Nitrogen-Containing Metabolic Stressors Stimulate High-value Compounds Accumulation in Arthrospira platensis

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Abstract

Suitable conditions for high-value compounds hyperaccumulation are the most challenging approach in Arthrospira platensis. In the current study, the co-production of specialty pigments and high-value metabolites was investigated with a varying concentration of well-known and candidate metabolic stressors in Arthrospira platensis. Supplementing Arthrospira platensis with a minimum of 0.50% of Monosodium glutamate (MSG) and a maximum of 2% of aspartate (ASP) increased Phycocyanin productivity by 83.36 and 64.77 percent, respectively. However, a maximum of 1.50% of MSG and a minimum of 0.50% ASP increased carotenoid content by 188.80 and 68.97 percent, respectively. These observations, coupled with those showing supplemented ASP stimulate unsaturated fatty acid accumulation, possibly through enhanced de novo biosynthesis, and essential amino acid biosynthesis to a greater extent in Arthrospira platensis. A network correlation analysis also indicated the distinct metabolite abundance and the corresponding associated metabolic networks linked to pigment production in Arthrospira platensis.

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Abstract

Suitable conditions for high-value compounds hyperaccumulation are the most challenging approach in *Arthrospira platensis* . In the current study, the co-production of specialty pigments and high-value metabolites was investigated with a varying concentration of well-known and candidate metabolic stressors in *Arthrospira platensis* . Supplementing *Arthrospira platensis* with a minimum of 0.50% of Monosodium glutamate (MSG) and a maximum of 2% of aspartate (ASP) increased Phycocyanin productivity by 83.36 and 64.77 percent, respectively. However, a maximum of 1.50% of MSG and a minimum of 0.50% ASP increased carotenoid content by 188.80 and 68.97 percent, respectively. These observations, coupled with those showing supplemented ASP stimulate unsaturated fatty acid accumulation, possibly through enhanced de novo biosynthesis, and essential amino acid biosynthesis to a greater extent in *Arthrospira platensis* . A network correlation analysis also indicated the distinct metabolite abundance and the corresponding associated metabolic networks linked to pigment production in *Arthrospira platensis* .

Keywords : *Arthrospira platensis* , Aspartic acid, Monosodium glutamate, metabolic stress

1 | Introduction

Microalgal cultivation, as a green technology, has now received great attention. The biomass of cyanobacterium *Arthrospira* (i.e.,*Spirulina*) studied as superfoods owing to contain about 50-70% protein (as the major component), 15-25% carbohydrates, and 6-13% lipids, as well as substantial amounts of vitamins, minerals, and chlorophylls, which make the *Arthrospira* of excellent nutritional profiles and high-quality proteins (Salla et al., 2016).*Arthrospira* also contains low cholesterols and is considered an easy-digested food due to cellulose's absence in the cell wall (Benelhadj, Gharsallaoui, Degraeve, Attia, & Ghorbel, 2016). Nowadays, microalgae are being incorporated into many food formulations as a coloring agent. However, *Arthrospira* shows potential for being used as ingredients in the development of novel functional foods, which are among the top trends in the food industry (Lafarga, Fernández-Sevilla, González-López, & Acién-Fernández, 2020).

Phycobiliproteins are the most abundant proteins in the structure of*Arthrospira* . Phycocyanin, as a blue chromoprotein-based dye with numerous functional properties, is the most common constituent of phycobiliproteins (7-14% dry weight). The biomass accumulation and productivity of high-value metabolites in microalgae are associated with several parameters such as proper microalgae species, environmental conditions, and the culture medium components of which the culture medium's composition is the most critical factor (Manirafasha et al., 2017). Recently, several investigations have been accomplished by the supplementation of ammonium sulfate and urea (Soletto, Binaghi, Lodi, Carvalho, & Converti, 2005), sugarcane vinasse (Dos Santos, Fernandes-Araujo, De Medeiros, & Moreira-Chaloub, 2016), sodium nitrate, and ammonium chloride (Johnson et al., 2016) as the nitrogen sources on *Arthrospira* to enhance the biomass accumulation, Phycocyanin productivity, and amino acid biosynthesis. It was previously shown that the utilization of monosodium glutamate (MSG) could boost the growth and accumulation of Phycocyanin in *Arthrospira* . MSG is one of the intermediate metabolites in the biosynthesis of tetra-pyrroles such as porphyrin and vitamin B_{12} in living cells, along with phycobilin and chlorophylls in cyanobacteria cells (Manirafasha et al., 2017). Likewise, aspartic acid (ASP) is the precursor to several amino acids, including four essential amino acids: methionine, threonine, isoleucine, and lysine in plants and microorganisms (Fornazier, Azevedo, Ferreira, & Varisi, 2003). ASP and MSG are available, green material, inexpensive, and naturally occurring compounds that serve as good nitrogen sources for cultivating microalgae or microorganisms. Besides, cyanobacteria's cell membrane has permeability for glutamate and aspartate via a periplasmic substrate-binding protein named 'N-II (the ABC-type amino acid uptake transporter), which is responsible for translocating acidic amino acids (Pernil, Picossi, Herrero, Flores, & Mariscal 2015). A thorough evaluation of the relevant literature yielded some data about applying MSG supplement in *Arthrospira* cultivation. But, no report has been reported on the use of ASP supplement in *Arthrospira*cultivation.

The metabolic stress is currently a promising approach for manipulating the cell pathway and elevating interest metabolites production and cell growth. In this regard, *Arthrospira,* with a flexible and unique tricarboxylic acid (TCA) cycle, is an appropriate candidate for examining the production of valuable metabolites using amino acids as the substrates. Different patterns of desired metabolic profiles with some minor changes in growth conditions or the medium composition are easily attainable in this cyanobacterium

(Steinhauser, Fernie, & Arau´jo, 2012). A wide range of regulatory mechanisms has been proposed for the TCA cycle in plants (Zhang and Fernie, 2018). Hence, it may be an utterly cost-effective production strategy. However, more studies are needed to understand the key metabolic chokepoints and regulatory sites in utilizing primary metabolic intermediates in *Arthrospira* . Likewise, culturing conditions are essential to biomass productivity and promote the content of high-valuable metabolites that have potential applications in the food industry as pigments, bioactive and functional compounds.

