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Research Article

# A Study on the Effect of Nitrate and Phosphate Concentrations on the Production of Mycosporine-Like Amino Acids by *Chlorella Vulgaris*

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**Background**: Cyanobacteria can produce compounds absorbing ultraviolet irradiation. Mycosporine like amino acids (MAAs) are some of these important metabolites, which can be potentially considered as a sunscreen agent in the pharmaceutical and cosmetic industry. Different factors have been reported that can affect the biosynthesis of MAA.

**Objective**: In this study, the influence of different concentrations of phosphate and nitrate under different environmental conditions on MAA production by *Chlorella vulgaris* was investigated using an experimental design method, in order to enhance MAAs production in this specious.

**Materials and Methods**: A $2^3$ full factorial design (FFD) using Design-Expert v7.0.0 software was used to optimize simultaneously all the three factors of nitrate and phosphate concentration and condition of incubation environment on the MAA production by this species of *C. vulgaris*. Two milliliter of organism stock were grown in 200 mL BG11 medium and after 21 days, the biomasses of all samples were separated. Then, the MAA was extracted from dried biomass using methanol extraction. The extracts were analyzed by reverse-phase high performance liquid chromatography (RP-HPLC). After complete analysis, four samples were then cultured at the optimized conditions and analyzed by liquid chromatohraphy coupled to mass spectrometry (LC/MS).

**Results**: The results showed that this microalga could produce compounds with λmax of 330 nm and a retention time of about 2 min. According to the central composite analysis, phosphate at 0.51 $g.L^{-1}$ and nitrate at 2.5 $g.L^{-1}$ can be considered as the optimum concentrations, resulting to the preferable conditions concerning the culture in germinator. Based on LC/MSS analysis, the major compound had a m/z of 332 at the optimum condition.

**Conclusion**: Thus, this species is expected to have the capability of MAA production (maybe Shinorine) or one of its glycosylated derivatives.

*Keywords: Chlorella vulgaris*, Culture, Environment, Mycosporine Like Amino Acids, Nitrate, Phosphate.

## 1. Background

Microalgae and cyanobacteria have inhabited a wide variety and a vast number of environments on earth. Each species has faced different conditions from high amounts of solar radiation or heat to different degrees of salinity and variant sources of nutrients. To survive and overcome such conditions, each species has developed some unique chemical adaptations, known as "secondary metabolites" (1). This chemical diversity found in microalgae and cyanobacteria has made them a rich source of novel molecules, and it is no surprise that why these have obtained special attention in the pharmaceutical and cosmetic industry. A number of new bioactive molecules with a variety of properties like antioxidant, antimicrobial, anti-inflammatory, and photoprotective have been reported for these organisms

(2,3). To confront the detrimental results of UVR exposure, like DNA and protein damage, increased level of reactive oxygen species (ROS), and photosynthesis inhibition, cyanobacteria, and algae have gained the capability of synthesizing a group of secondary metabolites known as "cyanobacterial sunscreens". Mycosporine-like amino acids (MAAs) are one of those secondary metabolites (4-6).

Mycosporin-like amino acids are one of the most important UV-absorbing compounds. MAAs are small molecules weighing less than 400 Da, water-soluble, and due to their special chemical structure are able to absorb ultraviolet light in the wide range of 309-362 nm. These biomolecules are made up of a ring of aminocyclohexenone, or aminocycloheximе, attached to an amino acid or immunoalkyl group. Some MAAs contain sulfate esters, while others bind to monosaccharides or disaccharides to form the glycosidic derivatives of MAAs. About twenty-five, well-characterized MAAs have been reported so far (7-9). Regulation of MAAs biosynthesis is mainly influenced by both the spectral distribution and intensity of solar radiation delivered to microalgae (7). But that is not the only leverage; research has shown that MAAs production is also controlled by other abiotic factors i.e., temperature, desiccation, salinity, osmotic pressure, and nutrient availability (10, 11). Circadian rhythm has its own impact on MAA production as well; one study showed that some MAAs are produced much more during light periods while some others increase in dark conditions (12). Regarding nutrients and essential elements, nitrogen (N) has been proved to be a necessity for MAAs biosynthesis (9). Different concentrations of nitrogen supplies in the medium affect both the quantity and composition of produced MAAs (13). Moreover, MAAs production is upregulated as the nitrate and phosphate concentrations of the medium increase (14, 15). Therefore, nitrate and phosphate availability play a major role in regulating the MAAs biosynthesis.

*Chlorella vulgaris* is a multi-cellular microalga, which has been reported as valuable source of bioactive peptides, proteins, polysaccharides, fatty acids, lipids, etc. This species has extensively used in nutritional, pharmaceutical, and cosmetic industry. Various pharmaceutical effects have been reported for bioactive compounds extracted from this microalga, such as anticancer and antimicrobial, anti-inflammatory (16, 17). Besides, a number of phycotoxins have also been reported from several microalgae that can induce adverse effects on humans (18). Although *C. vulgaris* has considered as safe nutraceutical species, the presence of toxin genes in it was also reported. These genes were expressed under specific induction. Hence, efficient harvesting of microalgal cellular contents has great importance in order to not trigger toxin activation (19).

Given that MAAs have been already commercialized and these are being used in sunscreen formulations like the one under the name of Helioguard® (20), the optimization of their mass production processes and culture conditions and increasing the yield of production, is a substantial work for the cosmetic industry.

## 2. Objective

In this study we used *Chlorella vulgaris*, a fresh-water green microalga, which is broadly used in the food, pharma, and water industry (21). The main purpose was to investigate the effect of different nitrate and phosphate concentrations and two different environmental conditions on the production of MAAs, as well as to determine the optimum conditions, using an experimental design method.

