ORIGINAL PAPER

Culture Optimization to Produce High Yields of Mycosporine-Like Amino Acids by *Fischerella* sp. F5

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Abstract

Fischerella sp. is a valuable source of active metabolites, including UV-protecting compounds, among which mycosporin-like amino acids (MAAs) can be mentioned. Mycosporine-like amino acids are attractive secondary metabolites of a wide range of microorganisms, including microalgae and cyanobacteria. Enhanced production of MAAs has been studied in different sources. This study aimed to optimize the phosphate and nitrate concentrations of the culture medium on BG11 to maximize MAAs production from *Fischerella* sp. F5, using response surface methodology. The extraction process from the cultures, grown in adjusted conditions, was also optimized. The results confirmed that increasing both, nitrate and phosphate concentration, in the culture medium had a positive effect on the MAAs production by *Fischerella* sp. F5. While, optimization of the extraction process was not led to a highly accurate predictive model; temperature, sonication time, methanol ratio, and solvent/biomass ratio exhibited significant effects on the final MAAs' concentration in partially purified extracts. In general, more optimization cultures studies need to complete these findings in reference to MAAs production and extraction from *Fischerella* sp. F5, for commercial-scale applications.

Keywords *Fischerella* sp. · Experimental design · Mycosporine-like amino acid · Optimization · Nitrate · Phosphate

Introduction

Mycosporin-like amino acids (MAAs) are bioactive secondary metabolites of a wide range of organisms, including macro- and micro-algae and cyanobacteria to show ultraviolet (UV)-absorbing properties. MAAs can be specially produced in highly UV-irradiated environments [1]. These natural protective compounds are low molecular weight chemicals and they commonly are less than 400 Da, water-soluble, and colorless. Meanwhile, cyanobacteria have been recognized as a significant source of MAAs, and initially in 1969 was reported as high production of MAAs in cyanobacteria for *Microcoleus* [2].

Nowadays, eco-friendly and eco-sustainable products have become more attractively well-known to consumers [3]. As for sunscreens, a growing number of consumers are becoming aware of the harmful effects of synthetic UV filters on ecosystems [4, 5]. Accordingly, requests for cosmetic products with biocompatible ingredients are increasing every single day; and MAAs have been introduced as the most-studied natural UV-absorbing agents with many beneficial effects on the skin [6, 7]. They are considered a promising alternative active ingredient for synthetic chemical sunscreens [3, 8]. In that, this growing global demand is driving the cosmetics industry to invest more in the research and development of natural algae-based products [9]. As a consequence, it seems as an essential to detect the parameters that increase the production yield of MAAs. Interestingly, MAAs have been used in emulsions for protecting plant organs, fruits, and vegetables against sunscald while

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(HPLC grade) at 4 °C and in dark condition. Before overnight incubation, a sonication step at 35 °C for 15 min was applied using an ultrasound bath (S 60 H, Elma, Germany). Right the next day, the mixture was centrifuged for 10 min at 6700×*g*. The supernatant was collected and dried. Subsequently, it was dissolved in distilled water and centrifuged at 13,200×*g* to remove the insoluble impurities in the water. The supernatant was decanted against 500 μL of chloroform and centrifuged again at 13,200×*g*. Finally, the upper aquatic phase was collected and filtered using a 0.22 μm syringe filter (MS® PTFE Syringe Filter), before being injected into the HPLC column.

Characterization of MAAs

HPLC was performed on a C_{18} HPLC column (5 μm, 25×4.6 mm), equipped with a photodiode array (PDA) detector, which scanned 200–800 nm (SPD-M10A-Shimadzu, Kyoto, Japan). The isocratic mobile phase contained 25% aqueous methanol plus 0.1% acetic acid (HPLC grade) at a flow rate of 1 mL/min. MAAs compounds were detected at 330 nm.

Experimental Design

The response surface methodology, using Design-Expert software version 2.0.1.0, was used to simultaneously optimize nitrate and phosphate (quantitative) concentrations. The central composite design (CCD) was applied to maximize MAAs production by *Fischerella* sp. F5. The characteristics of study factors, are shown in Table 2.