This research aimed to boost cell growth and the simultaneous co-production of high-valuable compounds in response to the varying concentration of two metabolic stressors in *A. platensis* under suitable culture medium conditions. Furthermore, a network correlation analysis was applied to identify biomarkers linked to pigment production in *Arthrospira platensis* .

2 | Materials and methods

2.1 | Microalgae cultivation

Arthrospira platensis (NIES-39) was purchased from the National Institute of Environmental Studies Collection, Japan. The pre-cultures were incubated into a photo-incubator under a continuous light intensity of 40-60 µmol photons $m^{-2}s^{-1}$ at 28 °C. Furthermore, the seed culture was irradiated for three days in 300 µmol photons m^{-2} s^{-1} white light-emitting diodes (LEDs, white Aqua Super LED-lamps, Singapore), followed by applying an overnight combination of red and blue LEDs each with 150 µmol photons m^{-2} s^{-1} intensity (Ooms, Dinh, Sargent, & Sinton, 2016). The Zarrouk culture medium was prepared as previously described by (Xie et al., 2015). Then, the medium was sterilized, and different concentrations of ASP (i.e., 0.5, 1, 1.5, & 2% w v^{-1}) and MSG (i.e., 0.25, 0.5, 0.75, 1, & 1.5% w v^{-1}) were added into the Zarrouk medium by a 0.2 µm filter. The temperature and pH were set to 28±2 °C and 9±0.2, respectively. Moreover, the aeration rate was 0.2 vvm. An initial inoculum of 0.350 g L^{-1} of 4 days old culture was inoculated to the liquid Zarrouk culture medium, and the cultures were irradiated for 12 days under continuous illumination of 300 µmol photons m^{-2} s^{-1} white LEDs. All pre-cultures and production cultures were grown in a 1 L glass vessel (SCHOTT DURAN), which was considered as a photobioreactor.

After 12 days, the gained biomass was collected from the liquid medium by centrifugation (Hitachi Himac CR-GIII, Tokyo, Japan) at 18,000 g for 15 min at 20 °C. Then, the pellet was washed twice with deionized water and lyophilized (FDB-5502, OPERON, KOREA) at -60°C to obtain the dry weight for further experiments.

2.2 | Growth measurements

The samples of the liquid culture broth were collected every 48 h and analyzed based on the study's aim. Next, the biomass was measured using the sample's optical density (OD) at 680 nm wavelength by a UV-1780 spectrophotometer (Cary300, Japan). The correlation of the biomass dry weight (BDW) and OD was determined by equation (1) as follows:

$BDW = OD_{680} * 0.64 - 0.047$ ($R^2 = 0.988$) (1)

The productivity (P), specific growth rate (SGR), and doubling time (td) of *A. platensis* were also estimated at the exponential phase by equations 2, 3, and 4 (Xie et al., 2015):

$P\ (g\ L^{-1}day^{-1}) = (X_2 - X_1) / t$ (2)

$\mu\ (day^{-1}) = (\ln X_2 - \ln X_1) / t$ (3)

$td\ (day) = \ln 2 / \mu = 0.693 / \mu$ (4)

Where X_2 = biomass density, X_1 = initial biomass, and t = time interval.

2.3 | Measurement of targeted metabolites

2.3.1 | Chlorophyll and total carotenoids

Chlorophyll *a* and the total carotenoid contents of 1 mL of the algal culture were determined spectrophotometrically (Cary300, Japan) by their extracting from the biomass with methanol 1:1 (V/V) at 662, 645, and 470 nm, respectively. The Chlorophyll *a* and total carotenoid concentrations in the extract were calculated using equations (5) and (6) according to Sido (2019).

Chl *a* (mg L^{-1}) = 12.94*(A_{665} - A_{720}) (5)

Total carotenoids (mg L^{-1}) = [1000 (A_{470} - A_{720}) - 2.86 (Chl *a*))/221 (6)

2.3.2 | Phycocyanin

Phycocyanin was extracted from 5 mL of the algal culture. Briefly, samples were centrifuged at 12000 g for 10 min at 4°C. Next, cell pellets were washed twice with distilled water, suspended in 1 mL of a 100 mM phosphate buffer saline (PBS, pH: 6.8), frozen overnight at -70 °C, and thawed at 4 °C. Then, 4 mL of a 100 mM PBS was added to the mixture, and the suspension was stirred at 400 rpm for 4 h at room temperature. The cell debris was removed by centrifugation at 18,000 g for 20 min at 4 °C and the blue-colored supernatant (phycocyanin crude extract) was collected (Fekrat, Nami, Ghanavati, Ghaffari, & Shahbazi, 2018), followed by measuring the phycocyanin concentration using equation (7).

Phycocyanin concentration (mg mL^{-1}) = [A_{618}-0.474 (A_{650})]/5.34 (7)

Where A_{618} and A_{650} are the maximum absorbance of Phycocyanin and allophycocyanin, respectively.

2.3.4 | Fatty acids

The fatty acid methyl ester (FAME) was extracted via the direct transesterification method based on the study by Lepage and Roy (1986) with minor modifications. Briefly, 300 µL of the extraction buffer containing methanol (MeOH) and 2% of concentrated H_2SO_4 were added to 4 mg of the algal sample. The sample was incubated for 2 h at 80 °C at 750 rpm in a thermomixer (Thermomixer compact, Eppendorf, the USA). Then, 300 µL of a 0.9% NaCl solution and 300 µL of hexane were added into the reaction tube. The sample was centrifuged at 3000 g at 20°C for 5 min, and finally, the supernatant containing hexane and FAME was used for gas chromatography analysis (Matthew et al., 2009). Next, fatty acids were analyzed using a Varian CP-3800 gas chromatography (Varian, Inc., Palo Alto, CA) equipped with a CP-Sill 88 fused silica column (100 m, 0.25 mm I.D., and film thickness of 0.25 µm). For fatty acid measurement, the oven temperature was set at 130°C for 4 min, increased to 180 °C at the rate of 5 °C/min, kept at 180 °C for 8 min. At last, the oven temperature was programmed to rise from 180 to 220 °C at the rate of 4 °C/min under the following conditions: Helium as the carrier gas (1 mL min^{-1}), a split ratio of 20:1, and the flame ionization detector (FID) at 280°C. Fatty acid peaks were identified by comparing the retention time with the FAME standards of Merck's fatty acid (Darmstadt, Germany).

