Chang et al. (1993) showed that a crude extracts of *D. primolecta* had antimicrobial activity against *Staphylococcus aureus, Bacillus cereus, Bacillus subtilis* and *Enterobacter aerogenes* and these extracts contained substances having antibiotic properties. *D. primolecta* was also investigated by Ohta, Shiomi, Kawashima, Aozasa, & Nakao (1995); in that study, it was found that microalga produced bioactive compounds against to *S. aureus* (MRSA); methanol extracts of microalga included linolenic acid which was bioactive. Mendiola *et al.,* (2008) studied the effects of different temperatures and pressures on the $CO_2$ extracts from *D. salina* and their antimicrobial activities; researchers pointed out that indolic derivate was found in the microalgal extracts having antimicrobial activity. Srinivasakumar & Rajashekhar (2009) performed experiments with *D. salina* and used different solvents to obtain microalgal extracts. In that study, they observed the most efficient bioactive compound in buthanol extracts. Krishnakumar, Bai, & Rajan (2013) cultivated *D. salina* to find antimicrobial activity of bioactive metabolites produced by this microalga; they found that extracts with chloroform and methanol mixture had the most efficient antimicrobial activity.

The effects of media and environmental conditions on antimicrobial activity of *D. salina* were not investigated in previous articles investigating the antimicrobial effect of *D. salina*. In these works, efficiency of antimicrobial activity was found lower than found in other algal extracts. In a previous study done by Krishnakumar, Bai, & Rajan (2013) with *D. salina*; environmental conditions like pH, temperature and salinity were studied as an effector factor affecting antimicrobial activity. But, in that study, antimicrobial activity was found similar that found in other studies explained as above.

Our group isolated *Dunaliella* species producing high biomass and showing resistance to various pollutants, and these species were used to investigate their antimicrobial activity capacities against bacteria in the current study. It was aimed to find new bioactive compounds in these species responsible for the antimicrobial effect. Since the synthesis of these compounds is directly related to exposure to stress, the effect of conditions as nitrogen concentration, light intensities, salt concentration, and incubation period are optimized for the highest efficient bioactive compound production by *Dunaliella* spp.

## Materials and Methods

### Microalgae, Growth Conditions and Selection

In this study, four different *Dunaliella* strains (*Dunaliella* sp. 1, *Dunaliella* sp. 2, *Dunaliella* sp. 3, and *Dunaliella* sp. 4) were obtained from Ankara University, Faculty of Science Laboratories' current culture collection (Dönmez & Aksu, 2002). *Dunaliella* strains were cultivated in 250 ml Erlenmeyer flasks including 100 ml of Johnson's medium (pH: 7.5) (Johnson, Johnson, MacElroy, Speer, & Bruff, 1968) at 30 °C, under continuous light intensity as 2400 lx at a growth chamber (BINDER, model: KBW 400 (E5.1), S.no: 15-13640) for 14 days.

Microalgae used in the study were tested to determine having the most effective bioactive compound. For these experiments, four different strains were inoculated in Johnson's medium and at the end of incubation period the effectiveness of bioactive compounds were determined. From these trials, *Dunaliella* sp. producing the most effective bioactive compound was found and further trials were done with this microalga.

### Production of Bioactive Compounds

Different environmental conditions could affect bioactive compound production by microalgae. Bioactive compounds having different contents might be produced under stress conditions (Skjånes, Rebours, & Lindblad, 2013). Therefore, production of bioactive compounds by *Dunaliella* species under different environmental conditions was investigated with regard to increasing nitrogen concentrations, different light intensities, NaCl concentrations, and different incubation periods. Unless other stated, microalgae were cultivated in Johnson's medium at 30 °C, under continuous light intensity as 2400 lx at a growth chamber for 14 days.

### Effect of Nitrogen Concentration On Bioactive Compound Production

In these experiments, nitrogen concentrations were used in media as 0.5 g/L, 1.0 g/L, 1.5 g/L, and 2.0 g/L.

### Effect of NaCl Concentration on Bioactive Compound Production

To determine the effect of different NaCl concentrations on the production of bioactive compounds by *Dunaliella* sp., microalgae was cultivated in Johnson's media with increasing NaCl concentrations as 10% (w/v), 15% (w/v), 20% (w/v), and 25% (w/v).

**Effect of Light Intensity on Bioactive Compound Production**

To find the most effective bioactive compound produced by *Dunaliella* sp. under different light intensities, experiments were done at 1200 lx, 2400 lx, 3600 lx, and 4800 lx.

**Effect of incubation period on bioactive compound production**

To understand the effect of incubation period onto bioactive compound production by *Dunaliella* sp., trials were performed with incubation period as 7, 14, 21, and 28 days.

**Effect of Different Solvent Type on Bioactive Compound Extraction**

It is known that bioactive compounds, which were obtained with using different types of solvents, the efficiencies of them are changing. Because of that, in this study solvents as ethanol, methanol, hexane, chloroform, Tris-HCl (pH:8; 0.5 M), and water were used to obtain bioactive compounds produced by microalga.