## 3. Materials and Methods

### *3.1. Chlorella vulgaris Cultivation*

20 mL of stocked culture (MCCS 014, Microalgal Culture Collection of SUMS, Iran) were cultivated in 200 mL of BG-11 culture medium (varies in the concentrations of nitrate and phosphate). The chemicals of $NaNO_3$, $K_2HPO_4 \cdot H_2O$, $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, Citric acid, Ferric ammonium citrate EDTA, $Na_2CO_3$, $H_3BO_3$, $MnCl_2 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, $Na_2MoO_4 \cdot 2H_2O$, $CuSO_4 \cdot 5H_2O$, and $Co(NO_3)_2 \cdot 6H_2O$ for preparation of BG11 medium were supplied form Merck Chemicals (Germany). The cultures were incubated for 21 days and then, MAA extraction was performed.

### *3.2. MAA Extraction and Partial Purification*

MAA was extracted using the method developed by Rastogi *et al.* with slight modifications (22). The biomass was separated by centrifugation (2-16KL, Sigma, Germany) at 6700 g for 10 min and then, lyophilized. The dried biomass was slightly soaked in water and then, extracted by 100% HPLC grade

methanol (Merck Chemical, Germany) overnight at 4 °C and dark conditions. Then, this mixture was sonicated in an ultrasound bath (S 60 H, Elma, Germany) at 35 °C for 15 minutes and centrifuged at 6700 g for 10 min. The supernatant was transferred to a new tube and dried under nitrogen gas. One mL of deionized Mili-Q water (Millipore, Germany) was added to the dried product and transferred to a new Eppendorf tube. In order to separate water-insoluble impurities, it was centrifuged at 13200 g for 10 min. The supernatant was transferred into a new microtube and a few drops of chloroform were added, followed by shaking and vortexing vigorously until the chloroform layer turned green. The tubes were again centrifuged at 13200 g (10 min) and the colorless supernatant was transferred to a new Eppendorf tube. Before injecting to the HPLC column, the samples were filtered through a 0.22 µm syringe filter (MS® PTFE Syringe Filter).

*3.3. Characterization of MAAs*

HPLC was performed using a Shimadzu HPLC system equipped with a photodiode array (PDA) detector (SPD-M10A- Shimadzu, Kyoto, Japan). The $C_{18}$ HPLC column (5 µm, 25 × 4.6 mm) with the isocratic mobile phase in the flow rate of 1 $mL.min^{-1}$, which contained 10% methanol in 0.02% Trifluoroacetic acid (TFA) aqueous solution plus 10% acetonitrile (all in HPLC grade) was applied. The absorbance spectrum was scanned at the wavelength range of 250 to 450 nm and MAAs compounds were detected at 330 nm.

*3.4. Experimental Design*

A $2^3$ full factorial design (FFD) using Design-Expert version 2.0.1.0 software was used to optimize simultaneously all the three factors of nitrate and phosphate concentration (quantitative) and condition of incubation environment (qualitative) affecting the MAA production by this species of *Chlorella vulgaris*. The central composite design (CCD) were used. In the case of the face center, the maximum and the minimum limits of the level codes (+1) and (-1), were provided according to previously published studies, for each variable. Thus, the third level, as the zero or central level (0), was a value between the minimum and the maximum limits. The characteristics of the factors studied here are presented in **Table 1**.

A total of 28 runs in two blocks were used to optimize the range and levels of the chosen variables. Each run was completed in 28 days. The area under the curve (AUC) extracted from MAA HPLC chromatograms was taken as the response. The value of correlation ($R^2$) indicated the quality of the developed model and the analysis of variance (ANOVA) was used to evaluate the statistical significance of the model by using the Fisher's statistical test (F test). The experimental data obtained from CCD model experiments can be represented in the form of the following equation:

$$y = b_0 + \sum_{i=1}^{n}(b_i X_i) + \sum_{i=1}^{n}\left(b_{ii} X_i^2\right) + \sum_{i=1}^{n}\sum_{j=1}^{n}\left(b_{ij} X_i X_j\right)$$

Where y is the predicted response; n is the number of factors; $X_i$ and $X_j$ are the independent variables; $b_0$ is the interception coefficient; $b_i$ is the linear coefficient; $b_{ij}$ is the interaction coefficient, and $b_{ii}$ is the quadratic coefficient.

*3.5. Validation of the Model*

Moreover, to evaluate the mathematical model generated by the CCD, two cultures with optimum culture condition were experimentally evaluated and the maximum predicted level of the MAA was compared with the experimentally obtained MAA.

**Table 1:** Characteristics of the Experimental Design model

| Factor | Name | Units | Type | Minimum | Maximum | Coded Low | Coded High | Mean | Std. Dev. |
|---|---|---|---|---|---|---|---|---|---|
| A | phospahte | $g.L^{-1}$ | Numeric | 0.0000 | 1.21 | -1 ↔ 0.01 | +1 ↔ 1.00 | 0.5189 | 0.3572 |
| B | nitrate | $g.L^{-1}$ | Numeric | 0.0000 | 3.52 | -1 ↔ 0.50 | +1 ↔ 3.00 | 1.75 | 0.9598 |
| C | conditions | - | Categoric | RT | Germinator | | | Levels: | 2 |

## 4. Results

*4.1. HPLC Analysis*

The partially purified extracts of each sample were analyzed by HPLC and the data regarding retention times (Rt) and the areas under the curve (AUC) were shown in **Table 2**.

Based on the chromatographic data, this species can produce an MAA which has a retention time of about 2 minutes. This peak has a UV absorption between 330-340 nm which is within the range of MAAs.

For instance, the chromatograph of sample 20 has a main peak at about 2 minutes which has a UV absorption at 330 nm, as a characteristic sign of MAA compounds. The other peaks have an absorption less than 300 nm which probably are related to MAA precursors. The ratio of main peak AUC compared to total AUC is about 97% which shows that this sample has fewer impurities.

In sample 14, which the microalgae were cultivated in the presence of the minimum amount of nitrate and phosphate, the main peak was appeared at 2 minutes but compared to the sample 20, more impurities and MAA precursors were detected. Also, the AUC of the main peak was less than sample 20.