Fourteen runs of 30-day cultures, in two blocks, were designed to optimize the range and levels of nitrate and phosphate concentrations. The MAAs content, as the primary response, was calculated based on the area under the curve (AUC) of associated peaks, extracted from the MAAs HPLC chromatograms. Additionally, dried biomass weight was measured and applied as the second response for the optimization process. For the optimization of MAAs extraction process, five factors including the solvent type and volume (solvent/biomass ratio), sonication time, extraction temperature, and duration were considered. Quantitative

measures were used to measure all factors. The central composite design (CCD) method was applied. A total number of 32 experiments in two blocks were run. Lastly, 2FI was the best model to predict the fractional effects of factors. The factors' features are presented in Table 2.

Validation of the Model

To validate the predicted model, two optimum cultures were run and analyzed. These runs were 30-day cultures, and the purification of MAAs was performed similar to the previous runs. The growth curves of *Fischerella* sp. F5 under both conditions (normal and optimized) were derived using two series of thirty falcons containing 10 mL cultures of *Fischerella* sp. F5, which were sampled on a daily basis. The initial inoculum consist of 1 mL of a 25-day culture of *Fischerella* sp. F5 was applied.

Statistical Analysis

The experiments were designed by Design Expert software using Response Surface Methodology. Experiments were run in two blocks. The value of correlation (R^2) indicated the quality of the developed model; and the analysis of variance (ANOVA) was conducted to evaluate the statistical significance of the model. The significant level was considered *at* $p \leq 0.05$.

Results

Optimization of Culture Nutrients

HPLC Analysis of Partially Purified MAAs from each Experiment

A total of 14 samples including partially purified extracts of the cultures that had grown under predetermined conditions were analyzed by HPLC and the data regarding retention times (Rt) and the AUC of MAAs peaks were shown in Table 3.

Table 2 The characteristics of different factors used in optimization process

Optimization of	Factor	Name	Units	Minimum	Maximum
Culture nutrients	A	phosphate	g/L	0.0000	1.21
	B	nitrate	g/L	0.0000	6.04
MAAs extraction	A	Time	Hour	24.00	72.00
	B	Temperature	°C	4.00	50.00
	C	Sonication time	Minutes	30.00	120.00
	D	Methanol ratio	%	10.00	100.00
	E	Solvent volume	mL	1.00	3.00

Table 3 The data of experiments designed for the culture condition optimization to maximize MAAs production by *Fischerella* sp

	Run	Phosphate(g/L)	Nitrate(g/L)	Biomass (mg)	MAA (AUC)	MAAs AUC%
Block 1	1	0	5	5.72	3521525	91.916
	2	0.5	2.5	8.82	1992172	61.129
	3	0	0	3.24	498150	32.694
	4	0.5	2.5	8.36	1673130	82.509
	5	1	0	5.72	1223955	76.849
	6	1	5	6.77	1428938	60.065
	7	0.5	2.5	8.79	2316563	82.257
Block 2	8	0.5	2.5	5.76	960943	55.556
	9	0.5	2.5	5.26	1006734	74.461
	10	0.5	0	3.62	537189	68.241
	11	0	2.5	4.69	1333983	71.939
	12	0.5	6.03553	3.74	1255329	68.868
	13	0.5	2.5	6.94	803198	47.859
	14	1.20711	2.5	3.38	466176	51.668

The HPLC chromatogram of run 1 was seen in Fig. 1. The main peak, with a retention time (Rt) of 2.929 min, had a maximum absorption at 330–340 nm, which earlier reported in a preliminary study of MAA production of this species [15]. However, in run 3, which contained none of phosphate and nitrate ingredients, the amount of MAAs content and purity identified in extracted samples were at the lowest value. The HPLC chromatogram of 3rd run was shown in Fig. 2. The peaks numbers, with maximum absorption between 300 and 400 nm, were increased that might indicate the presence of several MAA structures with close physicochemical characteristics.