**Determination of Effectiveness of Bioactive Compounds**

In these trials, at the end of the incubation period, biomass was collected by centrifugation (MPW-351R) at 10.000 rpm for 5 min. Obtained biomasses were freeze-dried (Millrock Technology, Inc., Kingston, NY 12401, USA) for overnight and 1 gram of dried biomass was exposed to 3 ml ethanol solvent with a purity of 96%. After incubation for 1 hour, the mixture was centrifuged for 10.000 rpm for 5 min and supernatant was used as algal extract. These solutions were kept at 4**°**C and used within 2 days (Rao, Reddy, & Aradhya, 2010; Pradhan *et al.,* 2012).

Microalgal bioactive compound effectiveness was designated with antimicrobial activity. Antimicrobial activity was determined by disc diffusion method (Murray, Baron, Apfalle, Tenover, & Yolke, 1995). For this purpose, standard bacterial strains like *Bacillus subtilis* ATCC 6633, *Brochothrix thermosphacta* ATCC 11509, *Escherichia coli* 0157:H7 ATCC 35150, *E. coli* ATCC 25922, *Enterobacter cloacae* ATCC 700323, *S. aureus* ATCC BAA 976, *S. aureus* ATCC 25923, *S. aureus* ATCC 1026 were used. These bacteria were cultivated in Nutrient Broth for 24 h, and were inoculated uniformly using sterile cotton swab onto Nutrient Agar to test the antibacterial activities of microalgal extracts. Algal extracts were applied to sterile disks (40 µl/per disc) and impregnated disks were placed on the plates using sterile forceps properly spaced at equal distance. Paper discs loaded with ethanol were also checked for its effect against the tested bacteria. The plates were stored for 2h to allow of the extracts into the agar. Then, these plates were incubated for 24 h at 30 °C for growth of bacterial strains. The zone of inhibition was measured and expressed in mm in diameter.

**Chlorophyll Analysis**

The chlorophyll (a + b) concentration in the media was determined in buffered aqueous 80% acetone solution. The concentration of chlorophyll was found with recording optical absorption at 646.6 and 663.6 nm by using Shimadzu UV 2001 model spectrophotometer (Japan) according to the equation given as below (Porra, Thompson, Kreidemann, 1989).

$$\text{Chlorophyll (a + b)} \left(\frac{\mu g}{ml}\right) = (17.76 \times A_{646.6}) + (7.34 \times A_{663.6})$$

**HPLC-DAD Analysis of the Extracts**

The pigment composition and polar compounds were analysed for the extracts having the highest bioactive property. Pigment composition analysis was done by HPLC using an Agilent 1260 Liquid Chromatograph equipped with a DAD. The separation was carried out in a (zorbax) C18 column (150mm×3.9mm, 4 µm particle size) from ACE. The mobile phase was a mixture of solvent A (methanol/ammonium acetate 0.1N; 7:3) and solvent B (methanol) at 0.9 mL/min according to the following step gradient: 25% B at initial conditions, changing to 50% in 1 min, and reaching 100% B at minute 20 at a wavelength of 450 nm. The identification of peaks was performed with comparison with data appearing in the literature.

Polar compounds were analysed with HPLC using an Agilent 1260 Liquid Chromatograph equipped with a DAD. The mobile phase was a mixture of solvent A (water/acetic acid 95:5) and solvent B (acetonitrile 100%) with a flow rate as 0.9 mL/min at 280 nm wavelength. According to the following step gradient lasting for 30 min, starting from 1% B, changing to 2% B at 6 min, increasing to 100% B at 20 min and keeping 100% B constant for the remainder of the 30 min run. The identification of peaks was performed with comparison with data appearing in the literature.

**GC-MS Analysis for Fatty Acids**

The fatty acid methyl ester analysis was done for the extracts having the highest bioactive property. Microalgal extracts were mixed with 0.1 M KOH dissolved in methanol, and then added hexane for transesterification. After transesterification step, 1 µL sample was taken from the upper phase and the methylated fatty acids were analysed by the GCMS-QP2010 Ultra gas-chromatograph (Shimadzu, Japan). The condition of GCMS-QP2010 Ultra analysis was as follows: flame ionization detector (FID) 250°C; column

SP-2560, 100 m × 0.25 mm × 0.20 µm (Sigma-Aldrich); carrier gas He. Fatty acid peaks were identified against the chromatogram of a mixed fatty acid methyl ester standard (37 Comp. FAME Mix 10 mg/mL in $CH_2Cl_2$; Supelco, USA).

## Results and Discussion

### Selection of *Dunaliella* sp.

To find *Dunaliella* sp., which produced the most effective bioactive compound, four different *Dunaliella* strains were tested. The results obtained from these series of the experiments were given in Figure 1. *Dunaliella* sp.1 had the highest antimicrobial activity to *S. aureus* ATCC BAA 976 with an inhibition zone of 13 mm. On the other hand, *Dunaliella* sp. 2 produced the most efficient bioactive compounds against *B. subtilis* ATCC 6633 and *E. coli* 0157:H7 ATCC 35150 with inhibition zones 18 mm and 14 mm, respectively. *Dunaliella* sp. 3 had also efficient bioactive compound and the highest antimicrobial activity was found 14 mm against *B. subtilis* ATCC 6633. *Dunaliella* sp. 4 had antimicrobial activity against only two bacteria (*B. thermosphacta* ATCC 11509 and *E. coli* 0157:H7 ATCC 35150; both inhibition zones 12 mm). According to these trials, further experiments were performed with *Dunaliella* sp. 2 related to its highest antimicrobial activity.