2.3.5 | Amino acids

Amino acids were extracted using 20 mg of frozen samples and 1 mL of EtOH (80%), followed by heating the mixture at 80 °C for 1 h and then centrifuging it at 13000 g. The extracted supernatant (250 µL) was mixed with 100 µL of *ortho* -phthalaldehyde solution containing 50 mg o-phthaldialdehyde, 4.5 mL of MeOH, 0.5 mL of a borate buffer (50 mM, pH: 9.5), 50 µL of 2-mercaptoethanol, and 200 µL of a borate buffer and kept for 2 min at room temperature. The reaction was quenched by adding 50 µL of hydrochloric acid 0.5 N to the solution. Next, amino acids were separated on a C_{18} column (HALO C_{18}, 4.6 × 50 mm, 5 µm particle size, Advanced Materials Technology, Wilmington, USA) by the high-performance liquid chromatography (Knauer, Germany) and then detected by a fluorescence detector (RF-10 AXL, Berlin, Germany). The excitation and emission wavelengths were 330 and 450 nm, respectively. In addition, a gradient of 20-70% MeOH in the sodium acetate buffer (50 mM, pH: 7.0) with a flow rate of 1.1 mL min^{-1} was used as the mobile phase. Finally, each amino acid amount was calculated by comparing its peak area with that of the corresponding standard (Zhu, Zhu, & Gao, 2008) .

2.4 | Statistical analysis

The variance analysis was performed using statistical software (SPSS 23.0) with three replications and presented as the mean ± standard deviation. Then, the means were compared using Duncan's multiple range test ($p<0.05$).

2.5 | Data visualization

The Visualization and Analysis of Networks containing Experimental Data platform was used to analyze and visualize the metabolic data in their metabolic maps (Junker, Klukas, & Schreiber, 2006).

3 | Results and discussion

3.1 | Effects of MSG and ASP on the growth kinetics of*A. platensis*

Table 1 and Fig. 1 (a, b) present the biomass production and growth characteristics, including the productivity, specific growth rate, and doubling time of the *A. platensis* cultivated under different ASP and MSG concentrations. The maximum biomass production of 3.67±0.26 g L^{-1} was achieved on 12th day of cultivation in the control medium. Based on the results, the supplemented medium with 0.5% and 1% ASP had no significant difference with respect to biomass production ($p<0.05$) while the maximal biomass production of 5.48±0.28 g L^{-1} was obtained at 2% ASP (Fig. 1a). However, a significant increase was observed in biomass production (6.03±0.25 g L^{-1}) when the medium was supplemented with 0.25% MSG (Fig. 1b), followed by a decrease in maximal biomass with a higher concentration of MSG, indicating that the optimum biomass production correlates with lower MSG and higher ASP supplements in the Zarrouk medium. In contrast, the minimum biomass production of 2.13±0.33g L^{-1} occurred with 1.5% MSG (Fig. 1b), which might be due to acidifying the growth medium by a high level of L-glutamic acid (Khan and Yoshida 2008). On the other hand, the maximum microalgae productivity of 0.299±0.021 g $L^{-1}day^{-1}$ was obtained in the control culture medium at 12 days of cultivation. Furthermore, the productivity of microalgae grown under 0.5-2% w/v of ASP and 0.25-1.5% w/v MSG showed a range from 0.302±0.013 to 0.450±0.024 and 0.498±0.021 to 0.171±0.028 g L^{-1} day^{-1}, respectively. It seems that productivity is more affected in a medium supplemented with different MSG concentrations compared to ASP. Similarly, the SGR values of *A. platensis* were augmented by increasing ASP levels ranging from 0.5 (SGR: 0.320±0.012) to 2% (SGR: 0.353±0.014) while MSG levels ranging from 0.25 (SGR: 0.361±0.010) to 1.5% (SGR: 0.273±0.024) decreased SGR values. Further, the maximum SGR value of 0.361±0.010 was found in 0.25% at 12 days of cultivation (Table 1). The data revealed that *A. platensis* had a minimum doubling time comprising 1.921±0.051 and 1.967±0.076 days, in a 0.25% MSG and 2% ASP, respectively. However, the doubling time of 2.174±0.073 was observed at the control condition. The results mentioned above indicated that lower MSG and higher ASP supplements correlate with biomass production, optimum growth indicators, SGR values, and doubling time in the Zarrouk medium (Table 1). It is well-documented that microalgae growth directly relies on perceiving the effects of various chemical compounds on the culture medium. Based on previous findings, some bioactive compounds in microalgae are accumulated under the conditions of deficiency or the abundance of nutrients in the culture medium or the addition of precursor and intermediate compounds (Salla et al., 2016). Moreover, some studies applied supplements as a metabolic regulator for cultivating various microalgae such as *Chlorella vulgaris* (Ji et al., 2014) and *Rhodotorula glutinis* (Xue, Miao, Zhang, Luo, & Tan, 2008). The present study's approach relied on the idea that applying supplements as a metabolic regulator enhances various microalgae's biomass production. It is noteworthy that the variance in biomass production is partly due to a varying concentration of supplements that have a significant impact on optimum growth cells. However, the present results affirm the growing evidence regarding the role of supplements in microalgae growth. Additionally, sodium glutamate enhanced biomass yield in *A. platensis* FACHB-314 (Manirafasha et al., 2017). However, the addition of a supplement in the culture medium of microalgae and directed sources in bioactive compound biosynthesis aimed to enhance the high content phycocyanin accumulation in *A. platensis* .

3.2 | Different concentrations of MSG and ASP supplements affected the physiological and metabolic traits of *A. platensis*

3.2.1 | Chlorophyll content

Table 1 provides data related to the Chlorophyll *a* content of the*A. platensis* cultivated under different ASP and MSG concentrations. The Chlorophyll *a* content was in the range of 0.86±0.12 mg L^{-1} to the maximum value of 1.87±0.08 mg L^{-1} in control and 2% ASP at 12 days in the culture medium, respectively. The maximum Chlorophyll *a* content of 1.72±0.10 mg L^{-1} was also yielded at 0.5% MSG at 12 days of cultivation. These findings are consistent with those of the previous study (Rangel-Yagui, Danesi, De Carvalho, & Sato, 2004), showing that the Chlorophyll *a* content is ameliorated with nitrogen-containing compounds in the culture medium.

