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Impact of Nitrate and Ammonium Concentrations on Co-Culturing of *Tetradesmus obliquus* IS2 with *Variovorax paradoxus* IS1 as Revealed by Phenotypic Responses

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Abstract

Mutual interactions in co-cultures of microalgae and bacteria are well known for establishing consortia and nutrient uptake in aquatic habitats, but the phenotypic changes in terms of morphological, physiological, and biochemical attributes that drive these interactions have not been clearly understood. In this novel study, we demonstrated the phenotypic response in a co-culture involving a microalga, *Tetradesmus obliquus* IS2, and a bacterium, *Variovorax paradoxus* IS1, grown with varying concentrations of two inorganic nitrogen sources. Modified Bold's basal medium was supplemented with five ratios (%) of NO_3-N:NH_4-N (100:0, 75:25, 50:50, 25:75, and 0:100), and by maintaining N:P Redfield ratio of 16:1. The observed morphological changes in microalga included an increase in granularity and a broad range of cell sizes under the influence of increased ammonium levels. Co-culturing in presence of NO_3-N alone or combination with NH_4-N up to equimolar concentrations resulted in complete nitrogen uptake, increased growth in both the microbial strains, and enhanced accumulation of carbohydrates, proteins, and lipids. Total chlorophyll content in microalga was also significantly higher when it was grown as a co-culture with NO_3-N and NH_4-N up to a ratio of 50:50. Significant upregulation in the synthesis of amino acids and sugars and downregulation of organic acids were evident with higher ammonium uptake in the co-culture, indicating the regulation of carbon and nitrogen assimilation pathways and energy synthesis. Our data suggest that the co-culture of strains IS1 and IS2 could be exploited for effluent treatment by considering the concentrations of inorganic sources, particularly ammonium, in the wastewaters.

Keywords Co-culture of bacteria and microalgae · Phenotypic changes · Inorganic nitrogen sources · Symbiotic interactions · Metabolomes

Introduction

Microalgae are photosynthetic organisms commonly associated with heterotrophic bacteria through mutual interactions to establish efficient consortia [1]. The symbiotic association of microalgal−bacterial consortium results in increased growth, nutrient uptake, and production of vital metabolites in both the organisms [2–6]. The presence of bacterial species alone appears to be inadequate for total nitrogen removal, and microalgal population involved in co-culturing enhance nutrient removal [7]. Furthermore, the consortia significantly help in improving soil health besides exhibiting great biotechnological potential in wastewater treatment, sustainable biomass, biodiesel production, etc. [1, 4, 8, 10]. Importantly, the success of this widespread symbiotic association depends on the extent of nutrient availability, particularly nitrogen that

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fosters maximum biomass production [3, 11, 12]. Nitrates and ammonium are the usually preferred nitrogen sources for both bacteria and microalgae in consortia [11, 13, 14]. Several studies, therefore, focused on the uptake of nitrate or ammonium by microalgae in view of their abundant occurrence in habitats, including wastewaters [14–17]. Moreover, it is noteworthy that nitrate and ammonium are simultaneously present in aquatic systems, which are the natural habitats for consortia of microalgae and bacteria [14].

Nitrogen assimilation into biomass achieves a significant accumulation of nutrients that facilitate cellular productivity [18, 19]. Nitrate is assimilated in microbial cells via reduced ammonium, mediated by nitrate reductase [20], and its uptake is reduced by the presence of ammonium [14]. However, the uptake mechanisms of both nitrate-nitrogen (NO_3-N) and ammoniacal nitrogen (NH_4-N) are interlinked to achieve higher nitrogen assimilation enabling several physiological changes [14]. Inorganic nitrogen regulates the nitrogen fluxes within the individual species of microalgae and bacteria, coordinating the metabolic pathways involved in energy yield and carbon skeleton [13, 14, 20, 21]. Also, the availability of nitrogen critically controls the synthesis of proteins and nucleic acids in both eukaryotes and prokaryotes [21, 22]. Therefore, these metabolic responses are greatly affected by the interactions between nitrate and ammonium that co-exist mostly in aquatic habitats [23].