### Bioactive Compound Production by *Dunaliella* sp. 2 Cultivated in Media with Different Nitrogen Concentrations

To examine the effect of different nitrogen concentrations onto production of bioactive compounds by *Dunaliella* sp. 2, microalga was inoculated in to Johnson media with 0.5 g/L, 1.0 g/L, 1.5 g/L, and 2.0 g/L nitrogen concentration. The data were given in Figure 2a. Extracts obtained from microalga grown in media with 0.5 g/L nitrogen, the highest antimicrobial activity was shown against *E. coli* 0157:H7 ATCC 35150 and *E. cloacae* 700323 (inhibition zones 13 mm). When the microalgae were grown in medium containing 1 g/L of nitrogen, the extract prepared form that biomass showed the highest antimicrobial effect, producing an 18 mm inhibition zone against *B. subtilis* ATCC 6633 bacteria. The highest antimicrobial activity was determined against *E. coli* ATCC 25922, *E. cloacae* ATCC 700323, and *S. aureus* ATCC BAA 976 bacteria (inhibition zones: 12 mm) with the extracts obtained from biomasses in which *Dunaliella* sp. cultivated in media with a nitrogen concentration of 1.5 g/L. On the other hand, when the nitrogen concentration was 2 g/L, extracts from microalgal biomass showed the highest antimicrobial activity against *B. subtilis* ATCC 6633, *E. coli* 0157: H7 ATCC 35150 and *S. aureus* ATCC BAA 976 bacteria with producing 12 mm inhibition zones.

In the current study, antimicrobial activity of biomasses obtained in media with nitrogen concentrations ranging from 0.5 g/L to 2.0 g/L. To our knowledge, such an approach has not been found in the literature. Previous studies on nitrogen limitation generally related to the increment of certain fatty acids accumulation when nitrogen concentration decreased (Byrd, Burkholder, & Zimba, 2017). On the other hand, in another previous study, *D. salina* was cultivated in media with nitrogen-free and 250 mM nitrogen; extracts from the biomass obtained from nitrogen-containing medium were more effective against the tested cancer cells then extracts obtained from nitrogen-free medium (Singh, Baranwal, & Reddy, 2016).

In these experiments, with an increase in nitrogen concentration from 0.5 g/L to 1.0 g/L, antimicrobial effect also increased for 63% of the tested bacteria. According to these results, further experiments were done in media with 1.0 g/L nitrogen related to extracts having the highest bioactive property.

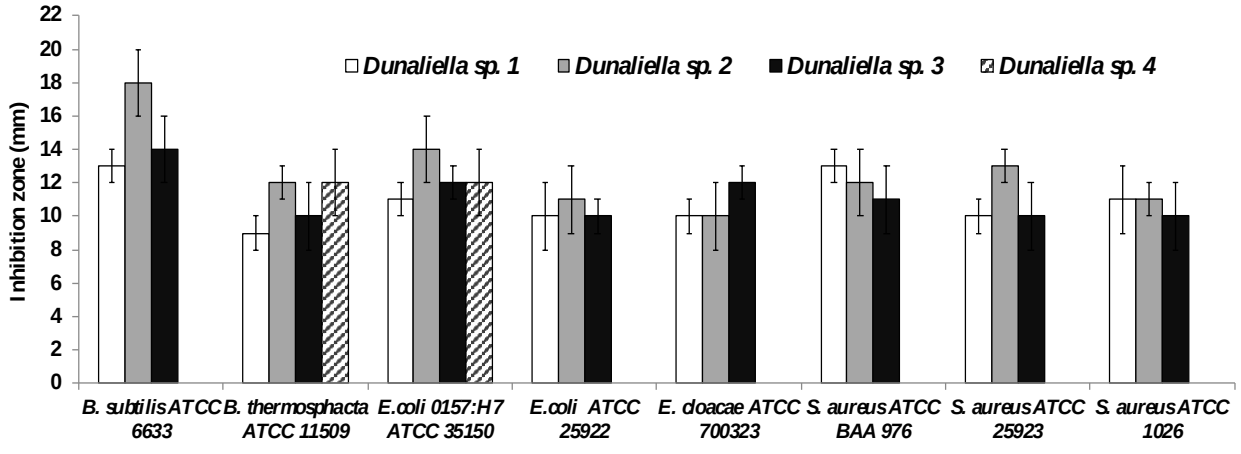


**Figure 1.** Antimicrobial activity [zone of inhibition (mm)] of bioactive compounds extracted from *Dunaliella* species (T: 30 °C; N concentration: 1 g/L; NaCl concentration: 10% (w/v); light intensity: 2400 lx; incubation period: 14 d).