**Table 2**: Culture conditions and results obtained from of the samples of block 1 & 2

| Sample | Phosphate Concentration ($g.L^{-1}$) | Nitrate Concentration ($g.L^{-1}$) | Temp | AUC of the main peak | AUC total | Percentage | Retention time of the main peak (min) | UV absorption of the main peak (nm) |
|---|---|---|---|---|---|---|---|---|
| 1 | 0.01 | 0.50 | RT | 4764746 | 12945767 | 36.805 | 2.050 | 340 |
| 2 | 0.51 | 1.75 | G | 7538292 | 11885904 | 63.422 | 1.931 | 340 |
| 3 | 0.51 | 1.75 | G | 6896713 | 10761443 | 64.087 | 1.995 | 340 |
| 4 | 1.00 | 3.00 | G | 3706590 | 6005582 | 61.719 | 2.046 | 343 |
| 5 | 0.01 | 3.00 | RT | 8148210 | 14092361 | 57.820 | 1.988 | 343 |
| 6 | 1.00 | 3.00 | RT | 8533007 | 12744230 | 66.956 | 2.004 | 342 |
| 7 | 0.51 | 1.75 | RT | 7615901 | 11888254 | 64.062 | 2.001 | 340 |
| 8 | 0.51 | 1.75 | G | 6556526 | 10334525 | 63.433 | 1.991 | 340 |
| 9 | 0.51 | 1.75 | RT | 9983130 | 15563154 | 64.146 | 1.988 | 342 |
| 10 | 1.00 | 0.50 | RT | 6569801 | 11607227 | 56.601 | 2.029 | 340 |
| 11 | 0.51 | 1.75 | RT | 8850381 | 14001406 | 63.211 | 1.924 | 340 |
| 12 | 1.00 | 0.50 | G | 6214444 | 10443615 | 59.505 | 1.981 | 341 |
| 13 | 0.01 | 3.00 | G | 2590946 | 4977529 | 52.053 | 2.060 | 340 |
| 14 | 0.01 | 0.50 | G | 4409789 | 8170532 | 53.972 | 2.000 | 340 |
| 15 | 0.51 | 1.75 | G | 12935122 | 13566157 | 95.348 | 1.969 | 335 |
| 16 | 0 | 1.75 | RT | 8206141 | 1192141 | 73.321 | 1.920 | 328 |
| 17 | 0.51 | 3.52 | RT | 12932072 | 13275833 | 97.411 | 2.010 | 332 |
| 18 | 0 | 1.75 | G | 8090713 | 14029523 | 57.669 | 1.945 | 329 |
| 19 | 0.51 | 1.75 | RT | 14599229 | 17869366 | 81.700 | 1.995 | 332 |
| 20 | 0.51 | 1.75 | G | 10542662 | 10852809 | 97.142 | 1.948 | 329 |
| 21 | 0.51 | 1.75 | RT | 13767118 | 16121693 | 85.395 | 1.999 | 333 |
| 22 | 0.51 | 1.75 | RT | 14454310 | 17499087 | 82.600 | 1.996 | 333 |
| 23 | 0.51 | 1.75 | G | 9396960 | 9598849 | 97.897 | 1.934 | 330 |
| 24 | 0.51 | 3.52 | G | 11106859 | 13930932 | 79.728 | 1.983 | 332 |
| 25 | 0.51 | 0 | G | 5992556 | 14040060 | 42.682 | 1.986 | 329 |
| 26 | 1.21 | 1.75 | RT | 5955875 | 8961688 | 66.459 | 1.987 | 329 |
| 27 | 0.51 | 0 | RT | 7278760 | 11958721 | 60.866 | 2.001 | 333 |
| 28 | 1.21 | 1.75 | G | 16461181 | 18946018 | 86.855 | 2.010 | 332 |

The AUC of the main peaks for samples 18 and 25 respectively cultured in the absence of phosphate and nitrate was less than sample 20 and more impurities were detected according to chromatograms.

*4.2. Best Model for MAA Production*

The data were analyzed using Design-Expert software and the central composite design method was applied. According to the results, the concentrations of 2.5 $g.L^{-1}$ and 0.51 $g.L^{-1}$ for nitrate and phosphate, respectively, can be considered as the optimum concentrations. The best model fitted to the experimental data was as Equation 1 for room temperature conditions and Equation 2 for cultivation in the Germinator.

And the general model was calculated as Equation 3 with p-value ≤0.05 (significant) for the model and its lack of fitness, which A is reported as the phosphate concentration, B as the nitrate concentration and, C as the culture conditions.

*4.3. Evaluation of the Predicted Optimum Culture*

Four cultures were evaluated under the optimized conditions and analyzed by the HPLC. According to the AUC of the main peak shown in **Table 3**, the germinator conditions were reported as the optimized conditions, which does not correspond to the suggestion of the software. Under this condition, the microalgae may produce a higher level of MAA.

The chromatograms of both samples related to germinator and room temperature conditions are shown in **Figures 1 and 2**, respectively, indicating that this microalga can produce an MAA compound with the λmax of 330 nm at the retention time of about 2 min.

*4.4. LC/MASS Analysis*

The optimized samples were analyzed by LC/MS. The main peak with the $\lambda_{max}$ of 330 nm has an m/z of 332, which may correspond to Shinorine. Thus, this microalga might be able to produce Shinorine under this circumstance. A compound with m/z of 132 was also detected, which may be related to a pentose unit. Therefore, this species might synthesis a glycosylated MAA, however, more analytical methods are required to prove and support further this claim. The LC/MS spectra are shown in **Figures 3 , 4**.