Fig. 1 The HPLC chromatogram of run 1 (**a**) and the UV spectrum of the main peak (**b**) which showed a maximum absorption between 300 and 400 nm as the specific feature of MAAs
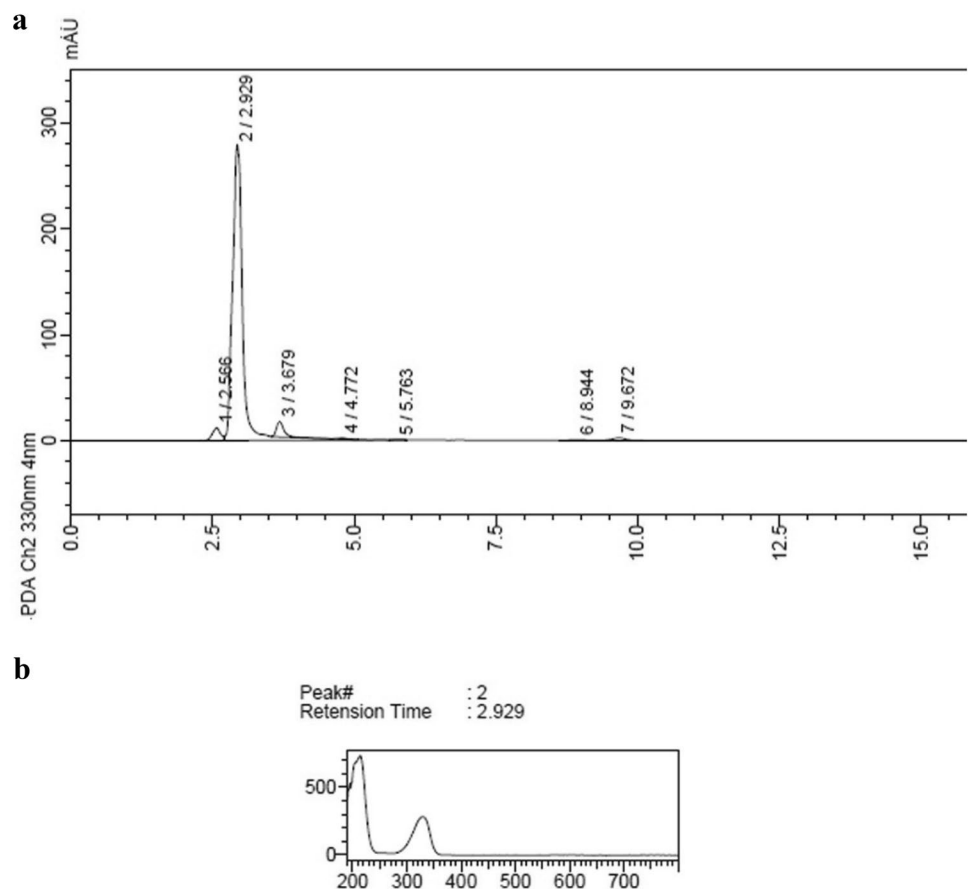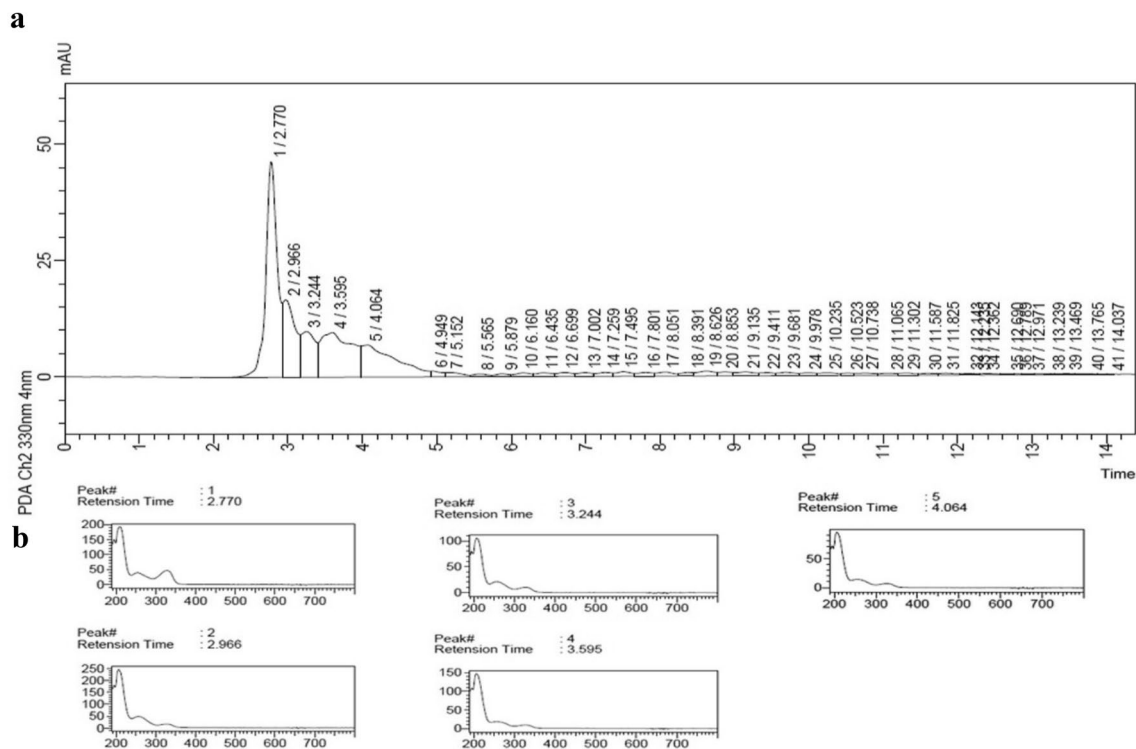
Fig. 2 The HPLC chromatogram of run 3 (**a**) and the UV spectrum of peaks (**b**) which showed a maximum absorption between 300 and 400 nm as the specific feature of MAAs