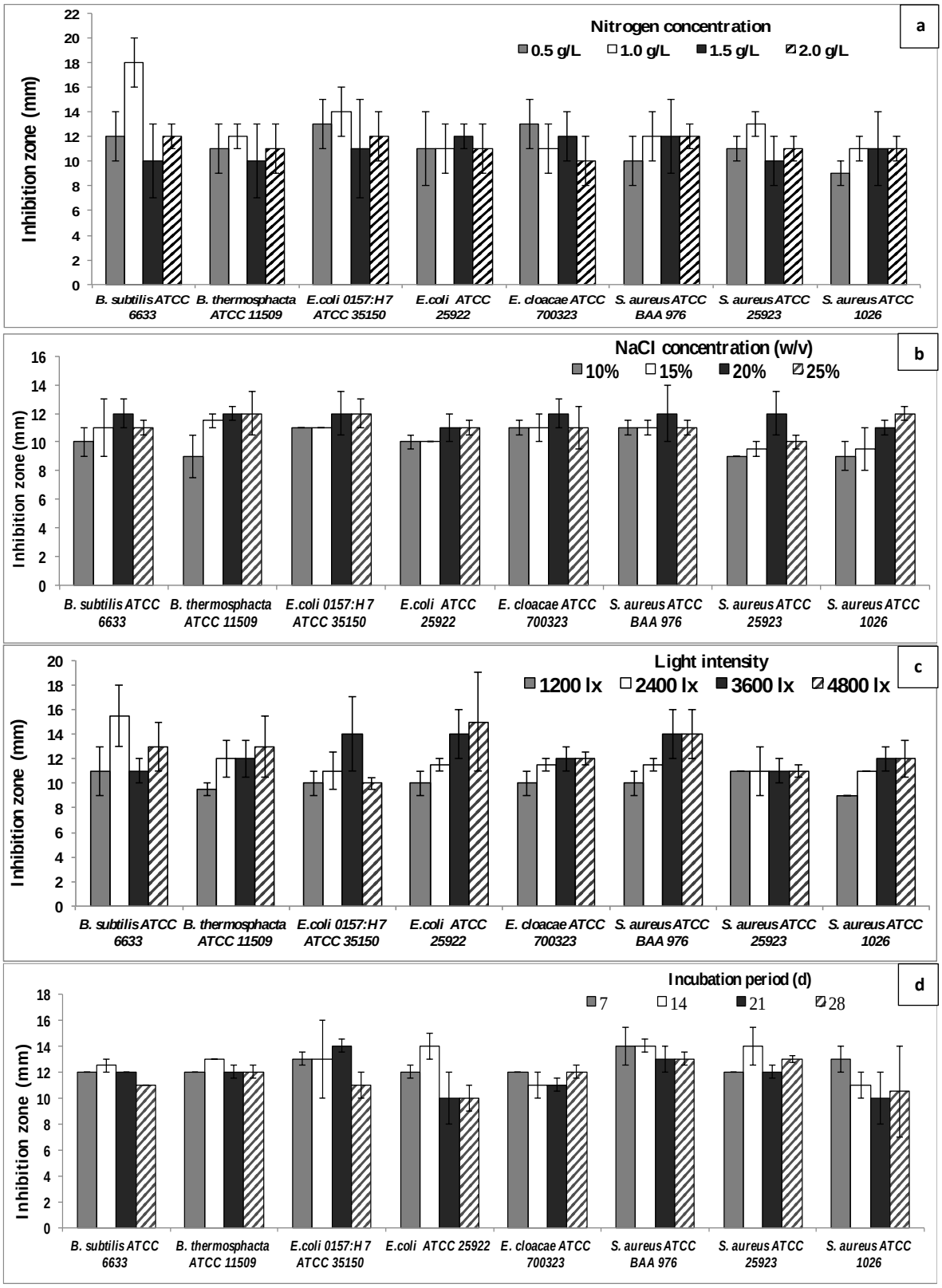


**Figure 2.** Antimicrobial activities [zone of inhibition (mm)] of bioactive compounds extracted from *Dunaliella* sp. 2 under different conditions (T: 30°C) (a) The effect of nitrogen concentrations (b) The effect NaCl concentrations (c) The effect of light intensities (d) The effect of incubation periods

Chlorophyll contents of the microalga were found adaptable with antimicrobial activities determined. Microalga had the highest chlorophyll content and bioactive property when it was cultivated in media with 1.0 g/L nitrogen. The amounts were 18.9 µg/ml, 26.9 µg/ml, 18.5 µg/ml, and 17.5 µg/ml when *Dunaliella* sp. 2 was cultivated in media under 0.5 g/L, 1.0 g/L, 1.5 g/L, and 2.0 g/L nitrogen concentrations, respectively.