**Equatuion 1 (Room Temperature)**:

$$Ln(MAA) = 15.3 + 1.062(\text{phosphate conc.}) + 0.565(\text{nitrate conc.}) - 0.0525(\text{phospahte} * \text{nitrate}) - 0.929(\text{phosphate})^2 - 0.107(\text{nitrate})^2$$

**Equation 2 (Germinator)**:

$$Ln(MAA) = 15.12 + 1.599(\text{phosphate conc.}) + 0.386(\text{nitrate conc.}) - 0.0525(\text{phospahte} * \text{nitrate}) - 0.929(\text{phosphate})^2 - 0.108(\text{nitrate})^2$$

**Equation 3:**

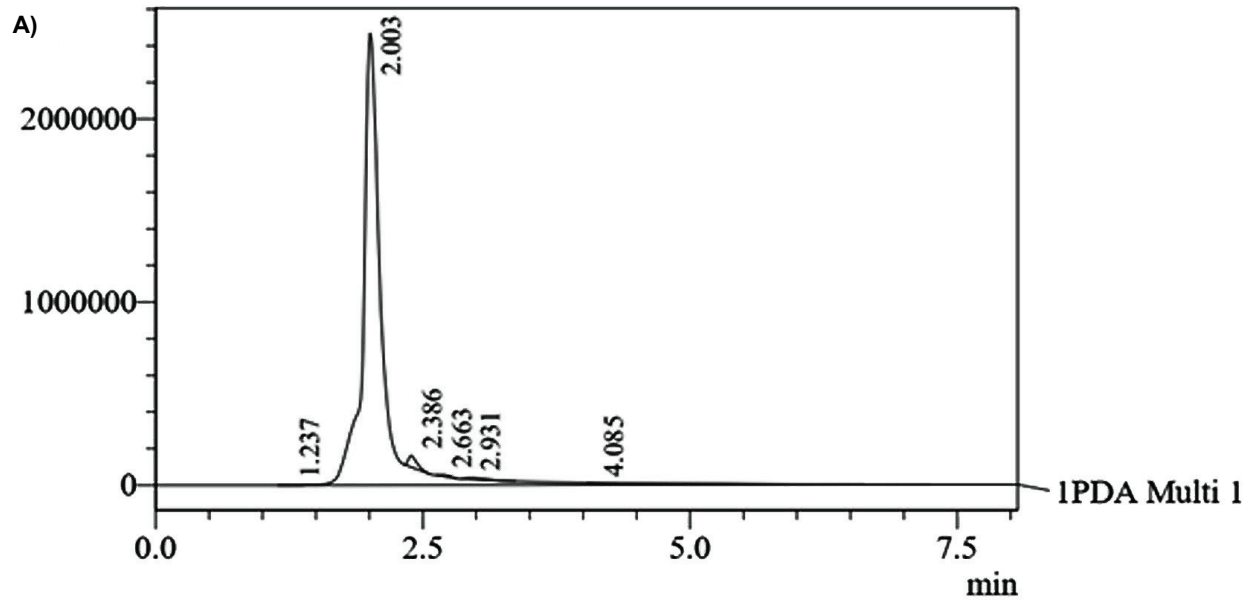
$$Ln(MAA) = 16.10 + 0.149(A)\ 0.091(B) - 0.11(C) - 0.0325(AB) + 0.133(AC) - 0.112(BC) - 0.228(A^2) - 0.168(B^2)$$

**Table 3**: Data of samples cultured under predicted optimum condition

| Sample | Phosphate concentration ($g.L^{-1}$) | Nitrate Concentration ($g.L^{-1}$) | Condition* | AUC of the main peak | % of AUC |
|---|---|---|---|---|---|
| G1 | 0.816 | 1.611 | G* | 30814018 | 98.34 |
| G2 | 0.815 | 1.595 | G* | 35658349 | 96.39 |
| R1 | 0.501 | 2.505 | RT** | 21190352 | 98.44 |
| R2 | 0.497 | 2.505 | RT** | 22435005 | 97.69 |

*G= Germinator (23 ± 2 ℃ constant humidity and light); **RT= Room temperature (20-25 ℃ variable humidity and light)

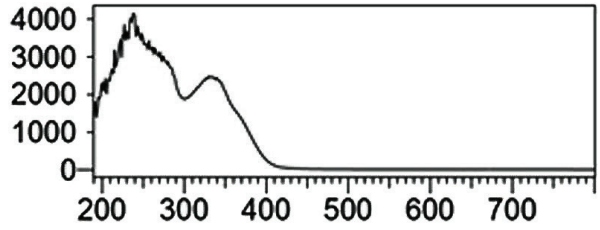
A)




1 PDA Multi 1 / 330nm 4nm

B)

Peak# : 2
Retension Time : 2.003

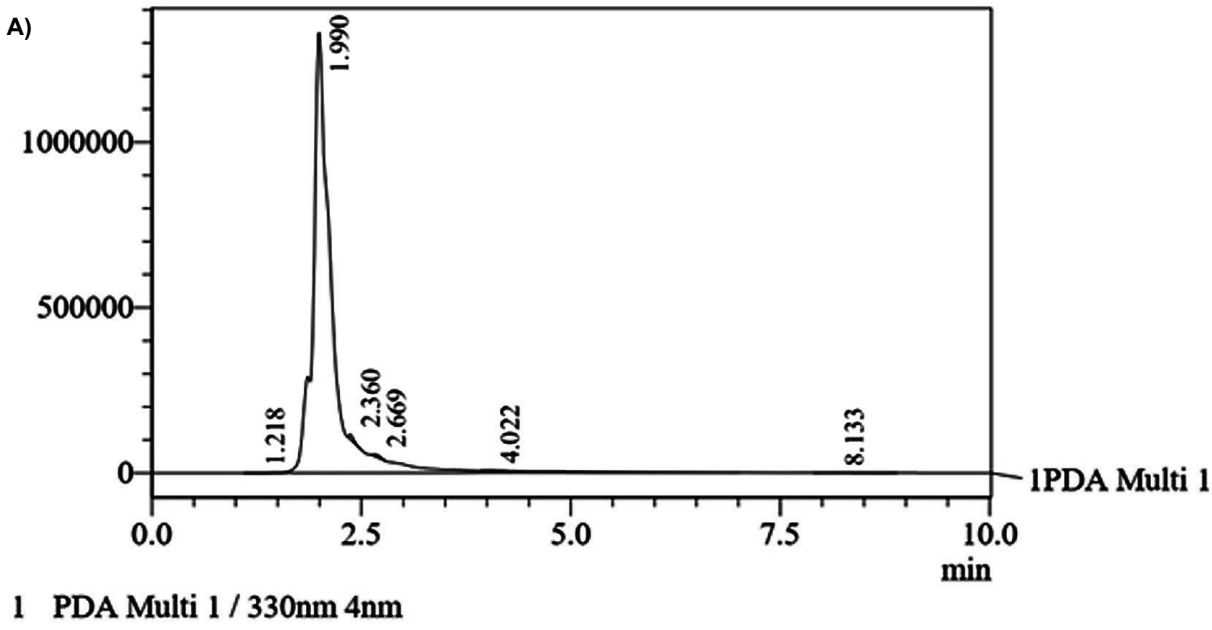



C)

