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Effects of menthone and piperitone on growth, chlorophyll a and β-carotene production in *Dunaliella salina*

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ABSTRACT

Recently, studying essential oils and secondary metabolites of plants and microalgae have received much attention. The biosynthesis of the secondary metabolites is strongly influenced by different environmental factors. Monoterpenes as a main fraction of essential oils of fruits and vegetables have many clinical applications. They could inhibit the carcinogenesis processes and therefore might be effective in treatment of cancers. *Dunaliella salina*, a photosynthetic green microalga is known as a rich source for β-carotene production. In this study, the effects of some monoterpenes including menthone and piperitone was investigated on yield of production of β-carotene were studied. Menthone and piperitone as parameters of stress can make tensions to the medium of *D. salina* increasing its β-carotene and chlorophyll *a* content in every single cell but on the other hand these two monoterpenes cause a decrease in the concentration of β-carotene and chlorophyll *a*.

INTRODUCTION

Essential oils helps the plants to easly preserve and adapt to the environmental stress conditions (Errafiy *et al.*, 2013, Figueiredo *et al.*, 2008). Monoterpenes are biosynthesised secondary metabolites that obtained from plants and microalgae. They are subgroup of terpenoids with anti-protozoan, antimicrobial, disinfectant, antiseptic, wound healing, anti-parasitic and anti-cancer properties (Bakkali *et al.*, 2008; Dorman *et al.*, 2000; Ghasemi *et al.*, 2007a; Leal *et al.*, 2013). They are widely used in food industry, clinical practices, cosmetics and agriculture (Burt, 2004, Edris, 2007). In fact, monoterpenes are hydrocarbons resulting from condensation of two isoprene units. They are well distributed in plant and vegetable essential oils (Knudsen *et al.*, 2006). Menthone and piperitone are the main fractions of *Menthapiperita* essence with anti-microbial properties and are widely used in fragrance and pharmaceutical industries (Iscan *et al.*, 2002; Kalemba and Kunicka, 2003). Microalgae are microorganisms of multi purposes, with a variety of potential applications in food, environmental biotechnology, chemical convertors and biofuel (Ghasemi *et al.*, 2007b; Ghasemi *et al.*, 2011a; Ghasemi *et al.*, 2008a; Ghasemi *et al.*, 2012; Shaker *et al.*, 2015; Yazdi *et al.*, 2005). *Dunaliella salina*, is a photosynthetic unicellular green microalga that lacks a rigid wall (Borowitzka, 2013). Among different species of microorganisms, *D. salina* has the most potential for production of β-carotene which can accumulate β-carotene up to about 13.8% of its total dry cell weight (Ye *et al.*, 2008). The global market of natural pigments has a high demand for carotenoids because of its wide use in coloring agents, medicine, cosmetics and food industries.

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Hence, different biochemical genetic and bioprocess experiments could be suggested to maximize the total carotenoid production in this microalga (Courchesne *et al.*, 2009). In this study, the effects of these two monoterpenes on chlorophyll *a*and β-carotene production in *D. salina* were evaluated.

MATERIALS AND METHODS

Strain and culture conditions

The microalgae *D. salina* was isolated from Maharlu Salt Lake, 27 kilometers southeast of Shiraz, Iran. Identification was done through morphological and taxonomical descriptions(Preisig, 1992). Besides, PCR reaction of 18S rRNA with universal primers were carried out and products were evaluated by 1% agarose gel electrophoresis(Ghasemi *et al.*, 2011b, Ghasemi *et al.*, 2008b).The single colony of *D.salina* was inoculated in modified Johnson broth containing 12% NaCl, 1.5 g $MgCl_2.6H_2O$, 0.5 g MgSO4.7H2O, 0.2 g KCl, 0.2 g $CaCl_2.2H_2O$, 1 g KNO_3, 0.043 g $NaHCO_3$, 0.035 g KH_2PO_4, by addition of distilled water to a total volume of 1 liter (pH adjusted to 7.5 by HCl). Microalgae growth was monitored under condition 37 µmol m^{-2} s^{-1} illumination and unlimited aerated condition during 35 days for *D. salina*. Temperature was adjusted at 25 ± 2 ºC. Cell number and optical density was measured by using anoptical microscope and UV/Visible spectrophotometer (U-0080D-Tokyo, Japan) at 620 nm respectively. For cell count, each time 20µL of microalgae suspension was removed through sampling tube and then direct count was performed using Neubauer hemocytometer and a light microscope (TCM400-Labomed, CA, USA).