Very recently, we identified a microalga, *Tetradesmus obliquus* IS2, and a bacterium, *Variovorax paradoxus* IS1, that can establish an efficient consortium [3]. Also, it has been observed that the symbiotic interactions among these microbial strains are mediated primarily by extracellular polymeric substances [24]. Reports available in the literature emphasized the crucial role of either NO_3-N or NH_4-N on the yield of biomolecules [13, 20, 25]. Although certain studies focused on identifying microalgal−bacterial consortia for higher nitrogen uptake for sustainable biomass production [5, 6], there is no information about the impact of NO_3-N and NH_4-N, in combination, on phenotypic responses in such co-cultures. In the present novel study, we investigated the effect of NO_3-N and NH_4-N by including five different ratios (percentages) of 100:0, 75:25, 50:50, 25:75, and 0:100 on morphological and physiological attributes in the co-culture of *T. obliquus* IS2 and *V. paradoxus* IS1. Overall, we used the Redfield N:P ratio of 16:1, which is common in phytoplankton that grows naturally and rapidly by altering fundamental cellular properties [26].

Materials and Methods

Co-Culturing of Microalga and Bacterium

T. obliquus IS2 (GenBank accession No. MN719511) and *V. paradoxus* IS1 (GenBank accession No. MN689266), both isolated from poultry slaughterhouse wastewaters [3, 24], were used in this study. Axenic culture of the strain IS2 was routinely maintained in Bold's basal medium (BBM) under continuous light (60 µmol photons m^{-2} s^{-1}) at 23 ± 1 °C with shaking (100 rpm) [24], and the bacterial strain IS1 was grown on Luria–Bertani agar medium at 37 °C. The culture medium (pH 7.0) was supplemented with 0.04 g L^{-1} glucose to initiate bacterial growth in the co-culture [3]. Inorganic nitrogen sources, in the form of $NaNO_3$ and NH_4Cl, were added to the culture medium to provide five different NO_3-N:NH_4-N ratios (%) of 100:0, 75:25, 50:50, 25:75, and 0:100. Both nitrogen and phosphorus in the culture medium were maintained at Redfield molar ratio of 16:1 as it plays a crucial role in algal physiology [26]. Logarithmically growing microalgal cells (1×10^6 cells L^{-1}) or bacterial cells (1×10^6 colony forming units (CFU) mL^{-1}) were used to inoculate, either alone or in combination in 100 mL of modified BBM contained in 250 mL Erlenmeyer flasks. The culture media used (Online Resource 1) were designated as M1, M2, M3, M4, and M5. All the culture flasks were incubated at 23 ± 1 °C in an orbital shaker (100 rpm) with continuous illumination of 60 µmol photons m^{-2} s^{-1} for 6 days. Each treatment included five flasks ($n = 5$), and each single flask was considered one replicate. All the experiments were repeated twice.

Microalgal Cell Morphology and Flow Cytometry

Samples from different cultures growing at exponential phase were collected for microscopic observation at × 1000 magnification in an inverted microscope (IX73, Olympus, Japan). Aliquots of the cultures were withdrawn after 96 h, centrifuged at 5000 × *g* for 2 min, washed in phosphate buffer solution (PBS, Sigma-Aldrich, St. Louis, MO), and resuspended in the same buffer. Microalgal cell granularity and autofluorescence were determined in BD FACS Canto flow cytometer (BD Biosciences, San Jose, USA). Sideward scatter (SSC) and forward scatter (FSC) signals acquired at the excitation on 488 nm blue argon laser were collected with a bandpass filter 695/40 nm. Cell granularity and cell size were determined using SSC-A and FSC-A in the scatter dot plot, respectively. An autofluorescence histogram was developed by red fluorescence of FL-3 channel (> 670 nm). The results of 10,000 events were collected and analyzed by FlowJo V10.7.1 (BD Biosciences, San Jose, CA, USA).

Analysis of Growth and Total Nitrogen Uptake

For determining microbial growth, *T. obliquus* IS2 and *V. paradoxus* IS1 were cultured either alone or in combination in different media as described above. At regular intervals of 24 h, 200 µL aliquots from each experimental flask were withdrawn and used for growth determination. Microalgal

growth was measured by direct cell counting using a Neubauer haemocytometer (Bright-line, Hausser Scientific, USA) in an optical microscope (CX31, Olympus, Japan) [27]. Viable bacterial cell count was determined following serial dilution and plating on Luria–Bertani agar, and colony-forming units (CFUs) were counted [5]. The specific growth rate at the logarithmic phase was calculated as described earlier [3].

Samples (1 mL) from each flask were withdrawn every 24 h and passed through 0.22 µm cellulose acetate syringe filters (Minisart®, Sartorius, Gottingen, Germany) to remove the biomass. The Orion AQUAfast nitrogen low range digestion tubes (Thermo Fisher Scientific, Waltham, MA, USA) were used for determining total nitrogen as per the manufacturer's protocol. The samples were assayed in Orion AquaMate 8000 UV–Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). MilliQ water (Elga LabWater, High Wycombe, UK) was used as a diluent as well as a blank.