Modeling of the Culture Nutrients and Optimization of MAAs Production

All AUCs of the peaks with maximum absorption between 300 and 400 nm were accumulated and considered as the total MAAs content. The best model [1] with Adjusted R^2 equal to 0.81 was calculated. The Model F value was 17.50, which implied that the model was significant. The results revealed the model was significant ($p = 0.0004$). In this case, A, B, and AB were significant model terms as Eq. 1 shows.

$$\begin{aligned} \text{MAAs content} = &406189.66 + 720010.27(A) \\ &+ 515885.76(B) - 563678.4(AB) \end{aligned} \quad (1)$$

For maximizing the biomass, another model was predicted (Eq. 2). The Model F value was 16.77, which implied that the model was significant. P values of 0.0009 indicated the model was significant. In this case, A, B, A^2, and B^2 were significant model terms [2].

$$\begin{aligned} \text{Biomass} = &7.389 + 0.567(A) + 0.837(B) \\ &- 0.357AB - 1.574(A^2) - 1.743(B^2) \end{aligned} \quad (2)$$

In Eqs. 1 and 2, A and B were phosphate and nitrate concentrations, respectively.

Table 4 The specifications of the best culture condition to maximize MAAs production by *Fischerella* sp

Phosphate g/L	Nitrate g/L	MAA	Biomass	Desirability	Desirability (w/o Intervals)
0.235	4.622	2348201.818	6.137	0.569	0.627

The best culture condition for maximum production of MAAs was reported in Table 4. The 3D surface model for the maximum production of MAAs was presented in Fig. 3.

The Optimized Culture Validation

The culture under the optimum condition (including 0.235 g/L of phosphate salt and 4.622 g/L of nitrate salt) and extraction process were performed. HPLC analysis of partially extracted MAAs showed a different pattern (Fig. 4). The main peak was seen around 3 min and there were at least four compounds with maximum absorption at 330–340 nm, although, all of them had similar maximum absorption wavelengths. Comparison of the growth curves of *Fischerella* sp. F5 cultured in normal and optimized conditions revealed a relatively increase in the growth and biomass production (Fig. 5).

Fig. 3 3D surface model for Phosphate and Nitrate concentrations to maximize MAAs production (**a**) and to maximize the MAAs content percent in final extract (**b**)

Fig. 4 The HPLC chromatogram of MAAs extracted from optimized culture (**a**) and the UV spectrum of peaks (**b**) which showed a maximum absorption between 300 and 400 nm as the specific feature of MAAs