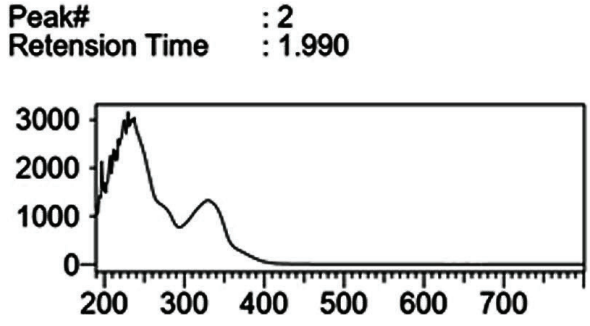
| Peak# | Ret. Time | Area | Height | Area % | Height % |
|---|---|---|---|---|---|
| 1 | 1.237 | 1302 | 182 | 0.004 | 0.007 |
| 2 | 2.003 | 30814018 | 2468347 | 98.349 | 97.012 |
| 3 | 2.386 | 338631 | 61716 | 1.081 | 2.426 |
| 4 | 2.663 | 52629 | 4810 | 0.168 | 0.189 |
| 5 | 2.931 | 122059 | 8982 | 0.39 | 0.353 |
| 6 | 4.085 | 2500 | 346 | 0.008 | 0.014 |
| total | | 31331139 | 2544384 | 100 | 100 |

**Figure 1. A)** HPLC chromatogram of sample G1, **B)** UV spectrum of the main compound of sample G1, **C)** HPLC chromatogram Data confirmed the high relative ratio of main compound with λmax=330 nm.

A)




B)




C)

| Peak# | Ret. Time | Area | Height | Area % | Height % |
|---|---|---|---|---|---|
| 1 | 1.218 | 1504 | 227 | 0.007 | 0.017 |
| 2 | 1.99 | 21190352 | 1330354 | 99.46 | 98.437 |
| 3 | 2.36 | 49691 | 12669 | 0.233 | 0.937 |
| 4 | 2.669 | 43168 | 6668 | 0.203 | 0.493 |
| 5 | 4.022 | 8813 | 1029 | 0.041 | 0.076 |
| 6 | 8.133 | 11801 | 526 | 0.055 | 0.039 |
| Total | | 21305330 | 1351474 | 100 | 100 |

**Figure 2. A)** HPLC chromatogram of sample R1, **B)** UV spectrum of the main compound of sample R1, **C)** HPLC chromatogram Data confirmed the high relative ratio of main compound with λmax=330 nm.