Analysis of Biochemicals

Cell suspensions (1.5 mL) were withdrawn from each flask on the fourth day of incubation, at which time the cultures exhibited maximum growth. Samples were centrifuged at $7000 \times g$ for 5 min, and the pellets were washed with sterile ultrapure water (Elga LabWater, High Wycombe, UK). Carbohydrates were extracted following the modified Anthrone method as described by Chen and Vaidyanathan [28]. For protein extraction, 2-mL aliquots from each culture flask were sampled on day 4, and centrifuged as mentioned above. The pellets were treated with 1 mL of 0.5 N NaOH at 80 °C for 10 min with stirring. The supernatants were withdrawn by centrifugation at $5000 \times g$ for 5 min and transferred into new vials. This alkali extraction was repeated thrice, and the final extraction was done at 100 °C for 10 min [28]. Protein in the supernatants was determined using the Bradford protein assay kit (quick start™, Bio-Rad, Hercules, CA) with bovine serum albumin (BSA, Sigma-Aldrich, St Louis, MO) as the protein standard according to the manufacturer's instructions.

Lipid content was assayed in aliquots of 2 mL, withdrawn on the fourth day from the experimental cultures. Portions of biomass were lyophilised in a Freeze dryer (John Morris Scientific, Osterode, Germany). Lipids from lyophilised biomass were extracted and quantified following the method described earlier by Perera et al. [3]. The chlorophylls (*a* and *b*) in the culture samples (2 mL) were extracted and estimated spectrophotometrically following the method of Chen and Vaidyanathan [28]. The samples were extracted with 80% aqueous acetone (v/v), and the total chlorophyll was expressed as the sum of chlorophyll *a* and *b*. The quantities of all the biochemical compounds were expressed as mg g^{-1} dry weight [3]. The analytical grade solvents were purchased from Sigma-Aldrich, St Louis, MO, or Merck, Darmstadt, Germany.

Analysis of Metabolomics by ^{1}H-NMR

The biomass from the remaining culture in each flask was harvested by centrifugation as described above and quenched with 70% aqueous cold methanol (v/v). The samples were centrifuged at $8000 \times g$ for 2 min at 4 °C to remove methanol and freeze-dried. Lyophilized biomass (10 mg) was used for extraction of polar metabolites with the solvent mixture, methanol: water (2:1), as described earlier by Perera et al. [3]. Concentrated polar extracts in Eppendorf tubes were reconstituted in 1.0 mL of 1:1 (v/v) CD_3OD:KH_2PO_4 buffer in D_2O (pH 6.0) that included 0.05% (w/v) 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid (TSP). The contents were sonicated at 20 Hz for 7 min at room temperature. The samples were transferred into 5 mm NMR vials, and ^{1}H-NMR spectra were obtained using Bruker BioSpin Avance III (600 MHz) NMR spectrophotometer [3]. All the AR grade solvents were obtained from Sigma-Aldrich, St Louis, MO.

The 1D spectra obtained were processed and integrated using TopSpin 4.0.5 (Bruker, Rheinstetten, Germany). Based on the chemical shifts assigned, the metabolites were identified using the 600 MHz chemical shift database of Chenomx profiler (Chenomx, Edmonton, AB, Canada) and other databases available in the literature [3, 29, 30]. The ^{1}H-NMR spectra were normalized against internal standard TMS and dry weights following log transformation and autoscaling using the Metaboanalyst 4.0 software [31, 32]. Heat maps were generated using 26 significant ($P \leq 0.05$) metabolites present in the samples following GraphPad Prism 9.0.1 (GraphPad Software, Inc., San Diego). Pathway analysis was performed with Metaboanalyst by comparing Kyoto Encyclopedia of Gene and Genome (KEGG) metabolic pathway libraries. Fisher's exact test enrichment method was used for over-representation analysis and pathway topological analysis following relative-betweenness centrality [24].

Statistical Analysis

Five biological replicates ($n = 5$) were included in all the experiments, and all the analyses were done with two technical replicates. Using Minitab 19 (Minitab Inc., State College, PA, USA), the data values (means ± SD) were analyzed by one-way ANOVA followed by Tukey's honestly significant difference (HSD) test at $P \leq 0.05$.