Essential oils treatment

Piperitone and menthone were added to 100 mL culture medium in concentrations of 0, 1, 1.5, 2, 2.5 µL/mL. concentrations over the range of 2.5 µL/mL caused at least the cell count in the defined medium. Erlenmeyers were incubated at 22°C in a room with light tension of 5000 lx. After treatment of cultures with piperitone and menthone, cell counting was done using Neubauer haemocytometer every 4 days.

Analytical procedure

For evaluation of the produced chlorophyll *a*, 3 mL of microalgae culture was centrifuged at 2500 rpm, for 10 min.The supernatant was replaced with 80% acetone solution and the suspension was shaked well by vortex, then after centrifugation (2500 rpm, 10 min), absorbance of the supernatant solutions in 668.2 and 664.8 nm were measured by using a spectrophotometer. According to Eijckelhoff and Dekker calculations, the amount of chlorophyll *a* was achieved (Eq. 1):

Eq. 1: $\text{Chl. a } (\mu g/mL) = 12.25_{A668.2} - 2.79_{A664.8}$

All experiments were done in triplicate with three time observations. For evaluation of produced β-carotene, 1 mL of microalgal culture was centrifuged in 3000 rpm for 10 min, and then the supernatant was replaced with 3 mL hexane/ethanol solution with the fraction of 1/2. After vortex the solution was centrifuged (3000 rpm, 10 min) and supernatant was separated into two isolated phases. The upper phase was hexane phase including β-carotene compounds. According to equation below (Eq. 2), the amount of β-carotene was calculated in µg/mL:

Eq. 2: $\beta\text{-carotene } (\mu g/ml) = 25.2 \times A_{450}$

Phylogenic analysis of the isolated strain

The gene sequence of our isolated *D. salina* was compared with other gene sequences in NCBI library by blast software. It was achieved a homology of 99% with the other *Dunaliella* strains. The phylogenic tree was drawn by the help of MEGA4 software according to neighbor joining method. According to phylogenic tree, *D.salina* has the most similarity to *D. bardawil* (Fig. 1).

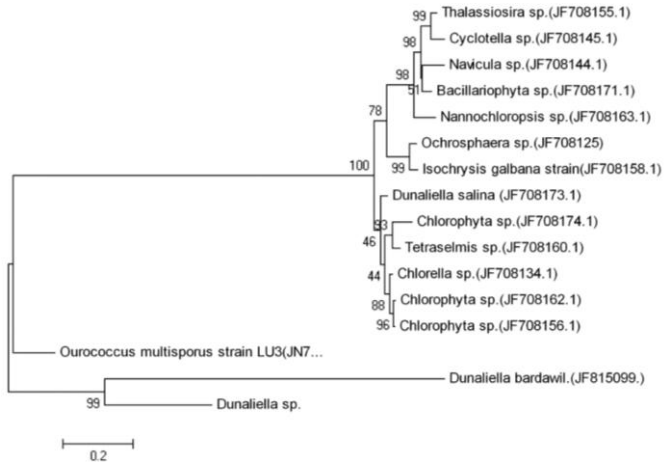

Fig. 1: Molecular phylogenetic analysis of the isolated *D. salina* strain with some related microalgal strains according to 18S rRNA gene. The evolution history was inferred by using the neighbor joining method. The bootstrap consensus tree inferred from 500 replicates. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The analysis involved 16 nucleotide sequences (accession numbers in parenthesis). All positions containing gaps and missing data were eliminated. Evolutionary analysis was conducted in MEGA4.

Statistical analysis

For the statistical analysis, identification of significant differences between the treatment groups was analyzed using ANOVA and the *t*-test. The chosen level of significance for all statistical tests was $p < 0.05$ Statistical computation was performed using the IBM SPSS16.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

The isolated microalgal strain found to be a *D. salina* strain. The results of phylogenetic studies are shown in Fig. 1. Growth curve of *D. salina* during 16 days after culture in Johnson medium was depicted in Fig. 2. As it has been shown, increasing the concentration of piperitone monoterpene from 0 to 2.5 µg/mL, will decrease the number of cells significantly. In high levels (>2.5

µg/mL), piperitone had extensive negative effect on growth and viability of *D. salina* cells. Even after treatment with 2.5 µg/mL of piperitone, between 12 and 16 days, the cell count was reduced from 192000 to 73000 cells/mL (Fig. 2).