**Bioactive Compound Production by *Dunaliella* sp. 2 Cultivated in Media with Different NaCl Concentrations**

With changing environmental conditions, contents of bioactive compounds produced by *Dunaliella* sp. 2 might also change. Thus, in these serial experiments, microalgae inoculated into media including 10-25% (w/v) NaCl. Extracts obtained from *Dunaliella* sp. 2 cultivated in media with 10%(w/v) had effective bioactive character against to *E. coli* 0157: H7 ATCC 35150, *E. cloacae* ATCC 700323 and *S. aureus* ATCC BAA 976 with inhibition zones as 11 mm (Figure 2b). In addition to this, under the same conditions, obtained extracts had lower inhibition zones against other bacteria tested. When NaCl concentration was increased to 15% (w/v), antimicrobial activity of the bioactive compounds extracted from microalga had the highest effectiveness to *B. thermosphacta* ATCC 11509 with an inhibition zone as 12 mm. With an increase in NaCl concentration to 20%(w/v), bioactive compounds produced by the tested microalga showed higher antimicrobial activity to all the bacteria used with an exception *B. thermosphacta* ATCC 11509. Under these conditions, antimicrobial activity was constant against to that bacterium. It was observed that extracts produced by *Dunaliella* sp. 2 in Johnson media with the highest NaCl concentration (25%(w/v) had slightly less or constant effective bioactive properties. The maximum inhibition zone was found 12 mm against *B. thermosphacta* ATCC 11509, *E. coli* 0157: H7 ATCC 35150 and *S. aureus* ATCC 1026.

At the end of these trials, it was determined that with an increase in NaCl concentration from 10%(w/v) to 20%(w/v), antimicrobial effect also increased for 100% of the tested bacteria. At the end of these experiments, further experiments were carried out in media with 20%(w/v) NaCl.

Under salt stress, *Dunaliella* sp. 2 had more efficient bioactive compounds, thus, antimicrobial activity increased. The bioactive compound by the tested microalga under salt stress might contain more carotenoid and fatty acids then it was under low salt conditions. Some previous studies also showed similar results. Xu & Beardall (1997) showed that with an increase in NaCl concentration from 0.4 to 4 M, saturated and monounsaturated fatty acids of *Dunaliella* sp. also increased. It was previously shown that fatty acids from microalga species were responsible for antimicrobial activity (Benkendorff, Davis, Rogers, & Bremner, 2005).

Chlorophyll contents of the microalga were 19.9 µg/ml, 21.1 µg/ml, 22.5 µg/ml, and 16.5 µg/ml when *Dunaliella* sp. 2 was cultivated in media with 10%(w/v), 15%(w/v), 20%(w/v) and 25%(w/v) NaCl concentrations, respectively. BenMoussa-Dahmen, Chtourou, Rezgui, Sayadi, & Dhouib (2016) showed that with an increase in salt concentration up to 3 M, *Dunaliella* sp. had more chlorophyll like in the current study. In that study, it was also found that because of salt osmotic stress, photosynthesis decreased and at the highest salinity, reduction of chlorophyll content was occurred. Similar effect was shown by Kirrolia, Bishnoi, & Singh (2011).

**Bioactive Compound Production by *Dunaliella* sp. 2 Cultivated in Media with Different Light Intensities**

In these experiments, when light intensity was changed effectiveness of bioactive compounds also varied. In Figure 2c**,** it was clearly seen that under 4800 lx light intensity, *Dunaliella* sp. 2 produced the most effective bioactive compounds against all the bacteria tested. When microalga cultivated at 1200 lx light intensity, the highest antimicrobial activity was shown against *B. subtilis* ATCC 6633 and *S. aureus* ATCC 25923 with an inhibition zones as 11 mm, while the lowest inhibition zone was 9 mm against *S. aureus* ATCC 1026. With bioactive compounds obtained from microalga grown at light intensity as 2400 lx, the highest antimicrobial activity was found as 15.5 mm against *B. subtilis* ATCC 6633. Under the same light intensity, bioactive compounds showed the lowest antimicrobial activity against *E. coli* 0157: H7 ATCC 35150, *S. aureus* ATCC 25923 and *S. aureus* ATCC 1026 (inhibition zones: 11 mm). *Dunaliella* sp. 2 was also grown at 3600 lx intensity and bioactive compounds produced under these conditions had the highest efficiency against *E. coli* 0157:H7 ATCC 35150, *E. coli* ATCC 25922 and *S. aureus* ATCC 976 with the same inhibition zone as 14 mm, while the lowest antimicrobial activity was 11 mm to *B. subtilis* ATCC 6633 and *S. aureus* ATCC 25923. When light intensity was increased to 4800 lx, *Dunaliella* sp. 2 produced its most effective bioactive compound against *E. coli* ATCC 25922 (inhibition zone: 15 mm). On the other hand, under the same conditions antimicrobial activity was its lowest value with an inhibition zone as 10 mm towards *E. coli* 0157: H7 ATCC 35150. In these serial experiments, it was determined that with an increase in light intensity from 1200 lx to 4800 lx, antimicrobial effect also increased for 63% of the tested bacteria. At the end of these experiments, subsequent trials were done under 4800 lx light intensity.