Results

Morphological Alterations in Microalga Grown in a Co-Culture

Alterations in cell morphology of *T. obliquus* IS2, when grown as a co-culture with *V. paradoxus* IS1 in modified BBM supplemented with varying ratios of NO_3-N and NH_4-N, were only observed as there were no changes in bacterial strain under the same culture conditions. Cell morphology, in terms of size and intactness of cells as revealed by microscopy, in *T. obliquus* IS2 grown as co-culture in the presence of both nitrate and ammonium (modified BBM such as M2, M3, and M4 with NO_3-N:NH_4-N:PO_4-P ratios of 12:4:1, 8:8:1, and 4:12:1, respectively), varied significantly compared to that observed in medium M1 that contained only NO_3-N (Fig. 1a). Increasing concentrations of NH_4-N in the culture medium enhanced the toxicity in microalga. Moreover, supplementing the modified BBM only with ammonium (M5) at a 16:1 ratio of NH_4-N: PO_4-P disintegrated microalgal cells, indicating that ammonium at this concentration is highly toxic to the microalga. Analysis of cell size by forward scatter (FSC-A) and intracellular granularity through side scatter (SSC-A) following flow cytometric analysis further supported the above morphological changes that resulted with the supply of the two inorganic nitrogen sources and their combinations (Fig. 1b). In fact, the cell size (FSC-A) also varied with the combination of NH_4-N and NO_3-N compared to the presence of the only nitrate as a nitrogen source. Also, the presence of NH_4-N in the modified BBM either alone or in combination with NO_3-N significantly enhanced intracellular granularity than with NO_3-N alone. Flow cytometric histograms of the co-cultures (Fig. 1c) revealed almost similar autofluorescence signals derived from microalgal strain IS2, as well as their overlap in the presence of NH_4-N, either alone or in combination with NO_3-N, while there was a slight shift in the histogram in case of the cultures grown only with sole NO_3-N. This observation clearly indicates that the nature of inorganic nitrogen supplemented to the culture medium is critical for changes in autofluorescence of microalgal species in co-cultures.

Growth, Nitrogen Uptake, and Biochemical Response of Microbial Strains

Growth response in *T. obliquus* IS2 and *V. paradoxus* IS1, when cultured alone or together, varied with the nature of

