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Untargeted metabolomics of the alkaliphilic cyanobacterium *Plectonema terebrans* elucidated novel stress-responsive metabolic modulations

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ABSTRACT

Alkaliphilic cyanobacteria are suitable candidates to study the effect of alkaline wastewater cultivation on molecular metabolic responses. In the present study, the impact of wastewater, alkalinity, and alkaline wastewater cultivation was studied on the biomass production, biochemical composition, and the alkalinity responsive molecular mechanism through metabolomics. The results suggested a 1.29 to 1.44-fold higher biomass production along with improved lipid, carbohydrate, and pigment production under alkaline wastewater cultivation. The metabolomics analysis showed 1.2-fold and 5.54-fold increase in the indole-acetic acid and phytoene biosynthesis which contributed to overall enhanced cell differentiation and photo-protectiveness. Furthermore, lower levels of Ribulose-1,5-bisphosphate (RuBP), and higher levels of 2-phosphoglycerate and 3-phosphoglycerate suggested the efficient fixation of CO_2 into biomass, and storage compounds including polysaccharides, lipids, and sterols. Interestingly, except *L*-histidine and *L*-phenylalanine, all the metabolites related to protein biosynthesis were downregulated in response to wastewater and alkaline wastewater cultivation. The cells protected themselves from alkalinity and nutrient stress by improving the biosynthesis of sterols, non-toxic antioxidants, and osmo-protectants. Alkaline wastewater cultivation regulated the activation of carbon concentration mechanism (CCM), glycolysis, fatty-acid biosynthesis, and shikimate pathway. The data revealed the importance of alkaline wastewater cultivation for improved CO_2 fixation, wastewater treatment, and producing valuable bioproducts including phytoene, Lyso PC 18:0, and sterols. These metabolic pathways could be future targets of metabolic engineering for improving biomass and metabolite production.

Significance: Alkalinity is an imperative factor, responsible for the contamination control and biochemical regulation in cyanobactera, especially during the wastewater cultivation. Currently, understanding of alkaline wastewater responsive molecular mechanism is lacking and most of the studies are focused on transcriptomics of model organisms for this purpose. In this study, untargeted metabolomics was employed to analyze the impact of wastewater and alkaline wastewater on the growth, CO_2 assimilation, nutrient uptake, and associated metabolic modulations of the alkaliphilic cyanobacterium *Plectonema terebrans* BERC10. Results unveiled that alkaline wastewater cultivation regulated the activation of carbon concentration mechanism (CCM), glycolysis, fatty-acid biosynthesis, and shikimate pathway. It indicated the feasibility of alkaline wastewater as promising low-cost media for cyanobacterium cultivation. The identified stress-responsive pathways could be future genetic targets for strain improvement.

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1. Introduction

Rapid progression in the urban and industrial activities related to paper and pulp, paints and dyes, food and beverages, textiles, and chloro-chemicals production have resulted in polluting the water bodies with elevated alkalinity [1]. Furthermore, the escalating environmental CO_2 levels due to anthropogenic activities have changed the water dynamics by altering dissolved inorganic carbon concentrations. Resultantly, changing levels of CO_3^{2-} and HCO_3^- in aquatic bodies that promote alkalinity and cyanobacterial blooms [2]. Though, these polluted alkaline water bodies (from herein will be referred to as alkaline wastewater) cause osmotic, oxidative, ionic stress, and have proven to be damaging for the most of biological resources. However, these wastewater bodies can be explored as valuable resources for microalgae and cyanobacteria cultivation which can help to remove the pollutants along with mitigating atmospheric carbon dioxide [1].

Alkaliphilic cyanobacteria are potential candidates to mitigate the wastewater and alkalinity related problems due to their substantial ability to uptake nutrients, scavenge CO_2, outcompete the microbial contaminations, faster cell proliferation, and improved lipid biosynthesis in response to alkalinity and wastewater cultivation [3,4]. Furthermore, cyanobacteria are valuable biofuel and biochemical production "chassis" due to their readiness for gene manipulation, indicating their feasibility as "Multiproduct CyanoFactory" [5]. However, metabolite biosynthesis is highly dependent on the abiotic factors which pose great challenge during large-scale cultivation of microalgae and cyanobacteria. Hence, it is important to understand the cellular metabolic changes in response to various abiotic factors including wastewater-derived alkalinity.

Elucidation of cyanobacterial cellular response against abiotic environmental factors such as nutrient concentration, nitrogen starvation, acidification, temperature modulations, oxidative stress, and osmotic stress have been areas of interest for many years because these data are necessary for the strain improvement and process upscaling [6]. In this regard, metabolomics offers promising potential to identify and quantify the low molecular weight metabolites in response to environmental conditions. It is an attractive approach to study the interactions between various biological constituents to design and analyze the integrated multi-omics studies [7]. Specifically, environmental metabolomics analyses have been employed to identify the stress-responsive metabolites from natural or laboratory-simulated conditions [8]. Metabolomic analysis of the wastewater-cultivated *Chlorella sorokiniana* and *Pseudomonas* showed upregulation of fatty acid, carbohydrates, and amino acids related metabolites. Furthermore, improved metabolite regulation and nutrient uptake were shown to be associated with the synergistic activity of these organisms [9]. Similarly, metabolomic analysis of *Chlorella* was performed in response to nitrogen limitation and lipid biosynthesis. It was found that *Chlorella* tolerates the nitrogen limitation by reducing the protein content and redirecting nitrogen (as amino acids) towards the TCA cycle, resulting in an improved lipid biosynthesis. Furthermore, photosynthetic activity enhanced the concentration of carbon-containing metabolites which were redistributed towards carbohydrate and lipid biosynthesis pathways [10]. Metabolomics of *Scenedesmus* in response to tris-phosphate (organophosphate flame retardant) showed an altered lipid metabolism which favored thylakoid-membrane strengthening instead of cell-membrane integrity that results in improved photosynthetic activity. Moreover, changes in polar lipids exhibited osmotic stress tolerance by improving the NO signaling [11]. Previously, transcriptomics analysis has also been employed to elucidate the global transcriptional response of the cyanobacterium *Synechocystis* sp. strain PCC 6803 to an alkaline pH using transcriptomics analysis [12]. Therefore, it is necessary to comprehend the impact of the environmental factors on the underlying molecular mechanisms [13] to fine-tune the genetically modified strains for strain improvement and their subsequent environmental and industrial applications.

However, at present, understanding the molecular mechanisms of cyanobacteria in response to wastewater and alkaline wastewater cultivation are needed to be explored [1] to harness their full potential. The current research was an effort for unveiling the impact of wastewater and alkaline wastewater on the cyanobacterial molecular mechanism through untargeted metabolomics. The aim was to understand the impact of wastewater and alkaline wastewater on the growth, CO_2 assimilation, nutrient uptake, and associated metabolic modulations of the alkaliphilic cyanobacterium *P. terebrans* BERC10. The data showed novel insights into the metabolic response of *P. terebrans* BERC10 under wastewater and alkaline wastewater which could be employed to select the pathways of interest as targets of metabolic engineering in the future.