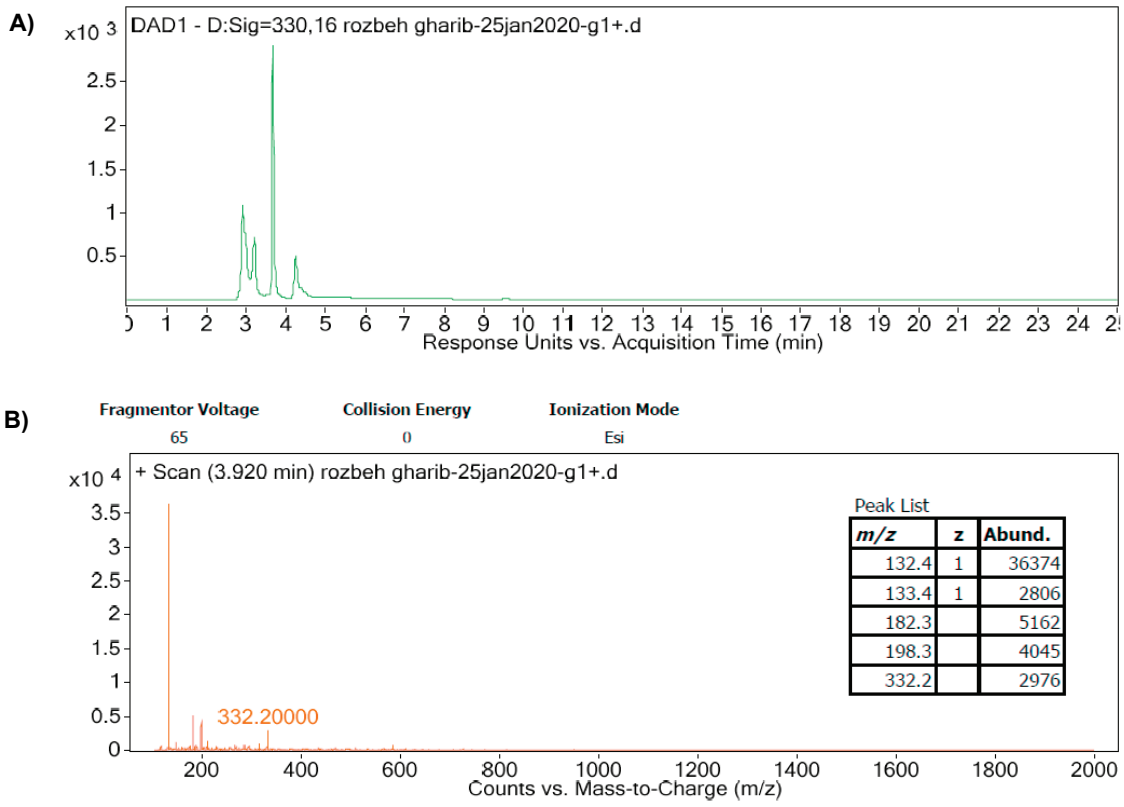



**Figure 3. A)** LC analysis of G1 at λmax= 330 nm, **B)** MS analysis and m/z of peaks

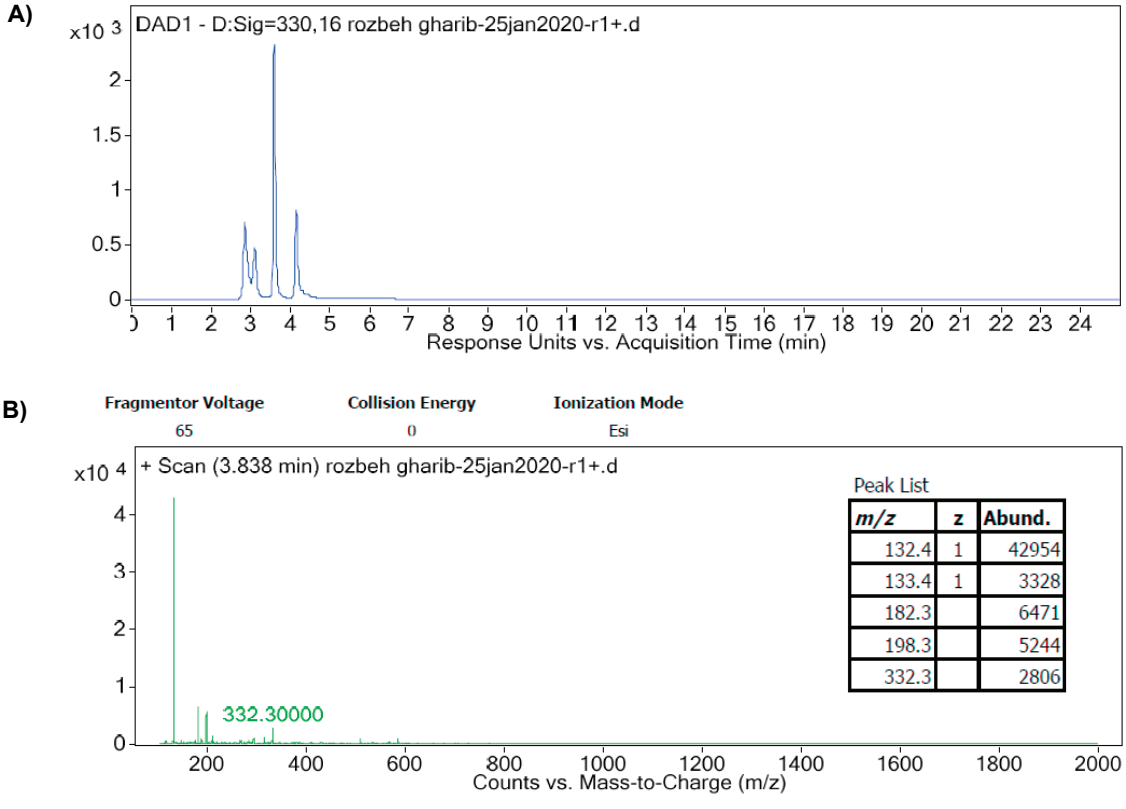



**Figure 4. A)** LC analysis of R1 at λmax= 330 nm, **B)** MS analysis and m/z of peaks

## 5. Discussion

MAAs are necessary means for microorganisms, to become acclimatized to live in difficult and harsh environments (23). For instance, microorganisms living in high-salt ecosystems tend to store MAAs in the intracellular space to preserve osmotic balance and prevent dehydration and the generation of oxidative stress due to hypertonicity of the environment. MAAs can also have osmoprotective action under freezing conditions in organisms of cold aquatic ecosystems (24). In recent years, researchers are making a great effort to find natural bioactive and biodegradable molecules for both cosmetic and therapeutic applications (2, 25), and this is becoming a global trend since there is a growing demand for natural raw materials for food, pharmaceuticals, and cosmetic products in order to have a green lifestyle (26, 27). Consequently, it is believed that natural sunscreens are more beneficial for human health and the environment, on account of their low toxicity and biodegradable feature (28). In addition, these not only have the negative effects of synthetic organic filters, like allergic reactions or endocrine disruption (29), but can possess more beneficial features, as we can see MAAs can reduce the inflammation of skin and have a direct anti-aging effect on skin by inhibiting the collagenase activity, which is responsible for formation of skin wrinkles after UV exposure (30, 31).

With increasing commercial demand for natural cosmetic products, microalgae and cyanobacteria are being cultivated at a large scale in different conditions. As a result, the chemical composition of raw materials from algal biomass might be different in every condition or even different yields might be achieved. Therefore, standardization and optimization of cultivation conditions (concentration of different nutrients, UV exposure, light and dark periods, salinity and etc.) must be considered in the processes of algal biomass production (32).

In the present study, we focused on investigating the effect of different concentrations of phosphate and nitrate in a culture medium in two different environmental conditions (outside and inside of the germinator) on MAA production in a green microalga *Chlorella vulgaris.*

In order to achieve the optimal conditions for MAA production and optimization, the Experimental design method was used. The central composite method was selected as the data analysis method This method was used to minimize the number of tests required to investigate the effect of three factors: nitrate and phosphate concentration and incubation conditions (laboratory environment and germinator). According to the equations obtained from the analysis of experimental data, the coefficient of phosphate concentration and the quadratic power of phosphate concentration in the equations have higher values, which indicates the noteworthy significance of this element in the production of MAA compounds. However, the calculated $R^2$ of the equations is about 60% that shows the relative efficiency of this model for the prediction of MAA production. According to the results of chromatographic findings, the studied species (*Chlorella vulgaris*) could produce UV absorbent compounds, due to the peak of the main compound being detected at the approximate retention time of about 2 minutes and its absorbance at the wavelength of 330 nm. This peak can be probably attributed to the MAA. It was observed that different concentrations of phosphate and nitrate affect the production of this compound and change the area below the corresponding peak in the chromatogram. These findings are in agreement with the results of previous studies (14, 15).