The higher antimicrobial effect was related to the content of bioactive compound produced in stress condition. In the current study, *Dunaliella* sp. 2 produced more efficient bioactive compounds under high illumination than in low light condition. Chlorophyll

contents of the microalga were 21.8 µg/ml, 21.0 µg/ml, 19.1 µg/ml, and 17.6 µg/ml when *Dunaliella* sp. 2 was cultivated in media under 1200 lx, 2400 lx, 3600 lx and 4800 lx light intensities, respectively.

High light intensity can cause strong harmful effects in the cell due to excessive stimulation of the photochemical apparatus (Skjånes, Rebours, & Lindblad, 2013). Under high light stress, chlorophyll can interact with oxygen to cause the formation of reactive oxygen species (ROS). Algae had defense systems against these ROS, of these, increasing to produce carotenoids was the most common one. On the other hand, Seepratoomrosh et al. (2016) demonstrated that under low light conditions, the chlorophyll content of *D. tertiolecta* was higher than in high light condition. In a study done by Simionata et al. (2011), under low light, cells of *Nannochloropsis gaditana* accumulated more chlorophyll than it was in high light. Since chlorophyll is essential for light harvesting (Li, Wakao, Fischer, & Niyogi, 2009), in low light the increment of the pigment was expected. It was known that carotene had antioxidant properties, while, phenolic compounds as a photo protective response were be useful as antimicrobial (Skjånes, Rebours, & Lindblad, 2013). *Dunaliella* sp. 2 most probably produced more phenolic compounds under high light and therefore antimicrobial activity was found more efficient under these conditions.

**Bioactive compound production by *Dunaliella* sp. 2 Cultivated in Different Incubation Periods**

In Figure 2d antimicrobial activities of bioactive compounds extracted from *Dunaliella* sp. 2 cultivated with different incubation periods were summarized. When microalga was grown with an incubation period as 7 days, obtained extracts showed the highest antimicrobial activity against *S. aureus* ATCC BAA 976 (inhibition zone: 14 mm). The most effective bioactive compound was produced by microalga after incubation for 14 days. Under these conditions, inhibition zone was found 14 mm against *E. coli* ATCC 25922, *S. aureus* ATCC BAA 976 and *S. aureus* ATCC 25923. In trials which *Dunaliella* sp. 2 was cultivated for 21 days, microalgal extracts had their highest antimicrobial activity towards *E. coli* 0157: H7 ATCC 35150 (inhibition zone: 14 mm). After incubation for 28 days, bioactive compounds obtained from *Dunaliella* sp. 2 had the maximum antimicrobial activity against *S. aureus* ATCC BAA 976 and *S. aureus* ATCC 25923 with an inhibition zone as 14 mm.

Bioactive compounds by microalgae can be sourced from primary metabolism, such as proteins, fatty acids, vitamins, and pigments, or can be produced from secondary metabolism (de Morais, Vaz, de Morais, & Costa, 2015). Therefore, the period of cultivation is an important parameter in producing bioactive compounds. Noaman, Fattah, Khaleafa, & Zaky (2004) performed that bioactive compounds from *Synecoccus leopoliensis* had the most efficient antimicrobial effect with extracts obtained when cyanobacteria was cultivated with an incubation period for 14 or 15 days. In the current study, it was also observed that *Dunaliella* sp. 2 had the most effective bioactive compound when microalga was grown for 14 days.

The highest chlorophyll amount was also found after incubation for 14 days. Chlorophyll contents of the microalga were 14.0 µg/ml, 21.1 µg/ml, 20.6 µg/ml, and 19.8 µg/ml when *Dunaliella* sp. 2 was cultivated in media under different incubation periods as 7 d 14 d, 21 d and 28 d, respectively. These results showed that the effectiveness of microalgal bioactive compounds was slightly affected from culture age. The most effective compounds were produced after incubation for 14 days. Therefore, incubation period was optimized as 14 d.

**Bioactive Compounds Obtained with Different Solvents**

Figure 3 showed antimicrobial activities of algal bioactive compounds obtained with different solvents. These extracts were tested against *B. subtilis* ATCC 6633 and *E. coli* 0157:H7 ATCC 35150 which microalgal extracts had the highest effect. For both bacteria methanol and chloroform extracts had the maximum antimicrobial activity; the inhibition zones were 20 mm and 19 mm against *B. subtilis* ATCC 6633, respectively. In addition to this, bioactive compounds obtained with chloroform formed 22 mm inhibition zone against *E. coli* 0157:H7 ATCC 35150, while methanol extract had an inhibition zone as 18 mm. Ethanol, methanol and hexane extracts had more activity against *B. subtilis* ATCC 6633, however, chloroform extracts had the highest effectiveness against *E. coli* 0157:H7 ATCC 35150. Lower antimicrobial activities were found for extracts obtained with Tris-HCI and water.

Several factors affect the yield of pigments extraction; of these the solvent to be used comes first. In the current study, methanol and chloroform were found the best organic solvents to obtain bioactive compounds from *Dunaliella* sp. 2. In a previous study, methanol and chloroform extracts of *D. salina* had also the most efficient antimicrobial activity like in the current study. On the other hand, Srinivasakumar & Rajashekhar (2009) showed buthanol extracts of *D. salina* had the most efficient bioactive compound. Herrero, Jaime, Martin-Alvarez, Cifuentes, & Ibanez (2006) showed that water extracts of *D. salina* had lower bioactive properties than it was obtained from organic solvents as it was in the current study.