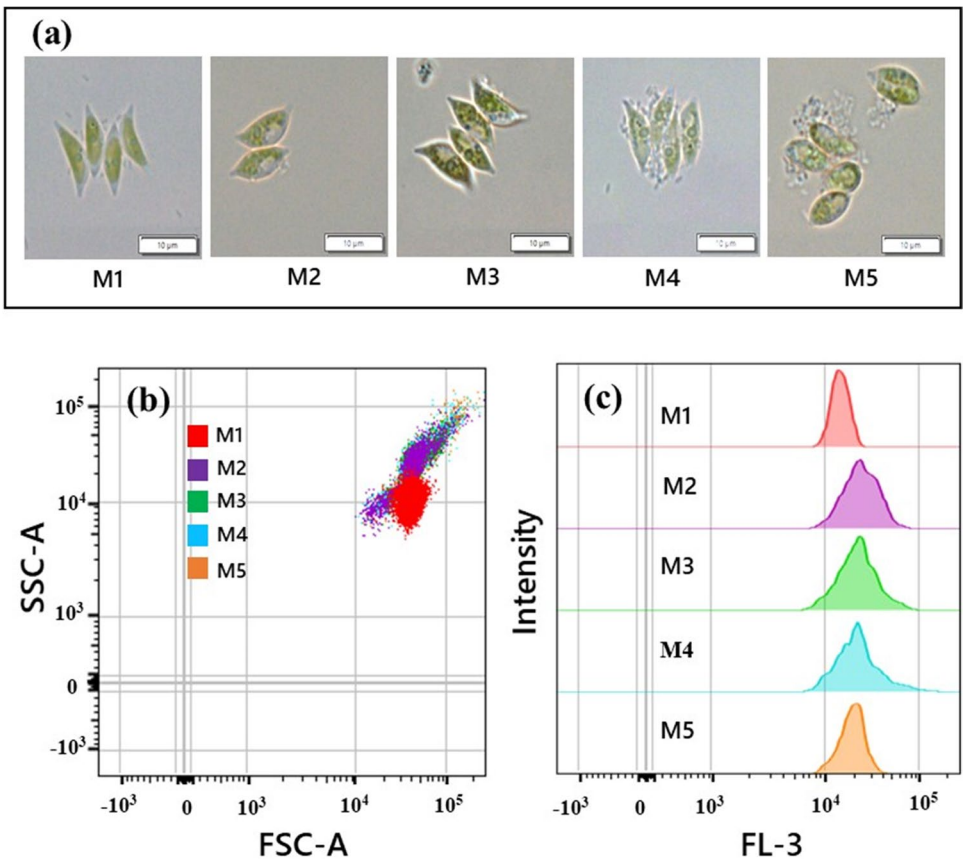

Fig. 1 **a** Morphological changes as revealed in light microscopy in *T. obliquus* IS2 when grown in modified BBM as a co-culture with *V. paradoxus* IS1 in presence of NO_3-N and NH_4-N alone or in combination. **b** Flow cytometric dot plot of SSC-A vs FSC-A depicting cell size and cell granularity of microalgal cells. **c** Flow cytometric histograms of autofluorescence signals excited from microalgal cells

inorganic nitrogen sources and their combination in the medium (Fig. 2). The growth rate in *V. paradoxus* IS1 was ˂0.46 day^{-1} when cultured alone in the presence of NO_3-N as a sole source of nitrogen in medium M1 or in combination with NH_4-N in medium M2 through M4 (Fig. 2a). However, the bacterial strain exhibited a maximum growth rate ($\mu_{max} = 1.41$ day^{-1}) when co-cultured in M5 that contained only NH_4-N as a sole nitrogen source. In contrast, strain IS2 showed the lowest growth rate (0.66 day^{-1}) when grown alone in M5 (Fig. 2b). The growth rate in strain IS2 in co-culture was significantly higher (1.05 day^{-1}) when it was grown in the presence of only NO_3-N. Of the three different combinations of NO_3-N and NH_4-N, their presence at 50:50 ratio in the culture medium resulted in a significant increase in the growth rate of bacterium (1.12 day^{-1}) and microalga (0.97 day^{-1}). The uptake of NO_3-N by microalga was significantly higher from medium M1 (30 mg L^{-1}) followed by M2, M3, and M4 (Table 1). The observed nitrogen uptake was only 10 mg L^{-1} in medium M5 that contained only NH_4-N. Almost complete removal of NO_3-N (~33 mg L^{-1}) was evident by the co-culture from medium M1 through M3. Again, increased concentrations of NH_4-N in the co-culture resulted in a decrease in total nitrogen uptake by the co-culture.

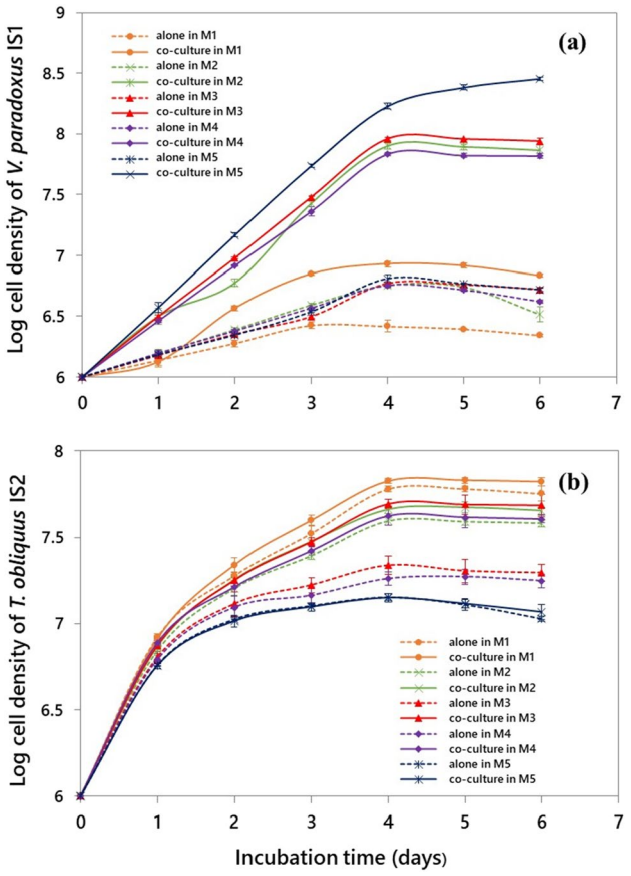

Fig. 2 Cell densities of the bacterial and microalgal strains grown alone or in co-culture in presence of NO_3-N and/or NH_4-N in modified BBM. **a** *V. paradoxus* IS1 and **b** *T. obliquus* IS2. Error bars represent means ± SD ($n = 5$)