2. Materials and methods

2.1. Algal culture and exposure to alkalinity

A newly isolated alkaliphilic cyanobacterium *P. terebrans* BERC10 [3] having a substantial growth potential in alkaline wastewater (pH > 10.0) was selected to elucidate the impact of respective cultivation conditions on the molecular modulations. Wastewater, being the ample nutritional source, was selected as the potential cultivation medium. However, to ensure the consistency during cultivation, the selected strain was cultivated in synthetic wastewater (MBG) as described previously [3]. MBG was devised by manipulating nitrate, phosphate, and carbonate concentrations of the standard BG11 media (hereafter called control for this study). The details of the compositional and physicochemical analysis are mentioned in the supplementary Tables S1 and S2. To study the impact of wastewater, the strain was cultivated in wastewater having pH 7.5–8.0 (hereafter called wastewater, CWW), while strain was also cultivated in wastewater having pH 10.0 (hereafter called alkaline wastewater, AWW) to study the combined impact of alkalinity and wastewater. Initial pH was maintained by 0.1 M NaOH. All experiments were performed using 15-day batch cultivation at 28 ± 2 °C in triplicate in a locally designed 10 L glass photobioreactor (90 cm long, 45 cm wide, and 15 cm deep) having 6 L of the cultivation media thus maintaining 40:60 air to media ratios. 12 h alternating light and dark periods were maintained by automatic light switching where 150 μmol m^{-2} s^{-1} of light intensity was provided by cool white fluorescent light (Philips, Japan) during the light period. Natural air with 0.04% CO_2 was provided in photobioreactors as influent gas to 0.05 gL^{-1} of initial biomass. The samples were harvested at their logarithmic phase at the end of the batch.

2.2. Alkalinity-responded growth and biochemical composition analysis

Wastewater and alkaline wastewater-cultivated cells were harvested, freeze-dried (Alpha 2–4 LSCbasic, Christ, Germany) and stored at −80 °C for further use. The gravimetric method [14] was applied on lyophilized samples to determine the impact of each condition on biomass production.

Ash content was determined as described by the standard protocol of ASTM E1755-01(2007). Briefly, the known amount of biomass was oven dried at 450 °C in muffle furnace using crucibles until constant weight was obtained. The difference in pre-weight and post-weight was used to determine the ash content.

Biochemical analyses were performed to measure proteins, lipids, carbohydrates, and pigments contents. For carbohydrate analysis, 20 mg of sample was extracted with 72% H_2SO_4 followed by treatment with furfural derivatives of the phenolic compounds as described by the modified phenol/sulfuric acid method [15]. Proteins were extracted by subjecting 10 mg sample to hot-alkaline (0.5 NaOH) treatment at 80–95 °C followed by the micro-biuret method [16] where the extract was treated with $CuSO_4$ solution (0.21% $CuSO_4$ in 30% NaOH). The absorbance of carbohydrate and protein samples were estimated at

λ490nm and λ310nm, respectively, by UV-VIS Spectrophotometer (Model:AE-S60-2 U, A&E Lab, UK). To measure the content, the absorbance was converted into metabolite concentration by employing the equations derived from the respective standard curves.

The lipid constituent of the sample was estimated by the modified Bligh and Dyer method [17]. Briefly, 50 mg sample was treated overnight performed with 1:2 methanol:chloroform for lipid extraction. The supernatant of the treated sample was then processed with 1% NaOH to remove non-lipid constituents. The organic phase containing lipid was then evaporated at 50 °C (until constant weight) to obtain pure lipids which were then measured gravimetrically.

The pigments including chlorophylls, carotenoids, and phycobilin were measured using absorbance-based methods as described previously (Shahid et al., 2021). Briefly, total chlorophylls were estimated to determine the photosynthetic efficiency while other pigments including carotenoids and phycobilins were estimated to evaluate the biotechnological applications. For chlorophyll estimation, 20 mg of freeze-dried (−80 °C) biomass was treated with 5 mL of methanol at 4 °C for 12–24 h to release the chlorophyll and carotenoids. The absorbance of the supernatant was measured at λ664nm, λ649nm, λ470nm, and the amount of chlorophyll-a, chlorophyll-b, and carotenoids were calculated by following formulae as described previously [18].

$$\text{Chl} - \text{a} = 13.36\ A_{664} - 5.19\ A_{649} \quad (1)$$

$$\text{Chl} - \text{b} = 27.43\ A_{649} - 8.12\ A_{664} \quad (2)$$

$$\text{C x} + \text{c} = (1000\ A_{470} - 2.13\ \text{Chl} - \text{a} - 97.63\text{Chl} - \text{b})/209 \quad (3)$$

where, "A" is absorbance, "Chl-a" is Chlorophyll a, "Ch-b" Chlorophyll b, and "Cx + c" is Carotenoids.

Additionally, 20 mg of freeze-dried biomass was extracted with 0.1 M phosphate buffer (3 mL) to extract phycobilins using Teflon-coated tubes, the debris was removed by centrifugation at 13,000 rpm for 10 min. The absorbance of the supernatant was measured at λ562nm, λ620nm and λ652 nm to estimate the amount of phycocyanin (PC), allophycocyanin (APC), and phycoerythrin (PE), respectively by using the following formulae [19].

$$\text{PC} = (A_{620} - (0.047 \times A_{652}))/5.34 \quad (4)$$

$$\text{APC} = (A_{652} - (0.208 \times A_{620}))/5.09 \quad (5)$$

$$\text{PE} = (A_{562} - (2.41 \times \text{PC}) - (0.849 \times \text{APC}))/9.62 \quad (6)$$

2.3. Metabolome extraction

Metabolites were extracted using methanol as described previously [20] with some modifications. Briefly, lyophilized sample (50 mg) was mixed in 1:10 ratio with 80% methanol and subjected to five repeated freeze-thaw cycles. 30 min of snap-freezing in liquid nitrogen was followed by 1 min of vortex-based thawing at room temperature. 1.0 mg mL^{-1} (*w/v*) of N-Fmoc-L-serine (OtBu) was used as internal standard. The supernatant containing metabolites was collected at 4 °C, filtered through a 0.22 μm microporous filter, and stored at −80 °C. The 1:1 diluted sample was prepared with pure methanol before running the metabolomic analysis.

2.4. Untargeted metabolomics analysis

Untargeted metabolomics was performed on DionexUltimate 3000 Liquid-Chromatogram (Dionex, Camberley, Surrey) attached with maXis II HR-QTOF Bruker (Bremen, Germany) Mass Spectrometer (LC-MS) as described previously [21] in the reverse and positive modes at 4 °C. For each run, the metabolite aliquot (5 μL) was injected at 40 °C in the NUCLEODUR C18 Gravity column attached with a guard column (Macherey-Nagel, Germany). Deionized water containing 0.1% formic acid was used as phase A, while methanol containing 0.1% formic acid was used as phase B for chromatographic separation. The mobile-phase B was maintained to 5% in the column for 3 min followed by sample elution at 5–95% linear gradient of mobile-phase B (5–95% for 3–17 min, 95% for 17–18 min, 5% for 19 min, and 5% for 19–20 min) with 0.3 mL min^{-1} of flow rate. Calibration was performed with 0.1 M sodium formate (HCO_2Na) at a flow rate of 0.17 mL hr^{-1} through the automatic switch valves. Mass spectrometer parameters were set accordingly, the MS was set as positive in DIA MS_{all} mode [22], the dry gas temperature was set at 270 °C, the flow rate was set at 12 $Lmin^{-1}$, the capillary voltage was 4500 V, collision energy was stepping from 20 eV to 55 eV, the mass range was 100–1200 *m/z*, and nebulizer pressure was set as 3.1 bar. The obtained data were recorded through the Compass HyStar 4.1 (Bremen, Germany) acquisition software. A quality control test was performed at the start and end of the batch to condition the column, assess the instrument stability, and ensure the data quality. Sample carryover contamination was assessed through random injection of blanks. The column was checked at the start and end of each batch to ensure column stability.