3.2.2 | Pigments content of Phycocyanin andcarotenoids accumulation

The maximum phycocyanin productivity of 0.18±0.016 mg L^{-1} was achieved at 12 days of cultivation in the control medium. Besides, phycocyanin productivity increased from 0.20±0.15 mg L^{-1} at 0.5% ASP and reached a maximum of 0.291±0.017 mg L^{-1} at 2% ASP at 12 days of cultivation (Fig. 2a). Contrarily, phycocyanin productivity decreased from 0.330±0.014 mg L^{-1} at 0.50% MSG and reached a minimum of 0.023±0.002 mg L^{-1} at 1.5% at 12 days of cultivation, indicating a sign of nitrogen depletion in the culture medium (Fig. 2a). The findings of a previous study showed that a lower nitrate concentration might enhance the final phycocyanin content in a fed-batch process (Kim, Mujtaba, & Lee, 2016). The data are perfectly in line with previous findings, implying that adding a proper concentration of stimulants (MSG) improves phycocyanin accumulation in*A. platensis* FACHB-314 (Manirafasha et al., 2017). Interestingly, there was no significant difference between 1.5% and 2% ASP, as well as 0.25% and 0.5% MSG (Fig. 2a). Thus, the results suggested that controlling nitrate concentration is necessary for improving phycocyanin production.

Furthermore, the maximum of the carotenoid productivity of 3.98 ± 0.24 mg L^{-1} was obtained at 1.5% MSG at 12 days of cultivation than the control condition. In contrast, the obtained carotenoid productivity decreased by increasing ASP concentrations with the minimum level at 2% ASP (1.63 ± 0.15 mg L^{-1}), the details of which are shown in Fig. 2b.

The changes in carotenoids and phycocyanin productivity seemed to have a reverse behavior in different ASP and MSG concentrations in the culture medium. The photosynthetic system of many cyanobacteria is highly adaptable to different environments. Likewise, stress conditions have a significant role in influencing the synthesis of physiological carotenoids and phycobiliproteins (Ojit et al., 2015). Thus, a particular stress condition probably increases the cytoprotective compounds like carotenoids while reducing phycocyanin accumulation (Lv et al., 2019).

3.2.3 | Fatty acids

Fatty acid compositions consist of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA). Table 2 summarizes the variation of the fatty acid compositions in *A. platensis* in control and under metabolic stress treatment. The observed SFA level was 52.29±2.21% of the total fatty acids (TFA) at 12 days of cultivation in the control condition. Moreover, the palmitic acid level, which is a major factor and precursor for the unsaturated C18 fatty acid, was 45.82±2.13% in the control condition. However, the SFA level decreased to a minimum of 35.91±0.93% and 39.92±1.09% in the presence of 1.5% ASP and 1% MSG compared to the control condition at 12 days cultivation in the culture medium, respectively. Consistently, the palmitic acid level significantly decreased in the culture medium supplemented with ASP (0.5 to 2%) and MSG (0.25 to 1.5%) compared to the control culture medium (Table 2). Conversely, the control sample's MUFA level was 10.99±1.28% of TFA at 12 days of cultivation in the culture medium and reached 22.26±0.45% and 20.46±0.91% in the presence of 1.5% ASP and 1.5% MSG, respectively (Table 2). This change led to an increase of 2.94- and 2.18-fold in the oleic acid level, which is the major MUFA in the presence of ASP and MSG, respectively, compared to the control condition at 12 days of cultivation in the culture medium. An increase in the oleic acid level might support the previous evidence that the de novo fatty acid synthesis was modulated in *A. platensis* in response to an increase in ASP and MSG in the culture medium (Kachroo, Singh Jolly, & Ramamurthy, 2006). Likewise, PUFA levels increased to 43.70 ±2.04 and 48.50±2.07% compared to the control sample (36.73±1.58%) at 2% ASP and 0.5% MSG 12 days of cultivation in the culture medium, respectively (Table 2). The content of γ-linolenic acid (GLA, C18:3n6)

was significantly ($p<0.05$) higher than that of other PUFAs in all treatments of ASP and MSG compared to the control. Similarly, GLA's maximum production was observed in the 2% ASP (29.04±2.69%) of TFA, which was 1.47-fold compared to the control sample. Omega-3, as a PUFA, is composed of ∝-linolenic acid (ALA, C18: 3 n-3) and two essential long-chain fatty acids, namely, eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3). Also, the maximum level of 0.61±0.11% and 0.14±0.02% was observed in 2% ASP and 1.5% MSG for ALA, respectively, showing a significant 7.40-fold and 1.70-fold increase compared to the control culture medium. However, the level of 0.57±0.03% and 0.20±0.10% was prominently found for EPA (C20:5 n3) and DHA (C22:6 n3) in 2% ASP in the culture medium, respectively (Table 2). The essential fatty acids of EPA and DHA are known to be beneficial for human health, reduce the risk of cardiovascular and blood cholesterol levels, provide maximal brain function, and increase anticancer and antimicrobial activities (Shakeri, Amoozyan, Fekrat, & Maleki, 2017). Therefore, the EPA and DHA content are nutritionally critical for evaluating the human health benefits (Arts, Ackman, & Holub, 2001).

The results further revealed that the UFA/SFA ratio increased in the presence of different ASP and MSG concentrations and reached 1.79±0.07 and 1.51±0.07 at 1.5% ASP and 1% MSG compared to the control (0.91±0.08), respectively (Table 2), which is higher compared to previous reports (Otleş and Pire 2001; Tokuşoglu and üUnal 2003). The minimum recommended PUFA/SFA ratio for the human diet is 0.45, suggesting that higher PUFA levels increase the food's nutritional value (Oliveira, Duarte, Moraes, Crexi, & Pinto, 2010). In the current study, the PUFA/SFA ratio was at a higher level of 1.17±0.07 and 1.19±0.07 at 1.5% ASP and 0.75% MSG compared to the control sample (0.70±0.06), respectively. Generally, *A. platensis* may respond to ASP and MSG by driving the fatty acid precursor pool (e.g., palmitic acid), possibly through enhanced de novo synthesis. Due to this robustness of *A. platensis* in the presence of metabolic stress, it could accumulate a higher amount of oleic and ∝ and γ- linolenic acids compared to the control culture.