The supply of NO_3-N and NH_4-N alone or in combination greatly influenced carbohydrates, proteins, and lipids in *T. obliquus* IS2 and *V. paradoxus* IS1, or total chlorophyll in *T. obliquus* IS2 when grown alone or in co-culture (Online Resource 2). The bacterial strain IS1 when cultured alone in the presence of NH_4-N exhibited significantly higher carbohydrates (35%), proteins (63%), and lipids (60%) than with NO_3-N. In contrast, strain IS2 preferred NO_3-N over NH_4-N and increased the synthesis of carbohydrates, proteins (~200%), and lipids (50%). Overall, the combination of NO_3-N and NH_4-N at 50:50 ratio in the medium used for co-culturing yielded significantly higher amounts of carbohydrates, proteins, lipids, and total chlorophyll in the co-culture than other combinations. All these biochemicals decreased when NH_4-N was supplied alone in high concentrations.

Metabolomic Response in Microbial Strains

Analysis of the polar extracts obtained from individual cultures and co-cultures revealed the significant occurrence of 26 significant primary and secondary metabolites

Table 1 Inorganic nitrogen sources (NO_3-N and NH_4-N) supplemented to the modified BBM and uptake of total nitrogen by *T. obliquus* IS2 grown alone or in co-culture with *V. paradoxus* IS1

	Medium ID				
Nitrogen	M1	M2	M3	M4	M5
Initial NO_3-N	33.0*	25.0	16.50	8.25	–
Initial NH_4-N	–	8.25	16.50	25.0	33.0
Total N uptake by strain IS2	30.0 ± 0.90^a	29.60 ± 1.30^a	27.40 ± 0.50^a	20.30 ± 1.40^b	10.10 ± 0.30^c
Total N uptake by strain IS2 + strain IS1	32.50 ± 0.50^a	32.20 ± 0.60^a	32.40 ± 0.10^a	26.20 ± 0.90^b	16.40 ± 1.10^c

*The values related to concentrations of total nitrogen in the medium and total N uptake are mg L^{-1}. Data values (means ± SD, $n = 5$) for total N uptake by the cultures in a row followed by the same letters are not significantly different ($P \leq 0.05$) as per one-way ANOVA followed by Tukey's honestly significant difference (HSD) test

(Fig. 3). The ^{1}H-NMR spectra clearly showed the prominent chemical shifts assigned to primary metabolites (δ 0.5–5.5), including amino acids, organic acids, and sugars. Secondary metabolites (δ 5.5–10.0) appeared mainly in the region for aromatic compounds (data not shown). Based on the spectra, amino acids observed in the extract included valine, alanine, cysteine, tyrosine, arginine, asparagine, proline, glutamate, glutamine, γ-aminobutyrate, and tryptophan. Organic acids such as acetate, succinate, citrate, oxoglutarate, and formate appeared in the region of δ 2.0–3.0 followed by carbohydrates like glucose, sucrose, ribulose-5-phosphate, and fructose as the most prominent metabolites in the region of δ 3.0–5.0. Other compounds such as nucleobases (adenine), antioxidants (glutathione), vitamins (thiamine), organic osmolytes, and lipid derivatives (ethanolamine and betaine) were also identified from the signals of ^{1}H-NMR spectra. Co-culture grown in all the media except in M5 exhibited an upsurge of amino acids, sugars, and organic acids. Again, the addition of NO_3-N and NH_4-N to the culture medium (M3) at an equimolar ratio greatly resulted in the expression of the above metabolites. The predominant metabolic pathways and the metabolites implicated in the co-culture of *V. paradoxus* IS1 and *T. obliquus* IS2 under the influence of inorganic nitrogen sources are presented in Fig. 4a and Online Resource 3. The most significant pathway was aminoacyl-tRNA biosynthesis, which was mainly affected by amino acids involved in nitrogen assimilation of the microbial strains of the co-culture. Glutamate and glutamine that originate through nitrogen assimilation seem to play a significant role in these prominent pathways (Fig. 4b). The uptake of NO_3-N and NH_4-N from the culture medium induced the synthesis of specific metabolites that regulate the key metabolic pathways of energy-yielding assimilation of nitrogen and carbon. Consequently, the intermediate metabolites synthesized via these pathways may have served as feedstock for amino acid biosynthesis, sugar metabolism, protein synthesis, lipid production, and nucleotide metabolism.