2.5. Data processing and statistical analyses

The LC-MS data sets (available at MetaboLights database; MTBLS2847) were calibrated and processed for features extraction using Bruker's Compass Data Analysis (version 4.4) and Bruker's MetaboScape (version 3.0) software, respectively. Feature extraction was performed on the following parameters; 0.7 EIC correlation 3000 counts intensity threshold, 100–1500 *m/z* mass range, and 0.3–20 min retention time. Extracted features were identified by comparing experimental MS/MS spectra with available mass spectral databases such as Mass bank of Europe, Mass bank of North America (MoNA) and NIST tandem mass spectral library (identification level 2 of metabolomic). Data normalization (*Z*-transform), scaling (Pareto), and log transformation were performed and exported to SIMCA (version 14.1, Umetrics, Umeå, Sweden) for the distinct groups' distribution and outlier detection using orthogonal partial least squares discriminant analysis (OPLS-DA). A combination of online available tools and software including XCMS [23] and MetaboAnalyst [24] were used for the statistical, functional, and integrative metabolomics analyses. Statistical significance was calculated using ANOVA for more than two groups, having 2 replicates per condition. Features with $p\text{-value} < 0.05$, after Benjamini-Hochberg corrections, were considered as significant. Fold change in intensity of features was also calculated with the threshold of ≥2.

3. Results and discussion

3.1. Impact of wastewater-derived alkalinity on biomass production

The stress tolerance potential of cyanobacteria is an interesting feature that can be exploited to maintain axenic cultures in open-pond cultivation without compromising biomass production. The cyanobacterium *P. terebrans* BERC10 was not only able to withstand the high nutrient (wastewater) and alkalinity (pH > 10.0) conditions but also produced 1.29-fold and 1.44-fold higher biomass in response to wastewater and alkaline wastewater cultivation, respectively, when compared to the BG11 control (Fig. 1). This response is in accordance with the fact that cyanobacteria tend to proliferate more efficiently in alkaline environments as compared to neutral and acidic conditions as observed for the *Synechocystis* which exhibited similar photoautotrophic behavior at pH 10.0 and pH 7.0. However, in response to high alkalinity (pH 11.0), the biomass production was reduced from 0.35 gL^{-1} to 0.24 gL^{-1} due to decreased light conversion efficiency [25]. Similarly, *Spirulina* produced 3.5 gL^{-1} of biomass at pH 12.0 with 300 mML^{-1} of bicarbonate concentration [26]. Whereas in the present study, *P. terebrans* BERC10 produced 2.0 gL^{-1} of biomass in alkaline wastewater (pH 10.0) without CO_2 supplementation (under 10 mML^{-1} bicarbonate) which indicated

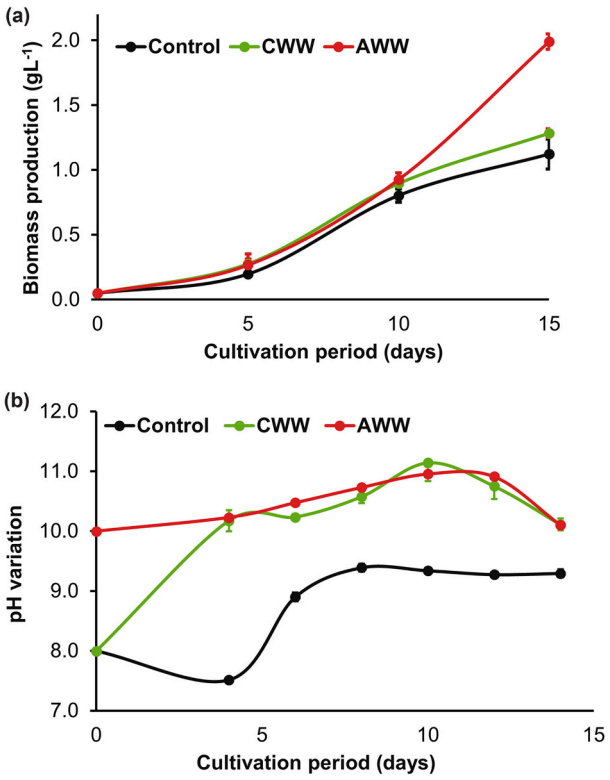

Fig. 1. Impact of stress conditions (Control, BG11; CWW, city wastewater; AWW, Alkaline wastewater) on the biomass production, and pH variation of *Plectonema terebrans* BERC10.

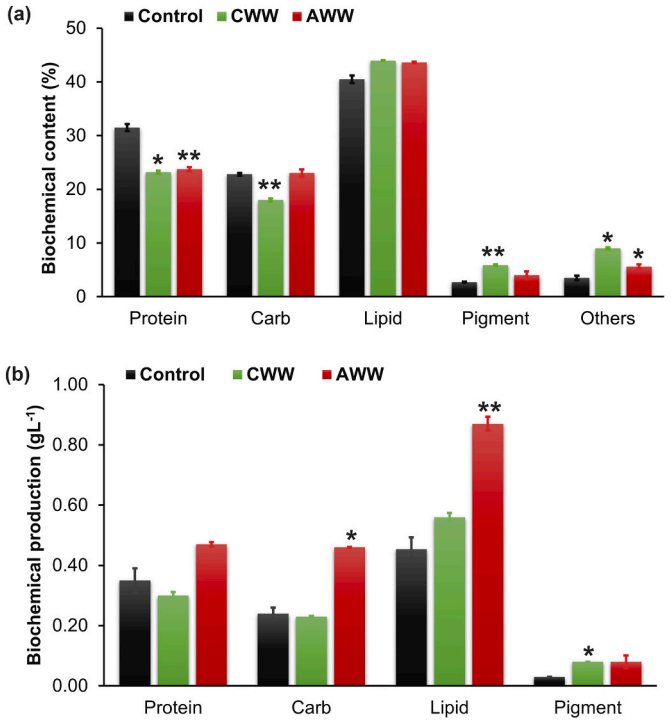

Fig. 2. Impact of stress conditions (Control, BG11; CWW, city wastewater; AWW, Alkaline wastewater) on the (a) biochemical content and (b) biochemical production of the *Plectonema terebrans* BERC10 (* for $p < 0.05$ and ** for $p < 0.005$).

the feasibility of the strain for biological CO_2 capture, storage, and utilization technology. Alkaline cultivation media offer an energy-inexpensive CO_2 concentration mechanism where CO_2 is dissolved as bicarbonates which are then processed by cyanobacteria for their growth and biomolecules synthesis [27]. Furthermore, integration of wastewater cultivation with alkalinity-based CO_2 capture offers the cost-effective and environment-friendly cultivation along with byproduct synthesis.

3.2. Impact of wastewater-derived alkalinity on the biochemical composition

The metabolite biosynthesis of cyanobacteria can be enhanced by manipulating abiotic growth conditions including nutrients and pH. The results indicated that wastewater (CWW) cultivation improved the lipid content from 40.5% to 44% whereas in alkaline wastewater lipid content shown to be 43.6%. Lipid production was 0.56 gL^{-1} and 0.87 gL^{-1} in wastewater and alkaline wastewater, respectively. The difference in lipid production is attributed to the higher biomass production of the *P. terebrans* BERC10 in alkaline wastewater. Wastewater cultivation decreased the carbohydrate content to 18% from 22.8%, whereas alkaline wastewater (AWW) cultivation did not affect the carbohydrate content because carbohydrate content was 23% in AWW, resulting in final carbohydrate production of 0.46 gL^{-1} (Fig. 2). A similar trend was observed for the *Spirulina* where aquaculture wastewater cultivation (supplemented with 25% Zarrouk's media) escalated the lipid and carbohydrate content by 1.25-fold and 1.0-fold, respectively, at pH 10.6 [28]. These results showed that alkaline wastewater is beneficial to improve the production of biofuel and biomolecules of the cyanobacterial strain BERC10.