For example, in samples with a minimum concentration of nitrate and phosphate or where one of these nutrients was not present, the area below the main peak was much lower than in samples with a higher concentration of nitrate and phosphate. On the other hand, in samples without phosphate or nitrate, peaks with UV absorption of less than 300 nm were more clearly and the area under the curve of these side peaks was higher. In samples with higher phosphate and nitrate concentrations, the AUC of the main peak and the main peak height had greater values than the AUC of the side peaks with UV absorption of less than 300 nm, which are probably related to the precursor molecules of MAAs.

In the second stage of the study, the chromatographic data and findings were analyzed by the central composite experiment design method, and according to the optimal values obtained from software analysis, the optimal conditions for nitrate and phosphate were calculated at 2.5 $g.L^{-1}$ and 0.51 $g.L^{-1}$, respectively. Similarly, the samples were cultured under the optimal conditions. The extraction process was performed once more and the samples were analyzed by positive ionization mass spectrometry. According to the mass

spectrometry findings, at the retention time of the main peak with maximum absorption of 330 nm, a compound with a mass to charge ratio of 332 m/z is released. Due to its mass similarity to the shinorine, one of the well-known mycosporine-like amino acids, and the maximum absorption at 330 nm, it can stated that *Chlorella vulgaris* can produce mycosporine-like amino acids with a structure similar to shinorine in the mentioned optimal conditions. This is in agreement with the results of a previous study, that production of shinorine was increased due to the increase in nitrate concentration of the medium in *Gracilaria tenuistipitata* (13). A fraction with a mass to charge ratio of 132 (m/z) was also observed in the mass spectrum that can be attributed to pentose (33).

## 6. Conclusion

According to the results of the present study, it can be concluded that the amount of *Chlorella vulgaris* metabolites with less than 300 nm absorption which is probably related to the precursor molecules of MAAs, are more than the MAAs compounds in the absence of/ or low availability of phosphate or nitrate nutrients and MAA compounds with maximum absorption at 320-360 nm raised in an optimum concentration of phosphate and nitrate. Further studies in order to complete deconvolution of this MAA compound should be performed. In addition, other effective factors such as UV irradiation and other salts compositions will be considered in future studies to achieve the best optimum conditions for MAA production in *Chlorella vulgaris*.

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### Disclosure Statement

No potential conflict of interest was reported by the authors.

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### References

1. Coates RC, Trentacoste E, Gerwick WH. Bioactive and novel chemicals from microalgae. In: Richmond A, Hu Q, editors. *Handbook of Microalgal Culture: Applied Phycology and Biotechnology*: *John Wiley & Sons Ltd*. 2013.**p**.504-531. doi:10.1002/9781118567166.ch26.
2. Vega J, Bonomi-Barufi J, Gómez-Pinchetti JL, Figueroa FL. Cyanobacteria and Red Macroalgae as Potential Sources of Antioxidants and UV Radiation-Absorbing Compounds for Cosmeceutical Applications. *Mar drugs*. 2020;**18**(12):659. doi:10.3390/md18120659.
3. Tabarzad M, Atabaki V, Hosseinabadi T. Anti-inflammatory Activity of Bioactive Compounds from Microalgae and Cyanobacteria by Focusing on the Mechanisms of Action. *Mol Biol Rep*. 2020;**47**(8):6193-6205. doi:10.1007/s11033-020-05562-9.
4. Leao PN, Engene N, Antunes A, Gerwick WH, Vasconcelos V. The chemical ecology of cyanobacteria. *Nat Prod Rep*. 2012;**29**(3):372-391. doi:10.1039/C2NP00075J.
5. Rastogi RP, Madamwar D. Cyanobacteria Synthesize their own UV-Sunscreens for Photoprotection. *Bioenergetics*. 2016;**5**(2):1000138. doi:10.4172/2167-7662.1000138.
6. Singh SP, Häder D-P, Sinha RP. Cyanobacteria and ultraviolet radiation (UVR) stress: mitigation strategies. *Ageing Res Rev*. 2010;**9**(2):79-90. doi:10.1016/j.arr.2009.05.004.
7. Rastogi RP, Sinha RP, Singh SP, Häder D-P. Photoprotective compounds from marine organisms. *J Ind Microbiol Biotechnol*. 2010;**37**(6):537-358. https://doi.org/10.1007/s10295-010-0718-5.
8. Carreto JI, Carignan MO. Mycosporine-like amino acids: relevant secondary metabolites. Chemical and ecological aspects. *Mar drugs*. 2011;**9**(3):387- 446. doi:10.3390/md9030387.
9. Chrapusta E, Kaminski A, Duchnik K, Bober B, Adamski M, Bialczyk J. Mycosporine-like amino acids: Potential health and beauty ingredients. *Mar drugs*. 2017;**15**(10):326. doi:10.3390/md15100326.
10. Joshi D, Mohandass C, Dhale M. Effect of UV-B Radiation and Desiccation Stress on Photoprotective Compounds Accumulation in Marine *Leptolyngbya* sp. *Appl Biochem Biotechnol.* 2018;**184**:35-47. doi:10.1007/s12010-017-2523-3.
11. Vale P. Effects of Light and Salinity Stresses in Production of Mycosporine-Like Amino Acids by *Gymnodinium catenatum* (Dinophyceae). *Photochem Photobiol*. 2015;**91**:1112-1122. doi:10.1111/php.12488.
12. Rastogi RP, Incharoensakdi A. Occurrence and induction of a ultraviolet-absorbing substance in the cyanobacterium *Fischerella muscicola* TISTR8215. *Phycological Res*. 2015;**63**:51-55. doi:10.1111/pre.12069.
13. Barufi JB, Korbee N, Oliveira MC, Figueroa FL. Effects of N supply on the accumulation of photosynthetic pigments and photoprotectors in *Gracilaria tenuistipitata* (Rhodophyta) cultured under UV radiation. *J Appl Phycol.* 2011;**23**:457-466. doi:10.1007/s10811-010-9603-x.
14. Briani B, Sissini MN, Lucena LA, Batista MB, Costa IO, Nunes JM, *et al*. The influence of environmental features in the content of mycosporine-like amino acids in red marine algae along the Brazilian coast. *J Phycol*. 2018;**54**(3):380-390. doi:10.1111/jpy.12640.
15. Biosci IJ, Saadatmand S, Zamani M. Investigating the effect of different concentrations of nitrate and phosphate on the quantity of mycosporine like amino acids production and