3.2.4 | Amino acids

High-performance liquid chromatography analyses were used to quantify 19 free amino acids including aspartate, glutamate, asparagine, serine, glutamine, histidine, glycine, threonine, arginine, alanine, ?-aminobutyric acid (GABA), tyrosine, valine, methionine, tryptophan, phenylalanine, isoleucine, leucine, and lysine, in *A. platensis* . The analysis of the amino acid spectrum of *Arthrospira* in control showed that the maximum available quantity was glutamic acid, followed by aspartic acid, asparagine, isoleucine, alanine, and arginine. In contrast, the least present amount was related to histidine and the Sulphur-containing amino acids tryptophan and methionine (supplementary Table 1). Similarly, somehow the same profile was previously determined for the amino acid composition of *A. platensis* , representing the highest amounts of glutamate and aspartate amino acids (Bashir, Sharif, Butt, & Shahid, 2016). The amino acid spectrum of *Arthrospira*appears to be a highly good source of the human diet except for cysteine and methionine (Shirnalli, Kaushik, Kumar, Abraham, & Singh, 2018).

The levels of all amino acids increased dramatically from 0.5% ASP to 2% ASP and reached the maximum amount of 3690.33±94.83 μmol g^{-1} DW at 2% ASP compared to the control culture medium (Fig. 3a and Supplementary Table 1). In contrast, the total amino acids' amount gradually decreased from 0.25% MSG and reached the minimum amount of 608.9±15.62 μmol g^{-1} DW at 1.5% MSG compared to the control culture medium (Fig. 3b and Supplementary Table 1). Furthermore, the maximum productivity of essential amino acids was 1394.74±21.11 μmol g^{-1} DW at 0.25% MSG, showing a 12.2-fold increase compared to the control culture medium. However, the maximum productivity of 797.47±57.89 μmol g^{-1} DW was achieved in the presence of 2% supplemented ASP, indicating a 6.98-fold increase compared to the control culture medium (Supplementary Table1). Glutamate, which plays an indispensable role in nitrogen metabolism and cell growth regulation in microalgae, is a precursor of ALA composition. Further, ALA is considered as the first main precursor in heme biosynthesis pathways in living organisms, and heme is an essential intermediate in the biosynthesis of phycobilins (Similar to the phycocyanobilin for phycocyanin biosynthesis).

Furthermore, the phycocyanobilin is biosynthesized by phycocyanobilin synthase that converts biliverdin to the phycocyanobilin, which can directly biosynthesize Phycocyanin (Fujisawa et al., 2010). In the current study, the maximum level of 474.10±41.53 and 494.17±28.96 μmol g^{-1} DW of glutamic acid was observed in

2% ASP and 0.25% MSG, respectively, demonstrating a significant 6.65-fold and 6.93-fold increase compared to the control culture medium (Supplementary Table 1 and Fig. 3). The two nitrogenous amino acids of glutamine and arginine are derived from glutamate synthesized from ammonia via glutamine synthase, playing a pivotal role in nitrogen metabolism. An increase in glutamine was accompanied by an increase in the total soluble protein content and other free amino acids. Moreover, the maximum level of 24.86±1.99 and 25.86±2.47 μmol g^{-1} DW was observed in 2% ASP and 0.25% MSG for glutamine, respectively, showing a significant 6.83-fold and 7.10-fold increase in comparison to the control culture medium (Supplementary Table 1 and Fig. 3). Asparagine could serve as essential nitrogen and carbon reservoirs. In the present study, the maximum level of 210.51±15.26 and 85.73±3.05 μmol g^{-1} DW was found in 2% ASP and 0.25% MSG for asparagine, respectively, which was 6.52-fold and a 2.65-fold increase compared to the control culture medium (Supplementary Table 1 and Fig. 3). According to Depraetere et al., (2015), the nitrogen associated with amino acids is recycled to provide a source of intracellular nitrogen regarding production for better development.

Based on the current knowledge besides MSG, ASP's availability as metabolic stress stimulated factors such as nitrogenous amino acids that may affect the cell growth and biochemical composition in microalgae cells. The storing of these nitrogenous compounds as soluble nitrogen or organic molecules may regulate growth and development by nitrogen uptake.

3.3 | Network correlation analysis of metabolites and bioactive compounds

3.3.1 | Metabolite-metabolite correlation

A network-based correlation analysis was conducted to identify metabolite correlations and interactions between two medium supplemented by ASP and MSG. This may reveal potential metabolic indicators and predict metabolic strategies. The correlation results indicated 222 positive and 21 negative correlations between metabolite contents in*Arthrospira* supplemented by ASP, and the correlations between amino acids were positive. Furthermore, amino acids were positively associated with UFA, MUFA, and PUFA, while the correlations between SFA and amino acids were negative (Fig. 4A). In contrast, fewer correlations were found between the metabolites of *Arthrospira* supplemented by MSG in the culture medium comprising six negative and 187 positive connections. Similar to ASP, the correlation between amino acids was positive. Also, amino acids were positively correlated with PUFA, while the correlations between SFA and MUFA with some amino acids were negative (Fig. 4B). Although metabolites with a similar chemical structure demonstrate a positive correlation (Ghaffari et al., 2016) globally, a negative correlation between amino and fatty acids may reveal an active trade-off to proteins or fatty acid biosynthesis.