**GC-MS Analysis for Fatty Acids**

In order to find the fatty acid profile of *Dunaliella* sp. 2, chloroform-extracts of the microalga showing the highest bioactive property were analysed. Table 1 shows

the fatty acid identification, the peak area contribution and the concentration of fatty acids of the microalgal extracts. As it was summarized in Table 1, stearic, capric, palmitic and oleic acids were determined to be the main fatty acids according to the chromatographic area as nearly 80% in total.

In previous studies, it was mentioned that antimicrobial activity of microalgae has been usually related with long-chain unsaturated fatty acids like oleic, linoleic, and linolenic acids (Benkendorff, Davis, Rogers, & Bremner, 2005). In the current study, a saturated fatty acid (stearic acid) was found to be the highest chromatographic area in chloroform-extracts. Thus, antimicrobial activity against bacteria might be caused by the presence of compounds rather than fatty acids. Mendiola *et al.,* (2008) also found similar observations in *D. salina* extracts as they determined saturated fatty acids like palmitic and stearic, acids having the highest chromatographic area with lower antimicrobial activity than their other samples with unsaturated fatty acids.

**HPLC-DAD Analysis of the Extracts**

In the present study, pigment profiles and polar compounds of chloroform-extracts were analysed by HPLC-DAD. In *Dunaliella* sp. 2 chloroform-extracts, it was observed that major component of carotenoid was lutein with its highest chromatographic area followed by α-carotene and zeaxanthin (Table 2). It is well known that *Dunaliella* species are good sources of β-carotene, however, in our study, the β-carotene content of carotenoid extracted from *Dunaliella* sp. 2 was lower than its lutein amount. Similar findings also showed by Deli et al. (2014); they found 53% lutein, while 13% β-carotene content in *D. salina*. Ahmed et al. (2014) found rich lutein content in *D. salina* as 65.2%, as well. In biosynthetic pathway of carotenoids, most of them enter the anabolism beginning with α-carotene and might be accumulated as lutein as the end product. Derivates of β-carotene such as violaxanthin, neoxanthin, zeaxanthin and β-carotene itself can also be

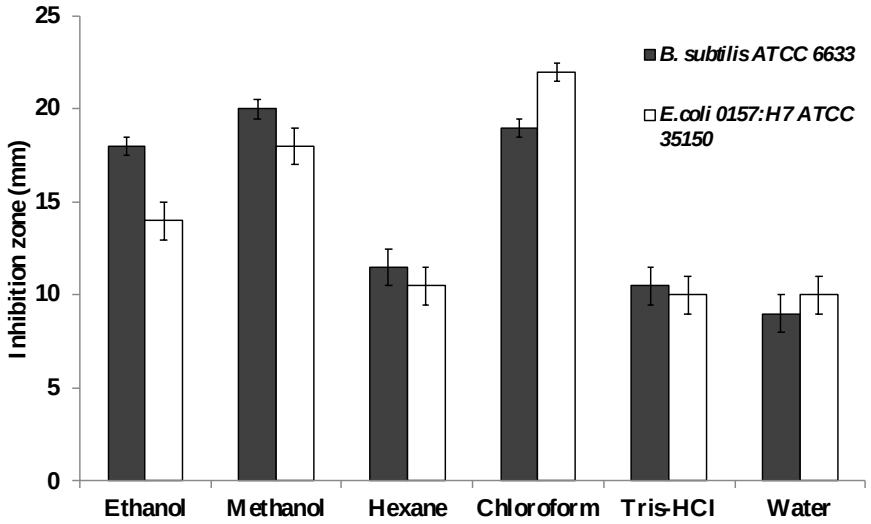


**Figure 3.** Effect of solvent type on production of bioactive compounds by *Dunaliella* sp. 2; antimicrobial activity against *B. subtilis* ATCC 6633 and *E. coli* O157:H7 ATCC 35150 (T: 30°C; N concentration: 1 g/L; light intensity: 4800 lx; NaCl concentration: 20% (w/v); incubation period: 14 d).

**Table 1.** GC-MS identification of fatty acids in chloroform-extracts of *Dunaliella* sp. 2

| ID | Retention time (min) | Fatty acids | % |
|---|---|---|---|
| 1 | 13.25 | Caprylic acid (C8:0) | 0.6 |
| 2 | 14.34 | Capric acid (C10:0) | 16.1 |
| 3 | 19.33 | Lauric acid (C12:0) | 3.2 |
| 4 | 26.61 | Palmitic acid (C16:0) | 8.3 |
| 5 | 26.83 | Palmiteloic acid (C16:0) | 0.5 |
| 6 | 28.83 | Heptadecanoic acid (C17:0) | 3.0 |
| 7 | 30.35 | Stearic acid (C18:0) | 53.5 |
| 8 | 31.53 | Oleic acid (C18:1n9c) | 5.9 |
| 9 | 32.66 | Linolelaidic acid (C18:2n6t) | 1.1 |
| 10 | 33.31 | Linoleic acid (C18:2n6c) | 3.3 |
| 11 | 35.36 | Heneicosanoic acid (C21:0) | 4.4 |

synthesized in biosynthesis of carotenoids. Environmental conditions or genetic effects might be the reason for different production of carotenoids. Microalgae originated bioactive compounds like pigments (β-carotene, lutein and zeaxanthin) have antimicrobial, antioxidant, anti-inflammatory properties in preventing or minimizing of diseases (de Morais, Vaz, de Morais, & Costa, 2015). In the current study, high amount of lutein was found as main carotenoid having bioactive property.