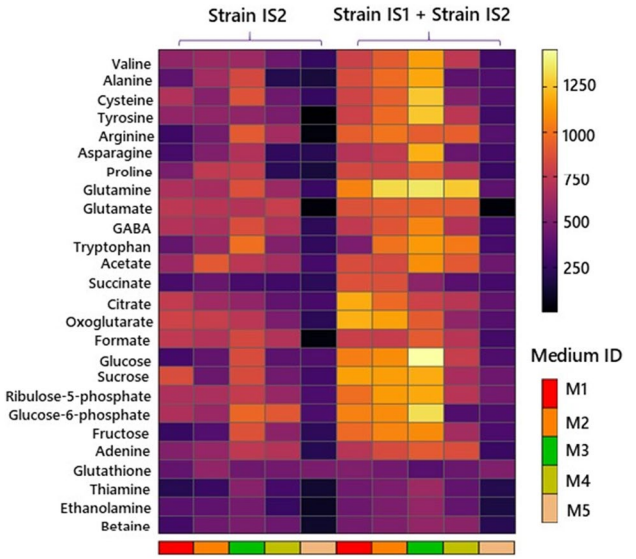

Fig. 3 Heat map of 26 significant ($P \leq 0.05$) cellular metabolites of *T. obliquus* IS2 grown alone or as co-culture with *V. paradoxus* IS1 in the presence of NO_3-N and NH_4-N supplemented alone or in combination. Each square represents the normalized value of integrated ^{1}H-NMR peak intensities. The color scale of the heat map, that ranges from pale yellow to black, indicates the highest to lowest metabolite expression, respectively

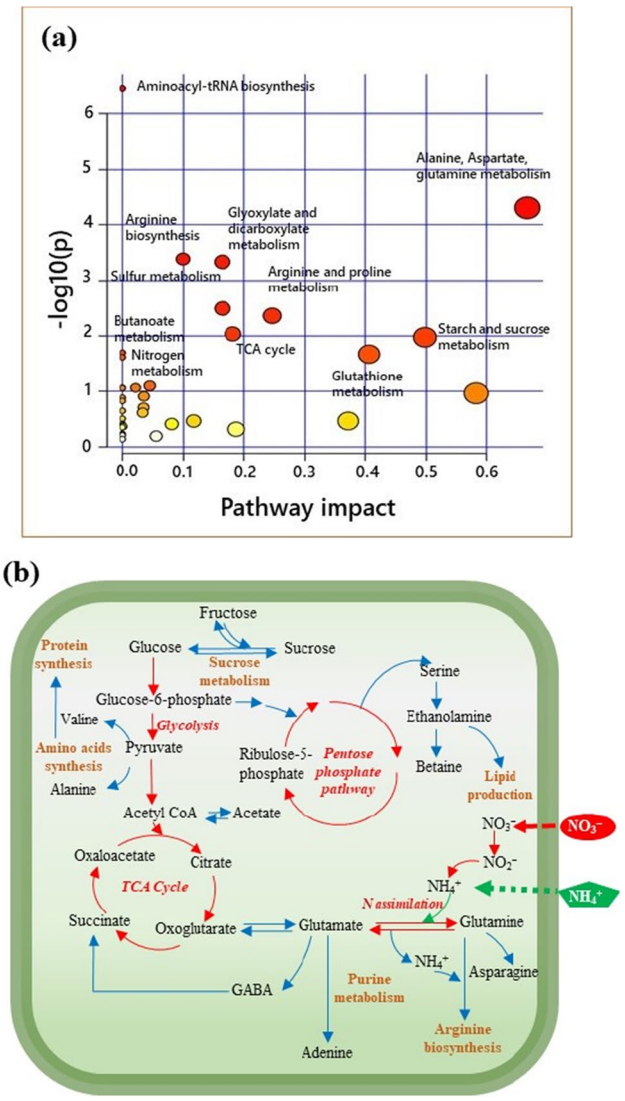

Fig. 4 **a** Pathway enrichment analysis of co-culture of *V. paradoxus* IS1 and *T. obliquus* IS2, grown in presence of NO_3-N and NH_4-N, as generated by the Metaboanalyst 4.0 software. The color gradient in each circle is indicated as per the *P* value. The most significant ($P \leq 0.05$) pathways are indicated in red. **b** Schematic metabolic pathways of the microalgal−bacterial co-culture as influenced by NO_3^- and NH_4^+ supplemented to the medium alone or in combination