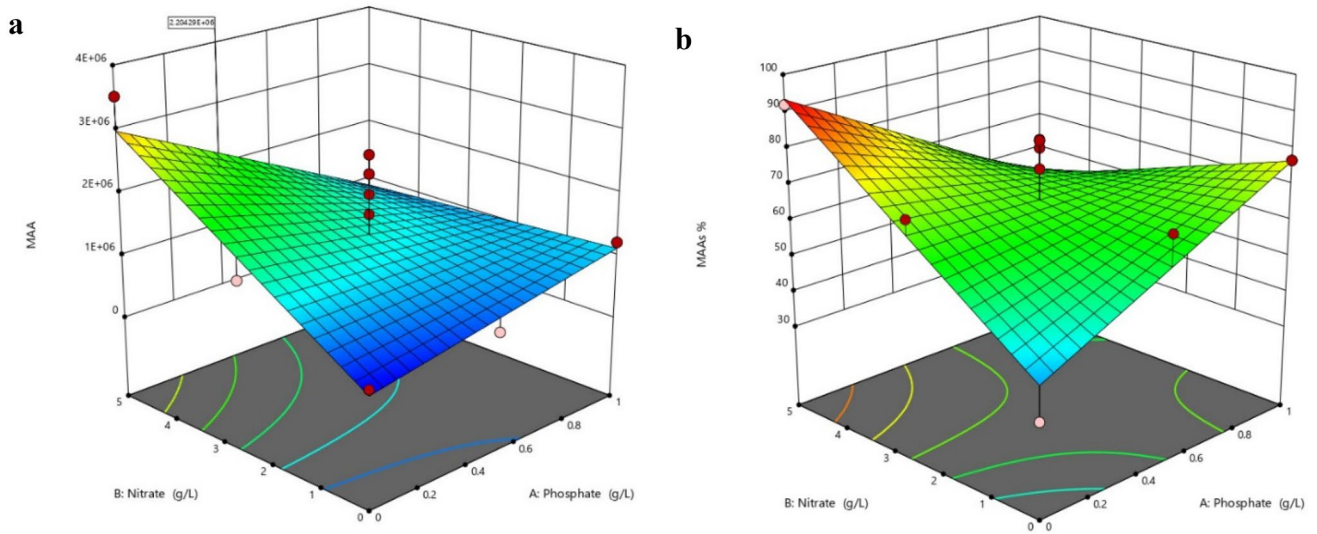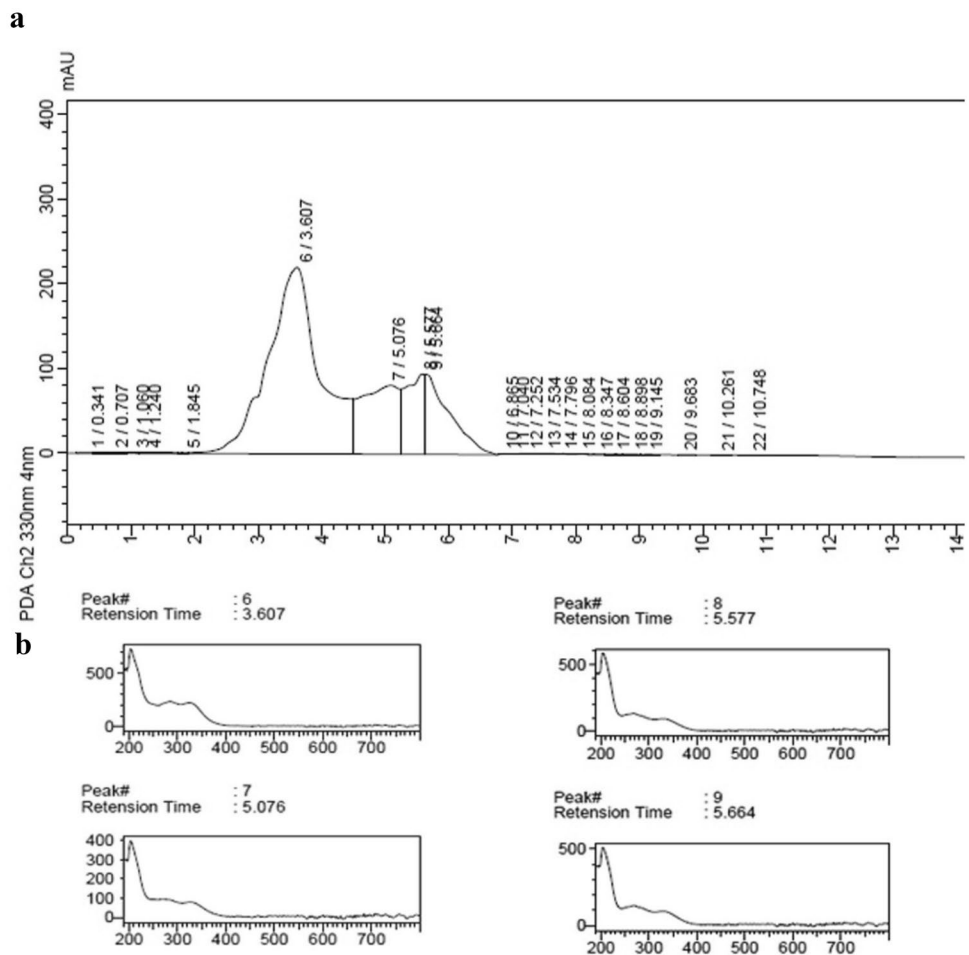## Optimization of MAAs Extraction Process

HPLC Analysis of Partially Purified MAAs from each Experiment

The culture under the optimum condition was performed and the extraction process was optimized by running 32 experiments, which were designed by 1/2 factorial design, considering five factors of time, temperature, sonication time, methanol ratio, and solvent volume. HPLC analysis of extracted samples was performed similar to the previous optimization process on the culture condition. Experiment features and results obtained from HPLC analysis were reported in Table 5.