3.3.2 | Metabolite-phycocyanin and carotenoids correlation

A network analysis resulted in a combined identification of the metabolite indicative of phycocyanin and carotenoids productivity. The outcomes of Pearson's correlation analysis are graphically illustrated in Fig. 5 and Supplementary Fig. 1. Twenty-four metabolites were significantly ($p<0.05$) correlated with phycocyanin accumulations in ASP-supplemented *Arthrospira* (Fig. 5a), including 23 positive (shown as blue edged) cases and one negative case (shown as red-edged). Contrarily, phycocyanin accumulation showed 23 correlations, of which 21 cases were positive (shown as blue edged) while two cases were negative in *Arthrospira* supplemented with MSG (Fig. 5b). In supplemented culture mediums, amino acid content was positively correlated with Phycocyanin, indicating a significant role of amino acids in their biosynthesis. Some studies reported the association between biomass and nitrogen and carbohydrate content in response to a varying nitrogen source in *A. platensis* (Depraetere et al., 2015; Kushwaha, Upadhyay, & Mishra, 2018). Fatty acid-related compounds demonstrated a diverse range of positive and negative correlations with Phycocyanin in culture media supplemented with ASP and MSG, indicating further increases of Phycocyanin at the expense of carbohydrate in the lower central metabolism.

Moreover, phycocyanin contents were positively correlated with biomass and Chlorophyll in culture media supplemented with ASP and MSG ($R>0.70$). The correlation between phycocyanin contents and carotenoids was negative in a culture medium supplemented with MSG. Additionally, the correlation analysis represented

that carotenoid accumulation was only correlated with five metabolites ($p<0.05$) in *Arthrospira* supplemented with MSG, including two positive and three negative cases (Supplementary Fig. 1). The carotenoid content and fatty acids were positively correlated with MUFA (R+0.85) while negatively associated with SFA (-0.69) and UFA (+0.69). There was no significant correlation between carotenoid accumulations in the supplemented *Arthrospira* and ASP, indicating a significant change in regulation in differentiating the two types of mediums for Carotenoid productivity. A recent study has reported that several genes are potentially associated with Phycocyanin biosynthesis in *Arthrospira* . There are still several genes that have an unknown function (Furmaniak et al., 2017). Therefore, the integrative analysis of metabolomic data with other 'omics' approaches will open up a way to further elucidate gene functions in *Arthrospira.* These efforts could reveal the vital roles of different metabolites related to important growth parameters in different culture medium conditions. Moreover, network analysis may help assess the genes contributing to metabolic variation and the contribution of metabolic variation to future studies of complex traits in *Arthrospira* .

3.4 | Metabolic stress and the cyanobacterial incomplete TCA cycle and its shunts

The TCA cycle is unusual and incomplete in cyanobacteria due to missing the 2-oxoglutarate dehydrogenase (OGDH) enzyme, and several shunts are identifiable. In Fig. 6, the TCA cycle and the known cyanobacterial TCA cycle shunts are depicted according to Steinhauser, Fernie, & Arau´jo, (2012). The GABA shunt is a variant of the TCA cycle that utilizes glutamate decarboxylase to convert glutamate to GABA. Then, GABA aminotransferase (GABA-T) converts GABA to alanine, and alanine aminotransferase (AlaAT) converts alanine to pyruvate and vice versa (Steinhauser, Fernie, & Arau´jo, 2012). Based on Fig. 3, increasing the ASP supplement significantly elevated GABA and alanine amino acid levels while MSG decreased GABA and alanine amino acid levels. The maximum alanine content was achieved at 0.25% MSG (546±17.2 μmol. g^{-1} DW) and 2% ASP (434±26. 5 μmol. g^{-1} DW), showing 21.84-fold and 17.36-fold compared to the control (25±1.6 μmol. g^{-1} DW), respectively. In addition, GABA levels were higher in all treatments except for 1.5% MSG (Fig 3 and Supplementary Table 1) compared to the control. It has been recently proved that an increase in the cellular GABA content promotes lipid biosynthesis under cadmium toxicity. In general, these findings revealed that GABA is one of the main components that provides carbon for the TCA cycle and increases lipid production in microalgae under abiotic stress conditions (Zhao, Song, Zhong, Yu, & Yu, 2020). According to recent reports, the TCA cycle intermediates such as succinate, malate, 2-oxoglutarate, and citrate levels and the levels of pyruvate and poly-3-hydroxybutyrate biosynthesis significantly decreased when the GABA shunt pathway was inhibited in cyanobacteria (Monshupanee, Chairattanawat, & Incharoensakdi, 2019). Hence, it seems that the level of pyruvate can be affected by the GABA shunt pathway. Further, an increase in GABA and alanine levels in the GABA shunt pathway probably can affect fatty acids and other metabolites induced by MSG and ASP supplements. Moreover, alanine and pyruvic acids can be interconverted via the GABA shunt pathway (Lv et al., 2016).

The aspartate transaminase reactions are the other critical variant pathways in cyanobacteria (the AspAT shunt), which are believed to permit the recycling of metabolites (Steinhauser, Fernie, & Arau´jo, 2012). Aspartate aminotransferase is considered a critical enzyme for biomass production, which catalyzes the reversible reaction of 2-oxoglutarate, glutamate, aspartate, and oxaloacetic acid produced by the TCA cycle, linking nitrogen assimilation with carbon metabolism (Ghaffari et al., 2016). The present study proposed that glutamate is converted to oxaloacetate and then to aspartate (by the involved AspAT enzyme), which is the precursor to many other amino acid substances (Fig. 6). It also seems via the citrate-malate shunt that oxaloacetate is converted to pyruvate by oxaloacetate decarboxylase, which is a precursor to acetyl coenzyme A (Acetyl-COA). Likewise, Acetyl-COA is a precursor for producing fatty acids and carotenoids (Fig. 6). It has been evidenced that the classic TCA cycle produces energy agents (i.e., NADH & FADH) and biosynthetic precursors (for producing amino acids, lipids, and "heme" synthesis and linking to nitrogen metabolism) whereas the variants with their oxidative and reductive branches generate more biosynthetic precursors (Araújo, Martins, Fernie, & Tohge, 2014; Steinhauser, Fernie, & Arau´jo, 2012). The obtained results represent that by exerting ASP and MSG supplements as metabolic stress, more precursors probably via GABA, aspartate transaminase reactions, and citrate-malate shunts are produced due to the flexibility of the TCA cycle in *Arthrospira* (Fig. 6). The presented strategy seems to provide an eco-friendly approach

for reducing the production cost of biomass and valuable metabolites in *Arthospira* .