In addition to bioenergy molecules, cyanobacteria accumulate food and health-relevant compounds in the form of proteins and pigments; especially phycobilin proteins. Wastewater cultivation shown to improve the pigment and lipid contents at the cost of protein biosynthesis. Protein content was reduced from 31.5% to 23% in wastewater and to 23.75% in alkaline wastewater. It might have happened due to the redirection of the nitrogenous compounds towards biomass production, and energy consumption [29]. Previously, reduction in protein content from 43.7% to 32% and reduction in pigment production from 41.3 mgL^{-1} to 10.6 mgL^{-1} was reported for wastewater-cultivated *Spirulina* [30]. Whereas, in contrast to that, wastewater cultivation escalated pigment production of *Pseudoanabaena* by approximately 1.0-fold resulting in a 237 mgg^{-1} of phycobilin yield [31].

It was interesting to note that wastewater alone or alkaline wastewater improved the pigment content by 2.17-fold and 1.5-fold, respectively (Fig. 2). The positive effect could be due to high bicarbonate availability at this condition which limits the CO_2 loss and favors the growth and biochemical composition of cyanobacterial cells [32].

3.3. Elucidation of the stress-responsive molecular mechanism

Cyanobacteria are also known to produce many valuable metabolites including pigments, alkaloids, sterols, polysaccharides, and phytohormones. Untargeted metabolomics of the wastewater (CWW) and alkaline wastewater (AWW) cultivated *P. terebrans* BERC10 was performed to determine the impact of these conditions on the metabolic response of the strain. Chromatograms of the three conditions are shown in supplementary Fig. S1. In total, 191 peaks were detected in the samples after normalization and peak extraction, out of which 61 peaks were identified at level 2 of metabolomics (Supplementary Table S3). Extracted ion chromatograms of three selected metabolites with their spectra are shown in Supplementary Fig. S2. Identified metabolites were shown to belong to various biochemical classes including alkaloids, carbohydrates, polysaccharides, lipids, sterols, amino acids, peptides, phytohormones, pigments, and chelators. Comparative analysis showed that 5 metabolites were unique to wastewater cultivation, and 6 were specific to alkaline wastewater (Supplementary Table S3). The

alkalinity-responded metabolites were important for promoting CO_2 sequestration, cell differentiation, light-harvesting efficiency, carbohydrate and lipid monomers biosynthesis, and stress-protective mechanism. The results reinforced the data related to growth and biochemical compositional analyses.

Normalized intensities of the 61 identified metabolites were then subjected to Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) (R2cum = 0.903, Q2cum = 0.618) (Fig. 3). This model is used to determine the reliability and significance of the obtained metabolites. The score plot is clearly showing variations between the groups as three distinct groups are formed on the plot (Fig. 3a). The variability among various groups indicated that media composition had a major impact on the metabolite composition of the cells. Detailed view of Fig. 3b showed that the group discrimination was mainly due to the 5 metabolites with the highest variable influence on projection (VIP) values which are shown in red. This signified the importance of cultivation media on the metabolite regulation. Metabolites with the highest VIP values included Anatoxin A, Chalcone, Glucose 6-phosphate, L-Histidine, and Phenylacetic acid (Fig. 3b).

Significance testing of the 61 identified metabolites was performed based on the volcano plots (Fig. 4) and ANOVA. It screened out 36 significant metabolites (Supplementary Table S3) with major upregulation or downregulation trends (relative to control) at all cultivation conditions. Alkaline wastewater cultivation upregulated 10 metabolites related to light harvesting, cell differentiation, CO_2 fixation, and storage compounds biosynthesis. In this condition cyanotoxin metabolites such as phenylacetic acid and anatoxins were downregulated (Fig. 4). Previously, wastewater-cultivated *Spirulina* modulated antioxidants, pigments, and biocomponent production [33] whereas, *Scenedesmus* altered the lipid profile [11] for cell membrane protection against stress. Similarly, *P. terebrans* BERC10 altered lipid profile, osmo-protectants, and antioxidant levels under wastewater and alkalinity stress for cell