Modeling of the Extraction Process and Optimization of MAA Partial Purification

The best model (Eq. 3) with Adjusted R^2 equal to 0.72 was calculated. The Model F value was 6.05, which implied the significance of the model. A P value of 0.0006 indicated that the model was significant. In this case, B, C, D, E, AC, AE, BE, and CD were significant model terms.

$$
\begin{aligned}
Ln_{(MAAs\ content)} = {} & 13.41 + 0.334(A) + 0.862(B) + 0.915(C) \\
& - 1.68(D) + 0.982(E) - 0.538(AB) \\
& - 1.012(AC) + 0.563(AD) - 1.08(AE) \\
& - 0.596(BC) + 0.42(BD) - 1.065(BE) \\
& + 1.254(CD) - 0.665(CE) + 0.575(DE)
\end{aligned}
\tag{3}
$$

In Eq. 3, A, B, C, D, and E were the time of extraction process, temperature, sonication time, methanol ratio, and solvent volume in the extraction process, respectively.

The best model had R^2 around 0.7 for MAA% and the numbers of MAA compounds and a significant model could not be adopted to data. The best predicted extraction process and the anticipated values for each factor were reported in Table 6.

Discussion

This study aimed to optimize the MAAs production and extraction as a natural sun-protective agent. Different organisms have developed unique chemical adaptations to confront the detrimental effects of the sun's ultraviolet radiation. Melanin in animals and humans, flavonoids in higher plants, and scytonemin and mycosporine-like amino acids (MAAs) in organisms dwelling in the marine environment; all are secondary metabolites produced to act as UV-absorbing agents in these species [25]. Using these natural UV protectants has been expanding in recent years. Therefore, finding new sources and optimizing the production and extraction of effective ingredients are attractive fields of study. In the current study, MAAs were considered as potent natural UV protectants, which used in sunscreens, and their production and extraction in *Fischerella* sp. F5, as one of MAAs source, was optimized using response surface methodology.

Regarding nutrient availability, nitrogen and phosphorus as the macronutrient, and iron as the micronutrient, are considered to be the most important resources affecting cyanobacterial growth. In a recent study on two N2-fixing cyanobacteria, it was observed that in *Halothece* sp., nitrate is the limiting factor, and a decreasing concentration of nitrate had a negative effect on its growth, while it was less influenced by phosphate or iron concentrations. On the contrary, *Fischerella muscicola*'s growth, was unaffected by the decrease in nitrate concentration, but phosphate and iron were more restrictive components in its growth [26]. While increasing the initial nitrate concentration resulted in enhanced MAA biosynthesis by *Glenodinium foliaceum*, it had opposite effect on increasing the initial phosphate concentration.. It should be mentioned that the nitrate and phosphate content
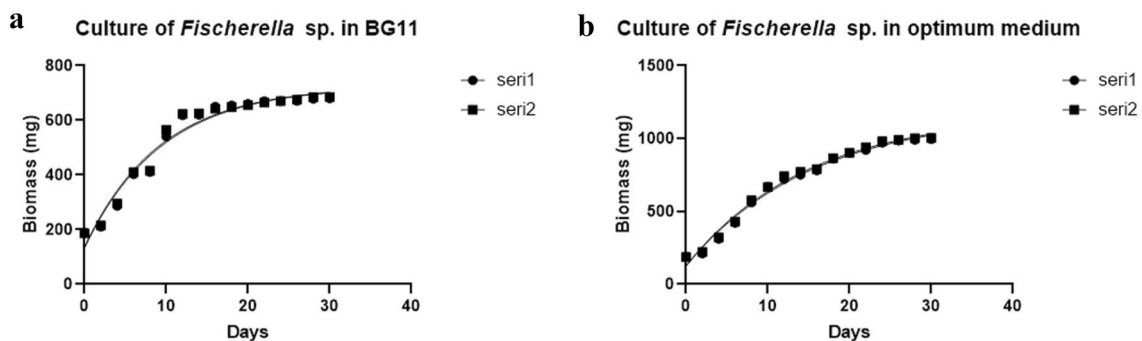