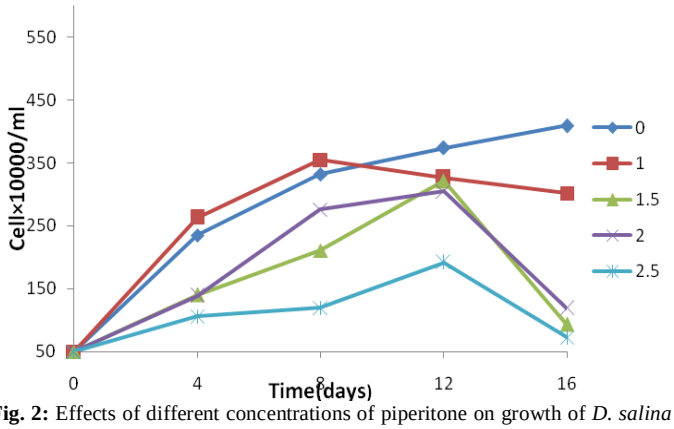

Fig. 2: Effects of different concentrations of piperitone on growth of *D. salina* during 16 days of experiment.

Overall, by increasing the concentration of menthone, the growth curve of *D. salina* showed a descending pathway. But the slope was gentler than piperitone. After treatment with high concentration of menthone (≥ 2.5 µg/mL), during the last four days, the cell count was reduced from 113000 to 92000 cell/mL (Fig. 3).

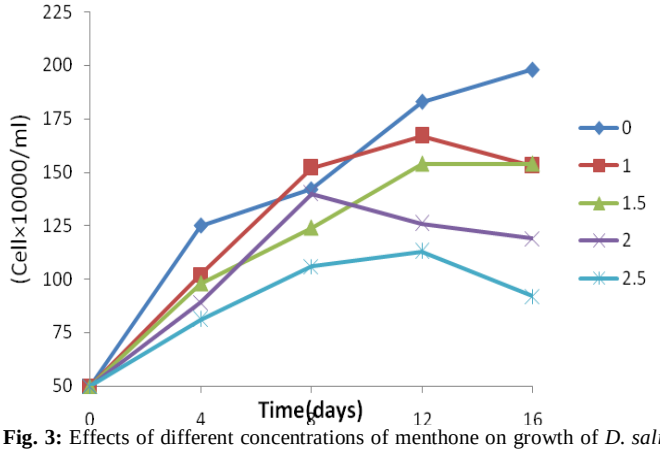

Fig. 3: Effects of different concentrations of menthone on growth of *D. salina* during 16 days of experiment.

By increasing the concentration of menthone from 0 to 2.5 µg/mL, the concentration/volume of chlorophyll *a* was decreased severely (Table 1). The most chlorophyll content was belonging to control group (without monoterpene) and the less chlorophyll *a*was for the highest concentration (2.5 µg/mL). After 16 days of incubation, the control group compared with the sample group, chlorophyll content in all groups was increased in every single cell, but the total content of chlorophyll was lower than the control group (Table 1).

The result of additional piperitone on chlorophyll *a* was as same as menthone. By additional concentration of menthone from 0 to 2.5 µg/mL, the concentration of chlorophyll/volume was decreased severally (Table 2).Generally, by increasing the concentration of menthone from 0 to 2.5 µg/mL, β-carotene production was increased and the total contents of β-carotene per volume was going decreased but the β-carotene contents in every cell was going increased (Table 3). But when the concentration of menthone was up to 2.5 µg/mL, β-carotene production was reduced (Table 3) with an inverse relationship.

Table 1: Effects of menthone on chlorophyll *a* concentration in *D. salina* during 16 days of experiment.

Menthone concentration (µL/mL)	4th day	8th day	12th day	16th day
0	1.177±.09	2.016±.01	4.508±.05	6.729±.11
1	1.155±.09	1.701±.06	4.327±.07	6.516±.16
1.5	1.086±.43	1.597±.08	3.844±.019	6.522±.11
2	0.688.06	1.597±.05	3.427±.07	6.398±.15
2.5	0.508±.04	1.301±.11	2.623±.09	5.432±.39

Table 2: Effects of piperitone on chlorophyll *a* concentration in *D. salina* during 16 days of experiment.