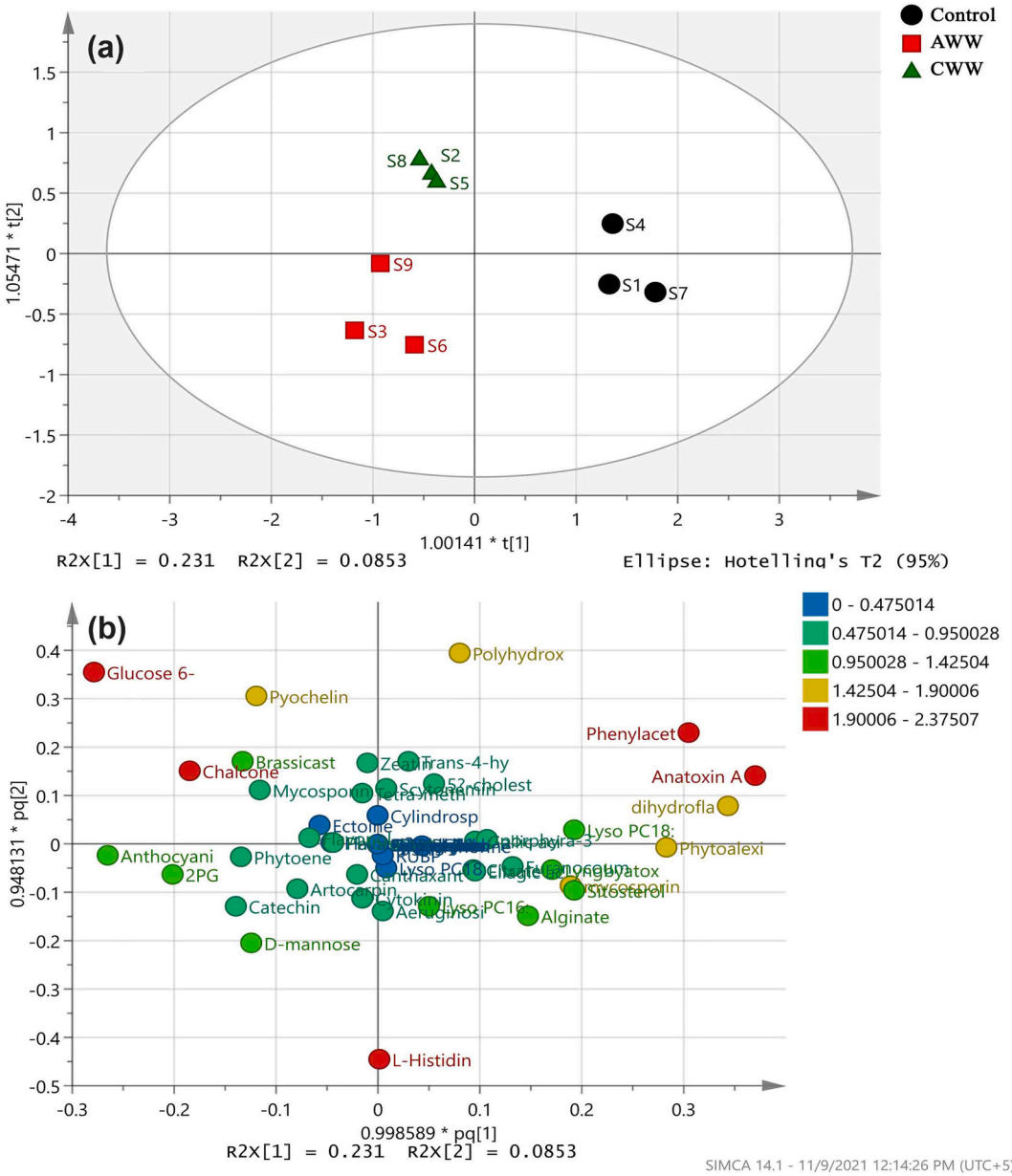

Fig. 3. Analysis of different stress conditions (Control, BG11; CWW, city wastewater; AWW, Alkaline wastewater) on *P. terebrans* BERC10. (a) OPLS-DA score plot showing variability within the groups (x-axis) and between the groups (y-axis). Black circle represents the control group, red squares AWW, green triangles CWW. (b) OPLS-DA loading plot colored according to the VIP (variable influence on projection) values. Metabolites with red color are showing highest VIP values. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

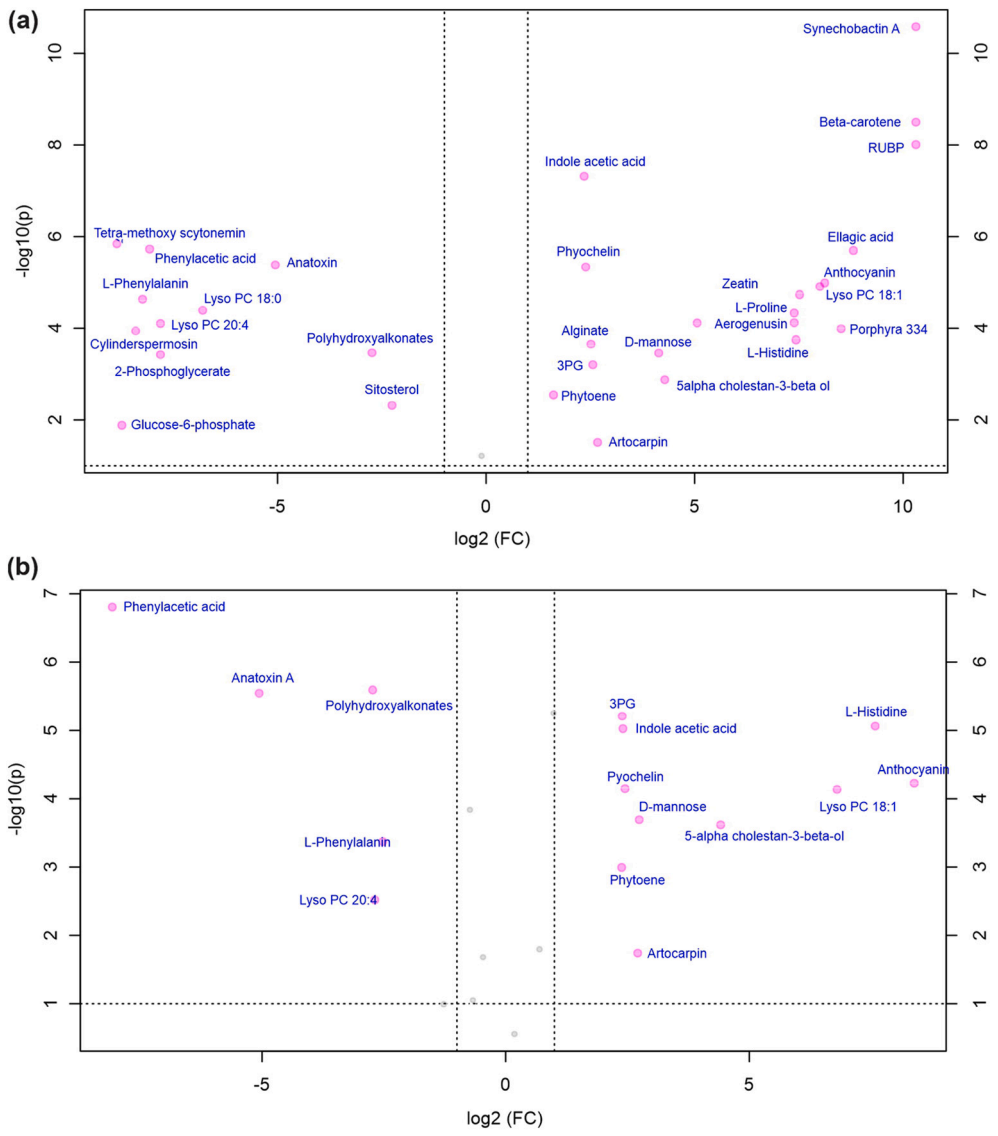

Fig. 4. Volcano plots of the (a) alkalinity and (b) alkaline wastewater cultivated *P. terebrans* BERC10 for significant features identification.

protection.

The up-regulation and down-regulation trend of the significantly identified metabolites was determined based on the fold-change (Log2_FC) analysis of the identified metabolites in response to different conditions (Figs. 5 and 6). Many features/metabolites with higher significance were unidentified which implies the need of detailed metabolite databases with refined analytical tools for the specific metabolite identification.

3.3.1. Molecular modulation in light-harvesting and signaling for improved biomass production in response to wastewater cultivation

Biomass production of *P. terebrans* BERC10 was observed to be higher in AWW when compared to CWW and control. Hence, correlation matrix analysis was performed to study divergence or coherence of data. Pearson correlation values for all identified metabolites were calculated against biomass production. Correlation values of metabolites higher than 0.80 were considered to be correlated with the biomass production (Table 1). Positive values in the table show positive correlation among variables while negative values show negative correlation. Values closer to 0 indicate poor negative or positive correlation while, values nearer to 1 show significant correlation. For significant analysis, ANOVA was performed and compounds with $P < 0.05$ were considered significant.

Cyanobacteria, being the unique chlorophototrophs perform photosynthesis through phycobilisome, photosystem I, and photosystem II for which complex photoreceptors have been evolved that control many metabolic and physiological aspects of the cyanobacteria [34]. Phytoene (an intermediate of carotenoid biosynthesis) is one of these industrially valuable photoreceptors with photoprotective and antioxidant properties [35]. Interestingly, in addition to hyperaccumulation of phycobilin proteins in *P. terebrans* BERC10, alkaline wastewater cultivation also improved the content of phytoene which might be responsible for improved light-harvesting, biomass production, and carotenoid accumulation. In the present study, the phytoene biosynthesis was improved by 5.54-fold in response to alkaline wastewater (Fig. 5) which was found to have positive correlation ($P < 0.05$) with biomass production (Table 1). A similar response was observed under irradiance [36] and alkalinity [1] stress which improved the phytoene content via upregulating the genes of the light-harvesting complex to combat the stress conditions.