Fig. 5 The growth curves of *Fischerella* sp. F5 in **a** the BG11 medium and **b** the optimum culture medium containing 0.235 g/L of phosphate and 4.622 g/L of nitrate salts

Table 5 The data of experiments designed for the optimization to MAAs extraction from *Fischerella* sp. optimized culture

Run	Block	Time (h)	Temperature (°C)	Sonication time (min)	Met. ratio	Solvent volume (mL)	MAAs content (AUC)	MAAs%	No. MAAs
1	1	24	4	120	100	3	2609000	81.354	3
2	1	24	4	120	10	1	530244	57.278	2
3	1	48	27	75	55	2	2787170	87.442	2
4	1	24	50	120	100	1	602515	56.556	2
5	1	72	4	120	10	3	728588	79.883	2
6	1	24	4	30	100	1	1000	0.01	1
7	1	24	50	30	100	3	111389	44.401	1
8	1	72	4	30	100	3	106972	37.01	1
9	1	72	4	120	100	1	120206	46.725	1
10	1	72	4	30	10	1	1876770	80.87	2
11	1	72	50	120	10	1	2825990	87.383	2
12	1	48	27	75	55	2	1342400	75.716	3
13	1	48	27	75	55	2	1374140	66.231	3
14	1	72	50	120	100	3	128759	35.672	1
15	1	72	50	30	10	3	5808970	93.536	3
16	1	72	50	30	100	1	169307	62.075	2
17	1	24	4	30	10	3	9658606	94.563	2
18	1	24	50	120	10	3	7105820	92.911	2
19	1	24	50	30	10	1	3145616	79.377	2
20	1	48	27	75	55	2	6531260	87.443	2
21	2	48	27	120	55	2	16664607	82.981	2
22	2	48	27	75	55	2	755471	61.543	2
23	2	48	27	75	55	2	287993	24.348	2
24	2	48	27	75	55	1	312093	44.765	3
25	2	48	4	75	55	2	245319	29.345	1
26	2	72	27	75	55	2	724187	56.151	4
27	2	24	27	75	55	2	557009	42.833	3
28	2	48	27	75	55	3	1381406	68.026	2
29	2	48	50	75	55	2	589976	52.568	2
30	2	48	27	75	100	2	3511316	73.951	4
31	2	48	27	75	10	2	915867	68.172	2
32	2	48	27	30	55	2	328572	40.429	2

Table 6 The best features of the extraction process to maximize MAAs extraction from *Fischerella* sp. biomass

Time	Temp	Sonic. Time	Met. ratio	Solvent volume	MAA extract	%MAA	Desirability
41	50	30	81.587	3.0	642353.307	57.070	0.896

of algal culture media had various effects on MAAs production and the composition of accumulated MAAs [27].

The findings revealed that increasing nitrate and phosphate concentrations had a positive effect on the MAAs production by *Fischerella* sp. F5. Concerning the best model fitted to data, increasing two-fold of nitrate and phosphate concentration resulted in a 2.64 and 2.28-fold increase in MAAs content, respectively. However, there was a superior effect of nitrate on the MAAs production compared to phosphate. Both nitrate and phosphate optimum concentrations were higher than their concentration in common BG11 culture medium composition (in which $NaNO_3$ is 1.5 g/L and K_2HPO_4 is 0.04 g/L). Similarly, Saadatmand and Zamani (2015) found that increasing of nitrate and phosphate concentrations could significantly enhance MAAs production by *Spirulina platensis*. They clarified that optimizing the macronutrients of culture medium, like nitrate and phosphate, could change the photosynthesis pathways

and stimulating shikimate pathway in increment of the UV absorbing metabolites such as MAAs [28]. Moreover, the characterization of MAAs from red marine algae of the Brazilian coast exhibited that high concentrations of phosphate and nitrate could enhance MAA contents [22].

Indeed, in certain cyanobacterial strains, phosphate-limited conditions are in favor of higher MAAs production. However, the increased nutrient availability caused better growth, in general [29]. In contrast to *Glenodinium* sp., *Lyngbya purpureum* was shown to produce more MAAs in a lower nitrogen concentration (0.3 mM compared to 1 mM) [30]. Therefore, it can be concluded that various microalgae and cyanobacteria species have responded different nutrient availability which resulted in varieties in growth patterns. In that, some cyanobacteria demand high concentrations of nitrogen and phosphate to grow better, while others prefer low-nutrient conditions [31].