growth in *Spirulina platensis*. *Int J Biosci (IJB).* 2015;**6**(5):63-69. doi:10.12692/ijb/6.5.63-69.
16. Sedighi M, Jalili H, Ranaei-Siadat S-O, Amrane A. Potential Health Effects of Enzymatic Protein Hydrolysates from *Chlorella vulgaris*. *Appl Food Biotechnol.* 2016;**3**(3):160-169. doi:10.22037/afb.v3i3.11306.
17. Arabian D, Amiri P. Improving Recovery Process of Omega-3 Fatty Acids from a Native Species of *Chlorella vulgaris* Using Integrated Method. *Appl Food Biotechnol*. 2020;**7**(4):273-282. doi:10.22037/afb.v7i4.29779.
18. Caruana AMN, Amzil Z. Chapter 13 - Microalgae and Toxins. In: Levine IA, Fleurence J, editors. *Microalgae in Health and Disease Prevention*: *Academic Press*; 2018. **p**. 263-305. doi:10.1016/B978-0-12-811405-6.00013-X.
19. Ng SL. The expression of yoeb and pezt bacterial toxin genes in microalga *Chlorella vulgaris* and the effects on cell viability/ Ng Shet Lee: University of Malaya; 2017. http://studentsrepo.um.edu.my/id/eprint/9329.
20. Figueroa FL. Mycosporine-Like Amino Acids from Marine Resource. *Mar Drugs*. 2021;**19**(1):18. doi:10.3390/md19010018.
21. Safi C, Zebib B, Merah O, Pontalier PY, Vaca-Garcia C. Morphology, composition, production, processing and applications of *Chlorella vulgaris*: A review. *Renew Sust Energ Rev.* 2014;**35**:265-278. doi:10.1016/j.rser.2014.04.007.
22. Gharib R, Tabarzad M, Hosseinabadi T. Effect of High Salinity on Mycosporine-Like Amino Acid Production in *Desmodesmus* sp. *Trends Pept Protein Sci (TPPS).* 2020;**5**:1-6. doi:10.22037/tpps.v5i0.28876.
23. Kageyama H, Waditee-Sirisattha R, Tanaka Y, Takabe T. Osmoprotectant and Sunscreen Molecules From Halophilic Algae and Cyanobacteria. In: Rastogi RP, Madamwar D, Pandey A, editors. *Algal Green Chemistry: Recent Progress in Biotechnology*. Amsterdam: *Elsevier*; 2017:1-16. doi:10.1016/B978-0-444-63784-0.00001-1.
24. Geraldes V, Pinto EJP. Mycosporine-Like Amino Acids (MAAs): Biology, Chemistry and Identification Features. *Pharmaceuticals.* 2021;**14**(1):63. doi:10.3390/ph14010063.
25. Alves A, Sousa E, Kijjoa A, Pinto MJM. Marine-derived compounds with potential use as cosmeceuticals and nutricosmetics. *Molecules.* 2020;**25**(11):2536. doi:10.3390/molecules25112536.
26. Udayan A, Arumugam M, Pandey A. Chapter 4 - Nutraceuticals From Algae and Cyanobacteria. In: Rastogi RP, Madamwar D, Pandey A, editors. *Algal Green Chemistry: Recent Progress in Biotechnology*. Amsterdam: *Elsevier*. 2017. **p**. 65-89. doi:10.1016/B978-0-444-63784-0.00004-7.
27. García JL, de Vicente M, Galán B. Microalgae, old sustainable food and fashion nutraceuticals. *Microb Biotechnol.* 2017;**10**(5):1017-1024. doi:10.1111/1751-7915.12800.
28. Saewan N, Jimtaisong A. Natural products as photoprotection. *J Cosmet Dermatol*. 2015;**14**(1):47-63. doi:10.1111/jocd.12123.
29. Wang J, Pan L, Wu S, Lu L, Xu Y, Zhu Y, *et al*. Recent advances on endocrine disrupting effects of UV filters. *Int J Environ Res Public Health*. 2016;**13**(8):782. doi:10.3390/ijerph13080782.
30. Hartmann A, Gostner J, Fuchs JE, Chaita E, Aligiannis N, Skaltsounis L*, et al*. Inhibition of Collagenase by Mycosporine-like Amino Acids from Marine Sources. *Planta Med*. 2015;**81**:813-820.doi:10.1055/s-0035-1546105.
31. Suh SS, Hwang J, Park M, Seo HH, Kim HS, Lee JH, *et al.* Anti-inflammation activities of mycosporine-like amino acids (MAAs) in response to UV radiation suggest potential anti-skin aging activity. *Mar Drugs*. 2014;**12**:5174-5187. doi:10.3390/md12105174.
32. Puchkova T, Khapchaeva S, Zotov V, Lukyanov A, Solovchenko A. Marine and freshwater microalgae as a sustainable source of cosmeceuticals. *Mar Biologic J. (MBJ).* 2021;**6**(1):67-81. doi:10.21072/mbj.2021.06.1.06
33. Nazifi E, Wada N, Asano T, Nishiuchi T, Iwamuro Y, Chinaka S, *et al.* Characterization of the chemical diversity of glycosylated mycosporine-like amino acids in the terrestrial cyanobacterium *Nostoc commune*. *J Photochem Photobiol B, Biol.* 2015; **142**:154-168. doi:10.1016/j.jphotobiol.2014.12.008.