4 | Conclusion

In conclusion, it was found *A. platensis* responded diffentially to varaying range of metabolic stress for high value compound productivity. Sodium glutamate and aspartate were found to be the best substrate for Phycocyanin and Cartoneid hyperaccumulation. However, to a greater extent, ASP was found to be the best substrate for stimulating unsaturated fatty acid accumulation and essential amino acid biosynthesis. Network analysis revealed the culture mediums supplemented with two metabolic stressors has distinct metabolite abundance and the corresponding associated metabolic networks. These findings provide important data for future metabolic studies to improve high-value compounds accumulation using metabolic engineering in *Arthrospira platensis.*

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Table 1 The biomass production and growth characteristics of*Arthrospiral platensis* cultivated under different levels of ASP (aspartic acid) and MSG (monosodium glutamate)

Samples	BDW ($g.l^{-1}$)	P ($g.l^{-1}day^{-1}$)	SGR (day^{-1})	td (day)	Chlorophyll ($mg.l^{-1}$)
Control	3.67 ± 0.26^{d}	0.299 ± 0.021^{d}	0.319 ± 0.011^{c}	2.174 ± 0.073^{b}	0.86 ± 0.12^{f}
ASP (%)					
0.5	3.71 ± 0.16^{d}	0.302 ± 0.013^{d}	0.320 ± 0.012^{c}	2.168 ± 0.081^{b}	1.20 ± 0.07^{e}
1	3.96 ± 0.24^{d}	0.324 ± 0.020^{d}	0.326 ± 0.008^{bc}	2.130 ± 0.049^{bc}	1.33 ± 0.06^{de}
1.5	4.75 ± 0.20^{c}	0.389 ± 0.018^{c}	0.341 ± 0.013^{abc}	2.036 ± 0.078^{bcd}	1.66 ± 0.08^{bc}
2	5.48 ± 0.28^{b}	0.450 ± 0.024^{b}	0.353 ± 0.014^{a}	1.967 ± 0.076^{cd}	1.87 ± 0.08^{a}
MSG(%)					
0.25	6.03 ± 0.25^{a}	0.498 ± 0.021^{a}	0.361 ± 0.010^{a}	1.921 ± 0.051^{d}	1.64 ± 0.13^{bc}
0.5	5.23 ± 0.20^{bc}	0.429 ± 0.017^{bc}	0.349 ± 0.013^{ab}	1.990 ± 0.075^{bcd}	1.72 ± 0.10^{ab}
0.75	5.00 ± 0.38^{bc}	0.410 ± 0.031^{bc}	0.345 ± 0.012^{ab}	2.012 ± 0.067^{bcd}	1.49 ± 0.09^{cd}
1	5.04 ± 0.49^{bc}	0.413 ± 0.041^{bc}	0.345 ± 0.011^{ab}	2.008 ± 0.063^{bcd}	1.41 ± 0.11^{d}
1.5	2.13 ± 0.33^{e}	0.171 ± 0.028^{e}	0.273 ± 0.024^{d}	2.550 ± 0.224^{a}	0.69 ± 0.10^{g}

Data shown are the averages of three runs ± SD (standard deviation).

Statistical significance in each column is indicated by different letters; $p < 0.05$

Table 2 Variation of the fatty acid compositions in *Arthrospira platensis* in control and under metabolic stress treatment: MSG (monosodium glutamate) and ASP (aspartic acid)

Fatty Acids	Control	MSG (%)	MSG (%)	MSG (%)	MSG (%)	MS
		0.25	**0.5**	**0.75**	**1**	**1.5**
Lauric acid (C12:0)	0.69 ± 0.01^{a}	0.61 ± 0.04^{ab}	0.60 ± 0.04^{ab}	0.49 ± 0.00^{c}	0.50 ± 0.13^{bc}	0.3
Myristic acid (C14:0)	0.08 ± 0.00^{bc}	0.12 ± 0.01^{bc}	0.10 ± 0.00^{bc}	0.15 ± 0.00^{b}	0.09 ± 0.00^{bc}	0.1
Palmitic acid (C16:0)	45.82 ± 2.13^{a}	40.85 ± 1.25^{b}	37.90 ± 1.52^{c}	35.87 ± 1.32^{c}	36.23 ± 1.18^{c}	36.
Palmitoleic acid (C16:1)	5.04 ± 0.68^{cd}	5.19 ± 0.60^{cd}	5.72 ± 0.10^{bc}	6.59 ± 1.03^{ab}	6.74 ± 1.00^{ab}	7.4
Stearic acid (C18:0)	5.69 ± 0.09^{a}	0.00 ± 0.00^{d}	2.91 ± 0.07^{c}	3.97 ± 0.78^{bc}	2.65 ± 0.27^{c}	3.3
Oleic acid (C18:1n9c)	5.95 ± 0.61^{ef}	6.69 ± 1.19^{e}	5.11 ± 0.82^{ef}	4.77 ± 0.87^{e}	8.86 ± 1.02^{d}	12.
Linolelaidic acid (C18:2n6c)	14.77 ± 1.61^{b}	17.42 ± 0.90^{a}	19.57 ± 0.60^{a}	19.64 ± 1.02^{a}	18.38 ± 2.93^{a}	14.
Arachidic acid (C20:0)	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.15 ± 0.01^{c}	0.00 ± 0.00^{d}	0.45 ± 0.04^{a}	0.4
Y-Linolenic acid (C18:3n6)	19.74 ± 1.01^{e}	28.06 ± 1.23^{ab}	27.12 ± 1.50^{abc}	27.57 ± 1.04^{abc}	25.03 ± 1.73^{cd}	23.
Linolenic acid (C18:3n3)	0.08 ± 0.01^{bcd}	0.03 ± 0.05^{d}	0.08 ± 0.00^{bcd}	0.08 ± 0.00^{bcd}	0.07 ± 0.01^{bcd}	0.1
Eicosatrienoic acid (C20:3n6)	0.52 ± 0.01^{a}	0.51 ± 0.05^{ab}	0.32 ± 0.02^{ab}	0.34 ± 0.01^{ab}	0.37 ± 0.10^{ab}	0.3