Piperitone concentration (µL/mL)	4th day	8th day	12th day	16th day
0	0.978±.09	2.595±.04	4.096±.06	5.419±.06
1	0.976±.08	2.343±.05	3.389±.07	5.271±.10
1.5	1.052±-06	2.738±.07	3.369±.04	5.071±.08
2	0.943±.08	2.727±.03	3.635±.06	4.951±.01
2.5	0.860±.06	1.995±.04	3.176±.07	4.661±.06

Table 3: Effects of menthone on β-carotene production in *D. salina* during 16 days of experiment.

Menthone concentrations (µL/mL)	4th day	8th day	12th day	16th day
0	0.5796	1.3188	3.4272	5.163
1	0.7896	1.3608	3.3459	4.3428
1.5	0.7056	1.4952	3.2467	4.8468
2	0.84	1.7388	2.94	3.654
2.5	0.4956	0.9996	2.4537	3.457

Despite increased levels of β-carotene after treatment with piperitone (≥2.5 µg/mL), there was a decrease in total content of β-carotene in control group (5.41 to 4.66 µg/mL).

The human cells are exposed to a wide range of oxidants in air, foods and surroundings (Brunekreef and Holgate, 2002, Valko *et al.*, 2005). These oxidants can harm proteins and nucleotides within the cells through free radicals. A surplus of plant reactions exist to find a way around the potentially harmful effects caused by salinity light, drought, pathogen infections, extreme temperatures, and other stresses. In 1940s, potency of phenolic compounds in prohibition of fatty acid oxidation was understood (Ghio *et al.*, 2012). Since then the usage of synthetic antioxidants has been developed but nowadays their application is reduced due to their disadvantages on human health (Kmiecik *et al.*, 2011). β-carotene as a natural and safe anti-oxidant is a good alternative.

Nowadays obtaining new methods for production of large amounts of β-carotene is very important. Application of stressful situations in culture conditions of *D. salina* increase the

amounts of β-carotene in cell contents. Addition of monoterpenes like piperitone and menthone through induction of stress can increase β-carotene contents of the cells (Yan *et al.*, 2011). Piperitone and menthone by prohibiting effect on growth of *D. salina* caused a reduction in cell counts, so the amounts of β-carotene in a defined volume was decreased but the β-carotene contents in each cell was increased. Considering the effects of monoterpenes menthone and piperitone on the pattern of *D. salina* growth curve it was revealed that piperitone had negative effects on microalgal cell growth. Monoterpenes could interrupt the microtubules dynamicity by joining to tubulin heterodimers. So the cell cycle will be arrested in G_0/G_1 phase (Choudhury *et al.*, 2010). Stressful conditions induced by different concentrations of monoterpenes piperitone and menthone (from 0.5 to 2.5 µg/mL) had a positive effect on chlorophyll *a* content in every single microalgal cell but by considering the descending pattern of cell growth curves, the amount of chlorophyll/volume was reduced.

Foyer *et al*. showed that increasing of photosynthesis and accumulation of chlorophyll in chloroplasts are coping strategies to combat with stress and ROS effects in microalgae and plants (Foyer and Shigeoka, 2011). Munne-Bosch investigated the role of a-tocopherol in plant stress tolerance (Munne-Bosch, 2005). His study indicated that the amount of a-tocopherol levels in a variety of environmental constraints change differentially and depending on the vastness of the stress and species sensitivity to stress. Khorasaninejad *et al*. have studied the effect of salinity stress on growth parameter essential oil yield and constituent of peppermint (Khorasaninejad *et al.*, 2010). The results showed that by attendance of high salinity in surrounding the ability of plants to survive was in pathway relationship.

Carotenoids and chlorophyllshave major roles to overcome with oxidative damages in living organisms (Roginsky and Lissi, 2005). β-carotene and chlorophyll are among the most important antioxidants in the living organisms. Stressful situations can increase the yield of their production as a prophylactic mechanism to overcome destructive effects of ROS and free radicals (Kara *et al.*, 2013). Monoterpenes piperitone and menthone can increase the production of β-carotene and chlorophyll by induction of stress to the microalgae cells, which could be useful to improve the carotenoid production.

In this study we showed that menthone and piperitone as parameters of stress can make tensions to the medium of *D. salina*, increasing its β-carotene and chlorophyll content in every single cell. On the other hand, these two monoterpenes cause a decrease in the concentration of β-carotene and chlorophyll. To increase the amount of chlorophyll and β-carotene in *D. salina* in the worth environmental condition, it is a special prominence for microalgae for more attention to exploit for food, industries and medical applications.

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Conflict of Interests: There are no conflicts of interest.

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