In Table 3, analyses of polar compounds in chloroform extracts of *Dunaliella* sp. 2 were shown. According to the chromatogram for polar compounds seven peaks were obtained, of these, third peak had the highest intensity. It was previously demonstrated that HPLC analysis of polar compounds at 280 nm in microalgal extracts might be related to phenolic compounds having bioactive properties (Rodriguez-Meizoso *et al.,* 2010). Simple phenols were also defined in other micralgal species such as *Chlorella vulgaris*, *Haematococcus pluvialis*, *Diacronema lutheri*, *Phaeodactylum tricornutum*, *Tetraselmis suecica*, and *Porphyridium purpureum* (Goiris *et al.,* 2014). In our study, the highest peak might be ferulic acid, which was shown previously in *D. salina* with a similar retention time by Safafar, Wagenen, Moller, & Jacobsen (2015). Borges, Ferreira, Saavedra, & Simoes (2013) also showed that simple phenols like ferulic and gallic acids had antimicrobial activity against *E. coli* CECT 434, *P. aeruginosa* ATCC 10145, *S. aureus* CECT 976, and *L. monocytogenes* ATCC 15313.

## Conclusions

The current study has shown different stress conditions, which may lead to increase in antimicrobial activity of bioactive compounds by *Dunaliella* species. *Dunaliella* sp.2 had the highest capacity in producing effective bioactive compounds against different bacteria. Further, antimicrobial activity increased in stress conditions, of these; osmotic stress was found the most effective one. Stearic acid from fatty acids, lutein from pigments and a simple phenol as ferrulic acid from polar compounds were determined with the highest amount in chloroform-extracts of the microalga. It can be concluded that lutein and ferrulic acid were responsible for higher antimicrobial activity under stress conditions. It can be concluded that *Dunaliella* sp. 2 is a safe beneficial biomaterial to be utilized in the field of pharmacology according to its bioactive properties.

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**Table 2.** Pigment composition in chloroform-extracts of *Dunaliella* sp. 2 (450 nm)

| ID | Retention time (min) | Compound | Area | Reference |
|---|---|---|---|---|
| 1 | 10.35 | Luteoxanthin | 15.89 | (Deli *et al.,* 2014; RT: 9.64; 446 nm) |
| 2 | 10.67 | Astaxanthin | 21.14 | (Ahmed *et al.,* 2014; RT: 10.6; 470 nm) |
| 3 | 10.87 | MV Chlorophyll c 3 | 33.20 | (Daigo *et al.,* 2008; RT:11.05; 451 nm) |
| 4 | 11.07 | Chlorophyll c2 | 10.35 | (Rodriguez *et al.,* 2002; RT 11.01; 452 nm) |
| 5 | 11.42 | Antheraxanthin | 6.81 | (Deli *et al.,* 2014; RT: 11.37; 447 nm) |
| 6 | 17.46 | 9Z or 9'Z lutein | 283.57 | (Deli *et al.,* 2014; RT: 17.47; 466 nm) |
| 7 | 19.47 | Neoxanthin | 19.36 | (Rodriguez *et al.,* 2002; RT: 18.70, 438 nm) |
| 8 | 20.30 | Violaxanthin | 11.93 | (Rodriguez *et al.,* 2002; RT: 20.68, 440 nm) |
| 9 | 21.56 | β-carotene | 5.19 | (Plaza *et al.,* 2010; RT:22.43; 452 nm) |
| 10 | 24.22 | Zeaxanthin | 65.36 | Louda *et al.,* 2008; RT: 24.3; 454 nm) |
| 11 | 25.15 | All-trans β-carotene | 25.29 | (Hu *et al.,* 2008; RT: 26.90; 458 nm) |
| 12 | 31.68 | α-carotene | 89.00 | (Deli *et al.,* 2014; RT: 31.93; 444 nm) |
| 13 | 34.61 | β-β carotene | 5.81 | (Rodriguez *et al.,* 2002; RT: 35.39; 452 nm) |

**Table 3.** Polar compound composition in chloroform-extracts of *Dunaliella* sp. 2

| ID | Retention time (min) | Area |
|---|---|---|
| 3 | 17.16 | 7065.37 |
| 4 | 18.49 | 9.96 |
| 5 | 18.74 | 22.87 |
| 6 | 19.44 | 6.02 |
| 7 | 20.02 | 48.60 |
| 9 | 21.17 | 12.97 |
| 10 | 25.10 | 2450.50 |

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