Phytohormones are small signaling molecules that regulate the adaptive stress response to trigger biomass production and metabolite accumulation. In recent years, phytohormone supplementation has emerged as an alternative strategy to improve biomass production and lipid biosynthesis. Recently, *Scenedesmus* cultivation supplemented with

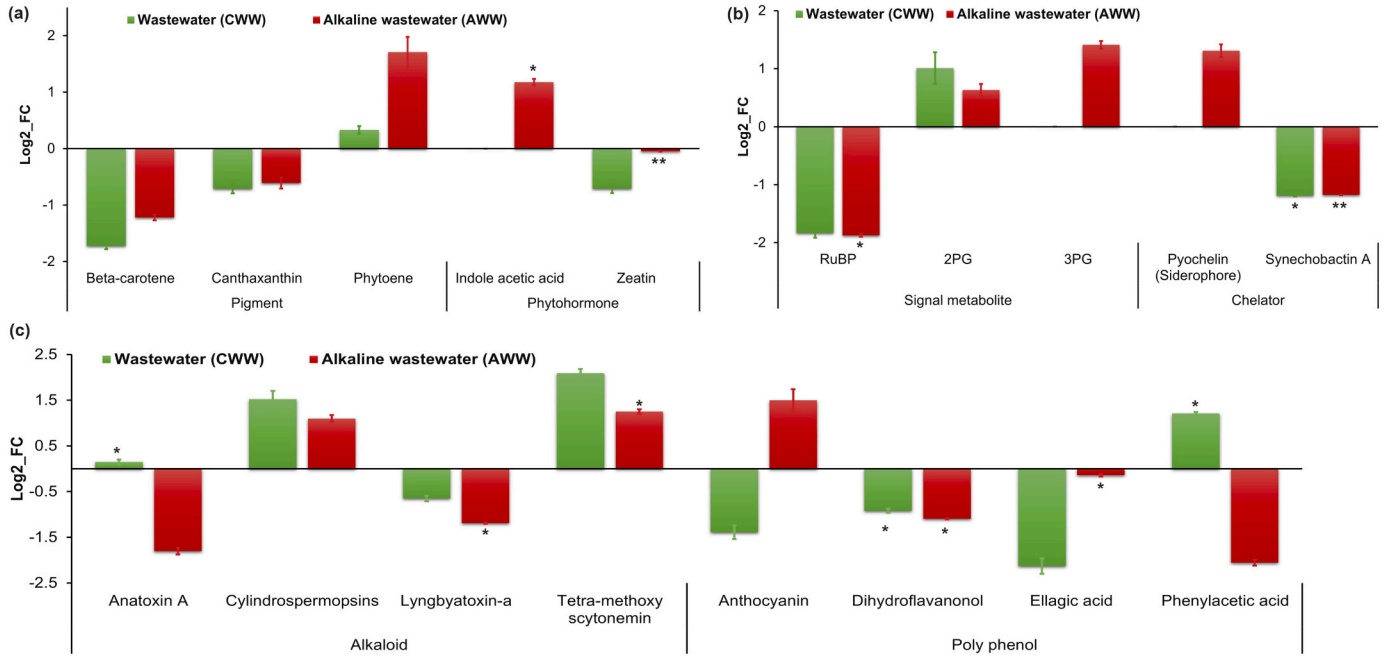

Fig. 5. Fold-change analysis of the significant metabolites (* for $p < 0.05$ and ** for $p < 0.01$) of the *P. terebrans* BERC10 in response to different stress conditions of wastewater, alkalinity, and alkaline wastewater (combination).

25 μM indole-acetic acid (IAA) showed an improved biomass productivity by 1.29-fold and lipid productivity by 1.95-fold. Whereas, 0.9 gL^{-1} of bicarbonate supplementation resulted in 1.48-fold and 1.86-fold improved biomass and lipid productivities, respectively [37]. A similar response was observed in the present study where alkaline wastewater cultivation improved the IAA content by 1.2-fold (Fig. 5) and IAA was found to be positively correlated ($P < 0.05$) with the biomass production (Table 1) which indicated it to be the possible reason for higher biomass production of *P. terebrans* BERC10 under alkaline wastewater cultivation.

3.3.2. *Stress-responsive molecular modulations in carbon and nitrogen metabolism enhanced CO_2 fixation and protein biosynthesis*

Carbon and nitrogen metabolism are pH-dependent tightly coupled mechanisms whose balanced activity is vital for subsequent assimilation and utilization of nutrients for the maintenance of biological processes such as cell growth, cellular conversion, and stress-tolerance in photosynthetic organisms. In response to wastewater and alkaline wastewater cultivation, the level of RuBP (Ribulose-1,5-bisphosphate) was reduced (Fig. 5b) which indicated an efficient photosynthetic conversion of CO_2 by RuBP into products like 3PG, glucose-6-phosphaste, and amino acids. It was interesting to note that the 2-phosphoglycolate (2PG); carbon limitation signal was upregulated in response to wastewater (neutral) and alkaline wastewater, whereas a higher concentration of 3-phosphoglycolate (3PG) was detected specifically in response to alkalinity and alkaline wastewater (Fig. 5). The 3PG was then utilized for the biosynthesis of amino acids and sugars in *P. terebrans* BERC10. Similarly, the cultivation of *Synechococcus elongatus* 11,802 in 1% CO_2 resulted in a reduced RuBP (CO_2 fixation substrate) level and an increased level of 3PG that improved the biomass production and biochemical (succinate and glutamate) synthesis [38]. In another study, enhanced 2PG concentration under CO_2-limited conditions was found to activate the ABC-type bicarbonate transporter for the cyanobacterial adaptation to CO_2 limited condition [39] which is consistent with the response of wastewater cultivated *P. terebrans* BERC10. This mechanism has been discussed in detail previously [40] that this could be an approach to enhance the photosynthetic efficiency and biomass production for crop improvement.

Amino acid and peptides are the direct products of nitrogen metabolism. In the present study, the higher activity of phenylacetic acid (a nitrogen binding metabolite) in the wastewater indicated the effective nutrient uptake by the *P. terebrans* BERC10 for efficient utilization of wastewater-derived nutrients. Interestingly, wastewater and alkaline wastewater exerted negative impact on protein biosynthesis (Fig. 6a). A similar response was observed in the wastewater-cultivated *Chlorella-Pseudomonas* consortia where nitrogen limitation reduced the amino acid content possibly due to diversion of nitrogen towards structural protein synthesis instead of nitrogenous metabolites [9]. Previously, It has been reported that higher amino acid content of *P.* is possibly due to higher 3PGA-levels which has been previously explained in *E. coli* where purine degradation genes activation (for nitrogen assimilation) through allantoin promote the 3PGA biosynthesis which then integrated into key energy metabolism [40]. However, it is not confirmed yet and require further investigations.