Fatty Acids	Control	MSG (%)	MSG (%)	MSG (%)	MSG (%)	MS
Eicosatrienoic acid (C20:3n6)	1.61±0.14a	0.52±0.01^{cd}	0.42±0.01^{cd}	0.52±0.00^{cd}	0.62±0.06^{c}	1.1
Eicosapentanoic acid (C20:5n3)	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.0
Docosahexanoic acid (C22:6n3)	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.0
saturated fatty acids (SFA)	52.29±2.21^{a}	41.58±1.26^{c}	41.67±1.47^{c}	40.48±1.46^{cd}	39.92±1.09^{cd}	40.
monounsaturated fatty acids (MUFA)	10.99±1.28^{d}	11.88±0.70^{d}	10.83±0.83^{d}	11.35±1.68^{d}	15.59±0.90^{bc}	20.
polyunsaturated fatty acids (PUFA)	36.73±1.58^{g}	46.54±1.39^{ab}	48.50±2.07^{a}	48.16±1.75^{a}	44.49±1.18^{bc}	38.
unsaturated fatty acids (UFA)	47.71±2.21^{e}	58.42±1.25^{c}	58.33±1.47^{c}	59.51±1.46^{bc}	60.08±1.09^{bc}	59.
UFA/SFA	0.91±0.08^{e}	1.41±0.07^{c}	1.40±0.08^{c}	1.47±0.09^{bc}	1.51±0.07^{bc}	1.4
PUFA/SFA	0.70±0.06^{c}	1.12±0.06^{a}	1.14±0.09^{a}	1.19±0.07^{a}	1.12±0.06^{a}	0.9

Data shown are the averages of three runs ± SD (standard deviation).

Statistical significance in each column is indicated by different letters; $p < 0.05$

Figure legends

Fig. 1. Biomass concentrations of *A. platensis* NIES-39 cultivated under different concentrations of (a) ASP and (b) MSG after 12 cultivation days.

Note . ASP: Aspartic acid; MSG: Monosodium glutamate; Medium: Zarrouk, pH: 9; Temperature: 28±2 ˚C; Continuous illumination of 300 μmol photons $m^{-2}s^{-1}$ white LED. The points are shown as the mean ± standard deviation (n=3).

Fig. 2. The contents of (a) phycocyanin and (b) carotenoids of*A. platensis* NIES-39 cultivated under different concentrations of ASP and MSG at 12 days of cultivation.

Note . ASP: Aspartic acid; MSG: Monosodium glutamate; Medium: Zarrouk; pH: 9; Temperature: 28±2 ˚C; Continuous illumination of 300 μmol photons $m^{-2}s^{-1}$ white LED. The points are represented as the mean ± standard deviation (n=3).

Fig. 3. Integrated pathway map of the quantitative regulation of amino acids, fatty acids, Phycocyanin, carotenoids, and Chlorophyll in the medium supplemented with different ASP and MSG concentrations visualized by VANTED.

Note . ASP: Aspartic acid; MSG: Monosodium glutamate; VANRED: Visualization and analysis of networks containing experimental data; Glc: Glucose; G-6-P: Glucose 6-phosphate; R-5-P: Ribose 5-phosphate; G-3-P: Glyceraldehyde 3-phosphate; PEP: Phosphoenolpyruvate; Crm: Chorismate; Prpn: Prephenate; Arn: Anthranilate; Pyr: Pyruvate; ATCoA: Acetyl Coenzyme A; SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids; UFA: Unsaturated fatty acids; OAA: Oxaloacetate; α-Keto: α-ketoglutarate; Succ: Succinate; ALA: δ-aminolevulinic acid. Data are represented as the mean ± standard deviation (n=3).

Fig. 4. Correlation-based networks of amino acids (a) and fatty acids (b) indicating the metabolite-metabolite correlation in the medium supplemented with different concentrations of ASP and MSG, respectively.

Note . ASP: Aspartic acid; MSG: Monosodium glutamate. Pearson's correlation was used to estimate correlation coefficients, followed by applying threshold tests for correlation coefficients (R) and p-values to detect significant correlations. In addition, thresholds were set for p[?]0.05. Further, positive and negative correlations are illustrated as blue and red edges, respectively.

Fig. 5. Correlations between phycocyanin content and metabolites in the medium supplemented with different concentrations of (a) ASP and (b) MSG.

Note . ASP: Aspartic acid; MSG: Monosodium glutamate. The positive and negative correlations are shown in blue and red colors, respectively, with Phycocyanin (yellow color). In addition, deep colors indicate the strongest correlations. Glc: Glucose; G-6-P: Glucose 6-phosphate; R-5-P: Ribose 5-phosphate; G-3-P: Glyceraldehyde 3-phosphate; PEP: Phosphoenolpyruvate; Crm: Chorismate; Prpn: Prephenate; Arn: Anthranilate; Pyr: Pyruvate; ATCoA: Acetyl coenzyme A; SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids; UFA: Unsaturated fatty acids; OAA: Oxaloacetate; α-Keto: α-ketoglutarate; Succ: Succinate; ALA: δ-aminolevulinic acid. Data are shown as the mean ± standard deviation (n=3).

Fig. 6. Schematic summary of the TCA cycle and its variants in cyanobacteria and its relation to other cellular metabolite production pathways.

Note. TCA: Tricarboxylic acid; (1) The closing reaction (without the glyoxylate shunt) of the incomplete cyanobacterial TCA cycle (AspAT shunt) – pink broken arrows. (2) the GABA shunt – broken green arrows; (3) The citrate-malate shunt – orange broken arrows; (4) Cyanobacterial complete TCA cycle shunt – broken violet arrows; TCA cycle intermediates are colored blue. In addition, the cross colored in red depicts the lack of OGDH in cyanobacteria. OGDH: 2-oxoglutarate dehydrogenase; AspAT: Aspartate aminotransferase; AlaAT: Alanine aminotransferase; GABA-T: GABA aminotransferase; GAD: Glutamate decarboxylase; CL: Citrate lyase; OADC: Oxaloacetate decarboxylase; OGDC: 2-oxoglutarate decarboxylase; SSADH: Succinic semialdehyde dehydrogenase; ALA: δ-aminolevulinic acid.

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