Volkmann and Gorbushina developed a universal method for the extraction and characterization of mycosporines and mycosporine-like amino acids of terrestrial, marine, and freshwater origin. The process included 20 mg of dried microorganism biomass suspended in 1 mL of 0.2% aqueous acetic acid with 0.5% methanol (v/v), followed by 10 s vortexing, and incubation at 4 °C for 12 h on a vertical shaker. The extracted samples were then centrifuged at 14,000×*g*, 5 min and the supernatants were filtered through 0.2 µm syringe filters. Final extracts were analyzed by HPLC and LC/MS/MS. The study approach was used for simultaneous extraction and analysis of MAAs. They could efficiently extract and detect glycosylated MAAs by HPLC, which were identified at the retention times of upper than 10 min [32]. However, in current study, MAAs extraction was performed followed the Rastogi et al. (2016) method [24, 33] with minor modifications including using methanol as extraction solvent, followed by chloroform decanting to remove impurities, such as proteins, from MAAs partially purified extract. The structural analysis was performed by HPLC using 25% aqueous methanol and 0.1% acetic acid as mobile phase.

Generally, a typical MAAs extraction was performed using absolute ethanol, up to ethanol 50%, or absolute methanol up to 20% in aquatic solution [1]. Ngoennet et al. (2018) reported a simple method for the MAAs extraction from the halotolerant cyanobacterium *Aphanothece halophytica*. They suspended the organism's cells in methanol and sonicated them using an ultrasound probe homogenizer. Then, the extraction mixture was centrifuged and the supernatant was dried using a rotary evaporator. Then, dried extract was solved in water, and decanted against chloroform to remove coloring compounds [34]. Another study reported that the extraction with water could increase the MAAs yields by twofold compared to absolute methanol. This study showed that 6 h extraction with water could lead to the best yield of MAAs extraction [35].

Here, we considered a similar strategy, and the factors involved in the extraction, including time, temperature, sonication time, methanol concentration, and solvent volume, were optimized. Results confirmed, except the time, all other factors could significantly affect the MAAs extraction yield. The best-predicted process would include extraction in 30 mL of 80% methanolic solution for 41 h at 50 °C, after 30 min sonication. In a similar study, Sultani et al. investigated the effect of different solvent including 100% and 20% methanol, 70% ethanol, distilled water and 100% ethyl acetate and the temperatures of 4 °C and 45 °C on the MAAs extraction from the cyanobacterium *Leptolyngbya fragilis* and red algae *Hypnea musciformis*. They found the best extraction form the cyanobacterium *L. fragilis* was when using the 100% ethyl acetate at 4 °C, and for red algae *H. musciformis* was in the 20% methanol extract at 45 °C [36].

It seemed that increasing the sonication time followed by incubation at higher temperature decreased the MAAs yield in the final extract, which probably resulted in MAAs instabilities against long time ultrasound exposure. Nevertheless, it was reported that MAAs were highly stable structures, such as MAAs extracted from the cyanobacterium *Gloeocapsa* sp. CU-2556. [37], *Nostoc* sp. R76DM [24], or cyanobacterium *Lyngbya* sp. [33], which all were stable against physicochemical stressors, including UV radiation, pH, heat, and a strong oxidizing agent.

Conclusion

The differences between cyanobacterial species should be considered for the optimization of their growth, as well as, the optimization of MAAs production, particularly for industrial scale. In this study, MAAs production by *Fischerella* sp. F5 was optimized, considering nitrate and phosphate concentrations in the culture medium, using response surface methodology. The optimized culture condition exhibited a slightly different HPLC pattern of partially purified MAAs compared to the culture in common BG11 medium. It should be well-thought-out that only the concentrations of two main macronutrients of cyanobacterial culture medium were optimized in present study, and the concentrations of other macro or micronutrients may also have partial effect on cyanobacterium growth or its MAA production. Further studies are needed to determine all influential factors in MAAs production, also. Subsequently the optimization of extraction process, based on the most important parameters in MAA extraction revealed that the optimum features to maximize the extraction yield are temperature, sonication time, and solvent type and volume. These factors significantly could affect the yield of MAAs extraction.

Declarations

Competing Interests All authors declare that they have no conflicts of interest.

Ethical Approval The work did not contain any human or animal stucy and it was approved by the Ethical committee of Shahid Beheshti University of Medical Sciences, Tran, Iran (IR.SBMU.PHARMACY. REC.1400.231).

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