3.3.3. *Stress-responsive molecular modulation of lipids and sterol metabolism*

Energy molecules such as lipids and fatty acids are of great importance due to their key role in metabolism. *P. terebrans* BERC10 mainly produced PC (Phosphatidylcholines) as lipid molecules. Results shown that wastewater cultivation promoted the production of metabolites related to lipids (PC 18:0, PC 18:2, and PC 20:4) and sugars (glucose-6-phosphate, D-mannose). Wastewater and alkaline wastewater cultivation improved the concentration of PC 18:0. Additionally, wastewater had positive impact on PC 20:4 and alkaline wastewater had positive impact on PC 18:1 (Fig. 6). It was observed that in microalgae and cyanobacteria Lyso-PC transferase has a higher affinity towards oleic acid (C18:1) when compared to palmitic acid (C16:0). It could be the reason for improved PC18 molecules as compared to C16 under all stress conditions molecules [41]. Short-term treatment of *Chlamydomonas* with 1000 ppm CO_2 resulted in the upregulation of myristic, palmitic, and oleic acids due to upregulated activity of the acyl-desaturase (AAD) [42]. Enhanced Lyso-PC biosynthesis is associated with chloroplast glycerolipids breakdown resulting in an enhanced TAG production [43]. Cyanobacteria are known to promote the biosynthesis of energy molecules under low-nutrient conditions [44] by redirecting carbon towards

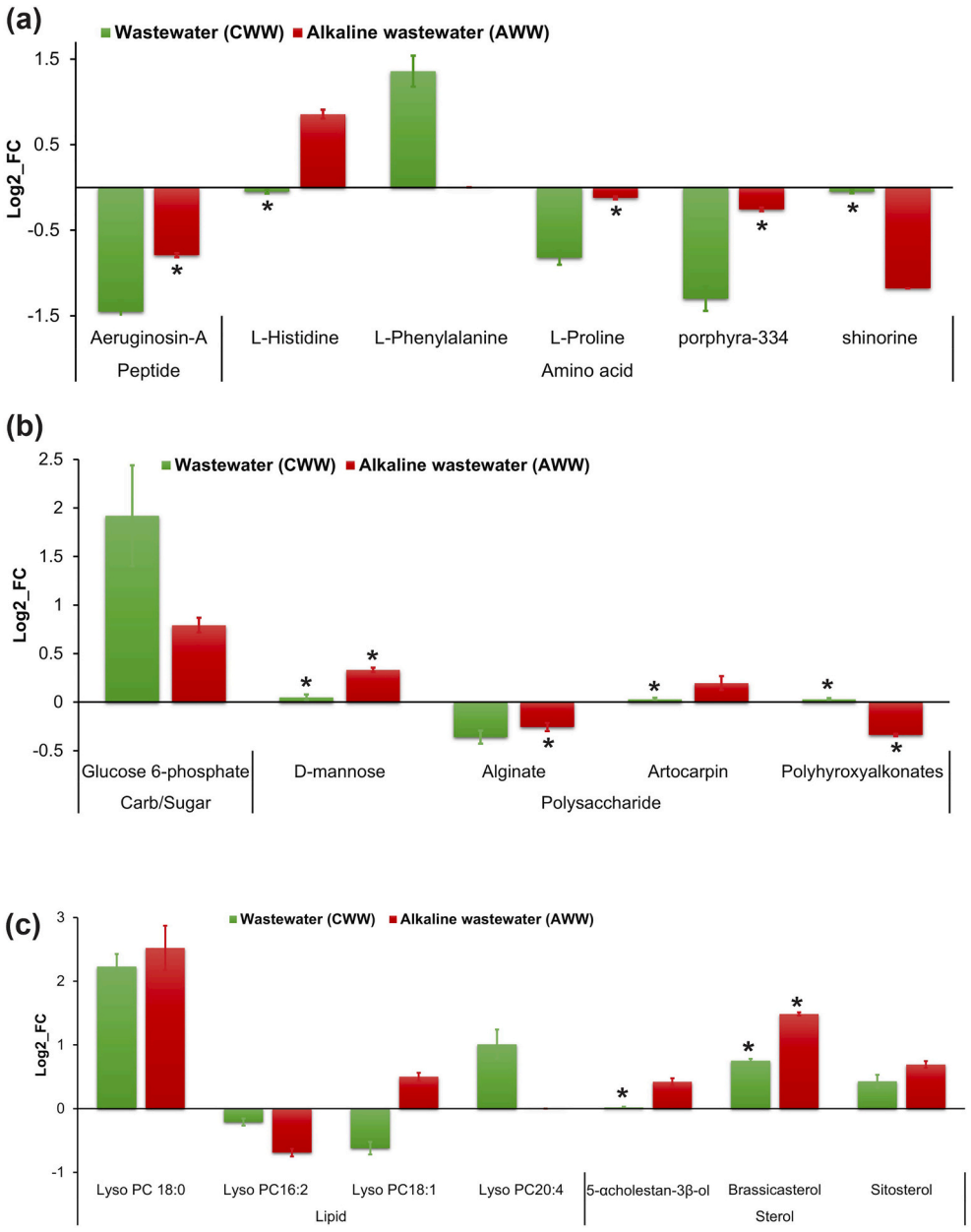

Fig. 6. Fold-change analysis of the significant biochemical primary metabolites (* for $p < 0.05$ and ** for $p < 0.01$) of the *P. terebrans* BERC10 in response to different stress conditions of wastewater, alkalinity, and alkaline wastewater (combination).

storage molecules (lipids and carbohydrates) to meet the energy demands of the cells. The similar response was observed in the present case, which signifies the phycoremediation (nutrient removal) ability of the *P. terebrans* BERC10.

Cyanobacteria can produce animal sterols (cholesterols) and plant sterols (phytosterols) with pharmaceutical, cosmetic, and aquaculture feed properties. Classification and exploration of these viable membrane lipids are necessary to elucidate the lipid metabolism and cell protection mechanism [45]. The metabolomics analyses of BERC10 showed an upsurging trend in the sterol biosynthesis except for stigmasterol in response to alkalinity followed by alkaline wastewater and neutral wastewater cultivation. A similar response had been observed in slaughterhouse wastewater-cultivated *Phormidium autumnale* which produced 455.3 ugg^{-1} of stigmasterol, and 279 ugg^{-1} of β-sitosterol [45]. Similarly, nitrogen limitation enhanced the sterol content of *Botryococcus braunii* from 5.8% to 11.8% [46]. These byproducts of the isoprenoid biosynthesis pathway are important for regulating the membrane fluidity and are being used as aquaculture feed, energy source, and cosmetic constituents [47].

3.3.4. Molecular modulation of carbohydrate biosynthesis in response to stress

Cyanobacterial carbohydrates are major carbon sink that act as energy reservoir, information system, and defense mechanism. Therapeutic and antioxidant properties of the polysaccharides are valuable to alleviate stress while, their immense functional versatility is attractive for industrial applications [48]. In the present study, intracellular concentration of glucose-6-phosphate (G6P) along with other polysaccharides was enhanced in wastewater (supplemented with bicarbonate) and alkaline wastewater cultivated samples as compared to control (non-supplemented with bicarbonate). Similar response was observed for *S. elongatus* under 1% CO_2 cultivation [38]. Similarly, high carbon (5% CO_2) concentrations improved the glucose and related phosphorylated sugar levels of the wild-type and mutant *Synechocystis*

Table 1
Correlation matrix analysis between biomass production and metabolite accumulation in response to different cultivation conditions. Metabolite with correlation value > +0.8/<−0.8 and *p*-value < 0.05 was found significant to be correlated with biomass production.

S. no.	Metabolite name	Correlation values	*p*-Values
1.	22-Dehydrocholestrol	**0.98447**	0.078746
2.	2PG	−0.04379	**0.036123**
3.	3PGA	**0.98447**	**0.049253**
4.	5α-cholestan-3β-ol	**0.98681**	**0.036122**
5.	Aerucyclamide D	−0.34025	0.054318
6.	Aeruginosin-A	−0.37873	**0.037126**
7.	Alginate	−0.43726	**0.028726**
8.	Anatoxin A	**−0.96640**	**0.044318**
9.	Anthocyanin	**0.82295**	**0.004538**
10.	Artocarpin	**0.99779**	**0.032299**
11.	Brassicasterol	**0.97380**	0.074572
12.	Campesterol	−0.64423	0.069559
13.	Canthaxanthin	−0.53104	0.06092
14.	Carrageenan-Lambda	−0.64423	0.06779
15.	Catechin	**0.95641**	0.098045
16.	Chalcone	**0.94562**	0.068597
17.	Citrate	**−0.99230**	0.060824
18.	Cylindrospermopsins	0.60926	**0.021139**
19.	Cytokinin (Kinetin)	**0.83667**	0.085827
20.	dihydroflavanonol	−0.74553	0.054279
21.	D-Mannitol	**0.98447**	0.061025
22.	D-mannose	**0.99984**	**0.026215**
23.	Ectoine	−0.36255	0.073203
24.	Ellagic acid	0.24282	**0.021917**
25.	Flavan-3-ol	0.77289	0.056803
26.	Flavone	−0.34025	0.054155
27.	Flavonol	**0.95077**	0.082985
28.	Fucophlorethol A	−0.64423	0.070638
29.	Furanocoumarins (Angelicin)	**−0.82888**	0.055003
30.	Gallic acid	0.24543	0.063907
31.	Glucose 6-phosphate	0.33537	**0.013924**
32.	Hydroxy-ectoines	**0.98447**	0.081187
33.	Indole-Acetic-acid	**0.98447**	**0.039314**
34.	L-Histidine	**0.97396**	**0.045101**
35.	L-Phenylalanine	−0.34025	**0.036122**
36.	Lyngbyatoxin-a	**−0.89901**	0.066118
37.	Lyso PC16:2	**−0.99429**	0.076909
38.	Lyso PC18:0	0.65042	**0.020125**
39.	Lyso PC18:1	**0.84042**	**0.042792**
40.	Lyso PC18:2	−0.34025	0.062383
41.	Lyso PC20:4	−0.34025	**0.041646**
42.	mycosporine	−0.15150	0.084647
43.	Mycosporin-glycine	**−0.85738**	0.05436
44.	Phenylacetic acid	−0.08541	**0.046514**
45.	Phlorotannins (Eckol)	**0.99989**	0.090557
46.	Phytoalexins (Camalexin)	0.33413	0.079589
47.	Phytoene	**0.99756**	**0.043411**
48.	Polyhydroxyalkanoates	**−0.97319**	0.054155
49.	porphyra-334	0.20715	**0.024776**
50.	Pyochelin (Siderophore)	**0.99120**	**0.041646**
51.	RuBP	−0.64423	**0.007041**
52.	Schizokinen	−0.64423	0.074423
53.	Scytonemin	−0.72722	0.086033
54.	shinorine	**−0.98774**	0.065792
55.	Sitosterol	0.78059	**0.042793**
56.	Stigmasterol	−0.64423	0.072221
57.	Synechobactin A	**0.98447**	**0.039313**
58.	Tetra-methoxy-scytonemin	0.10611	**0.026253**
59.	Trans-4-hydroxy-L-proline	0.18358	**0.043411**
60.	Zeatin	0.27492	**0.042715**
61.	β-Carotene	−0.64423	**0.042793**

Correlation values of metabolites higher than 0.80 were considered to be correlated with the biomass production and are shown as bold text in Table 1. The $P < 0.05$ were considered significant and are shown as bold text in Table 1.

strain [49]. An increase in carbohydrate and polyhydroxybutyrate production has been observed in wastewater-cultivated cyanobacteria upon bicarbonate supplementation at pH 7.0. This increasing trend is possibly due to the high C:N ratio where carbon is utilized for the carbohydrate biosynthesis as storage molecules [50]. In present study, lower G6P and polyhydroxyalkonates levels in response to the alkaline wastewater condition (pH >10.0) have been observed. Alkaline wastewater surged the biosynthesis of arotcarpin, alginate, and D-mannose by 5.5-fold, 0.37-fold, and 3.5-fold, respectively, in the *P. terebrans* BERC10 (Fig. 6b) as compared to control.

3.3.5. Stress-responsive protective mechanisms

Cyanobacterial secondary metabolites including alkaloids and polyphenols are of pharmaceutical importance due to their antioxidant, antimicrobial, and UV-protectant properties. Cyanobacteria usually produce these compounds as a protection strategy against stress conditions [51]. It was interesting to note that alkaline wastewater cultivation restricted the biosynthesis of toxin molecules like anatoxin, Lyngbyatoxin, and furanocoumarins and produced poly-phenolic compounds to curb the contamination. Whereas in wastewater cultivation, the *P. terebrans* BERC10 depended on the cylindrospermopsins (cyanotoxin) and tetra-methoxy-scytonemin for cell protection (Fig. 5c). A similar response was observed in *Raphidiopsis raciborskii* [2] and many members of the *Oscillatoria* sp. [52] where elevated nitrogen, phosphorous, and carbon reduced the alkaloid (saxitoxin) biosynthesis and improved the polyphenols. This response was possibly due to the redirection of nutrients towards amino acids biosynthesis and biomass production instead of its utilization in nitrogen-shunting toxic compounds [2]. Wastewater-cultivated sample of *P. terebrans* BERC10 showed down-regulated polyphenols (ellagic acid, anthocyanin, dihydroflavanol) biosynthesis and thus depended on alkaloid biosynthesis (except Lyngbya-toxin) for cell protection (Fig. 5c). In most of the cases, these compounds had a negative impact on the cyanobacterial growth [53], but in the present case no such effect was observed which indicated the non-toxic stress-tolerance potential of *P. terebrans* BERC10 under alkaline wastewater cultivation.

4. Conclusions and prospects

Alkaline wastewater reservoirs are damaging but worth exploring biological resources for the biotransformation of wastewater-derived nutrients to valuable compounds. A newly isolated cyanobacterium *P. terebrans* BERC10 exhibited remarkable alkaliphilic and wastewater treatment potential. Untargeted metabolomics assays revealed that *P. terebrans* BERC10 tolerates the alkalinity by improving protein biosynthesis, polysaccharide accumulation, and antioxidant metabolites. Whereas upregulation of the metabolites related to glycolysis, carbon-nitrogen metabolism, and lipid metabolism along with other protective metabolites like phytoene and sterols enabled the cells to tolerate alkaline wastewater conditions. Hence, these growth conditions can be particularly employed for the enhanced biosynthesis of these valuable metabolites by simply diverting the metabolic flux through nutrient manipulation. These stress-responsive pathways could be the future targets of strain improvement through genetic engineering. Besides, alkaline wastewater has proven to be a beneficial resource to produce industrially valuable commodities. The stress-tolerance potential of *P. terebrans* BERC10 makes it suitable biorefinery chassis and worth exploring candidate for wastewater treatment, alkalinity alleviation, and to become a feedstock for the multiproduct biorefinery.

CRediT author statement

Ayesha Shahid: Investigation, Writing-original draft preparation, Methodology, Funding acquisition. *Amna Jabbar Siddiqui:* Methodology, Software, Validation. *Syed Ghulam Musharraf:* Writing-reviewing, software, validation. *Chen-Guang Liu:* Writing-reviewing, software, validation. *Sana Malik:* Methodology, Software, Validation. *Achmad Syafiuddin*: Methodology, Software, Validation. *Raj Boopathy:* Writing-reviewing, software, validation. *Nesrin Ibrahim Tarbiah:* Methodology, Software, Validation. *Munazza Gull:* Methodology, Software, Validation. *Muhammad Aamer Mehmood:* Conceptualization, Supervision, Writing-

reviewing & editing, Funding acquisition.

Declaration of Competing Interest

It is declared that authors have no competing interests.

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Appendix A. Supplementary data

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