Discussion

The cellular elemental composition of phytoplankton biomass is primarily comprised of carbon, nitrogen, and phosphorus in a ratio of 106:16:1 [26, 33, 35]. In the present study, we investigated the versatile performance of a co-culture of *V. paradoxus* IS1 and *T. obliquus* IS2 in presence of NO_3-N and NH_4-N, supplemented to the culture medium at different ratios. The medium supplemented with NH_4-N alone at a higher concentration resulted in structural disintegration in microalga, confirming its significant toxicity [36]. The morphological changes in microalgae affect the uptake of nutrients like nitrogen as well as biomass productivity [37]. Our present observations support the negative expression of nitrogen assimilation genes with increased supply of ammonium and down-regulation of ammonium uptake that results in decreased microalgal growth [14, 38]. Nitrate alone increases the transport rate per unit area of the cell membrane [38], which may contribute to higher microalgal growth. Under the impact of NO_3-N and NH_4-N, in combination at equal concentrations, the optimal uptake of both the sources of nitrogen must have been governed by the rate of internal transport, internal pool of glutamine and nitrate to achieve increased growth of both the partners in co-culture [38, 39].

Nitrogen uptake, either from nitrate or ammonium, governs the turnover of the macromolecules that regulate metabolic pathways, which affect energy production and carbon skeleton [25, 40]. Even though a higher nitrogen pool governs the upsurge of protein and amino acids [20], their synthesis is induced by the co-occurrence of nitrate and ammonium than their availability alone [41]. In fact, ammonium conversion into protein is rapid compared to nitrate [20], and abundant amino acids follow the same oxidation level as ammonium [18]. Glutamate (Glu) and glutamine (Gln) are the primary amino acids in the GS-GOGAT nitrogen assimilation pathway, serving as a nitrogen acceptor and a donor, respectively [13, 14]. Since higher glutamine concentration governs through greater ammonium uptake [42], the significant glutamine production observed in both the strains grown in medium M2 through M4 indicates the higher ammonium uptake in co-culture. Although biomass obtained from the cultures grown only with NH_4-N contained low levels of all these metabolites, the accumulation of glutathione was significant, indicating the scavenging mechanism for stress caused by higher ammonium levels [43]. However, a high ammonium pool of the cell suppresses further ammonium assimilation [14], resulting in low glutamine content as evidenced in cultures grown in M5. Organic metabolites formed are likely to trigger heterotrophic or mixotrophic growth of both species under nitrogen limiting conditions [44]. It is, therefore, no decline in growth of microalgal strain was observed even though complete nitrogen uptake occurred after four days. Our present finding of higher amounts of adenine produced under increased levels of Glu and Gln indicates that these two amino acids are the precursors for the synthesis of nucleobases [45].

Acquisition of nitrate is a more energy-demanding process than ammonium, inducing energy-deriving pathways such as glycolysis and tricarboxylic acid cycle (TCA) [25, 40]. Higher nitrate uptake and accumulation of low levels of carbohydrates observed in co-culture grown in medium M1 suggest the onset of the glycolytic pathway that rapidly utilizes sugars and produces organic acids by upregulation of the TCA cycle. On the other hand, ammonium uptake by the co-culture in all the media excepting M1 probably required lesser energy, resulting in the accumulation of carbohydrates. Furthermore, complete nitrogen uptake by the co-culture resulted in enhanced lipid production since nitrogen availability is directly proportional to the synthesis of lipids [22, 25, 46]. In fact, enhanced nitrogen uptake in the co-culture may have triggered the synthesis of both ethanolamine and betaine, which are the membrane-derived lipids observed abundantly in *Scenedesmus obliquus* under elevated nitrogen levels [47]. However, further lipidomic studies are required to understand how nitrate and ammonium precisely contribute to polar and nonpolar lipids of the total lipid profile in the co-culture. The co-culture grown in media M1, M2, and M3 also exhibited higher amounts of total chlorophyll, which supports the complete nitrogen uptake and accumulation of glutamate [20]. Ammonium at the highest level included in the present study (≥ 33 mg L^{-1}) might have caused severe damage to photosystem II and the synthesis of chlorophyll *a* [14, 48].

In conclusion, we report here the changes in phenotypic traits in a co-culture of *T. obliquus* IS2 with *V. paradoxus* IS1 that govern the survival and metabolic activity when grown in the presence of varying levels of NO_3-N and NH_4-N. The addition of NO_3-N to the medium, either alone or together with NH_4-N, up to equimolar concentrations, significantly enhanced not only the growth of the two species in a co-culture but also their potential in nitrogen removal from the culture medium. Also, our data suggest that the microbial strains in a co-culture could tolerate the higher concentrations of NH_4-N well by altering their physiological and biochemical processes to achieve optimal cellular nitrogen, carbon, and energy balance. Hence, the co-culture of *T. obliquus* IS2 with *V. paradoxus* IS1 could be used as a potential system for wastewater treatment by considering the concentrations of inorganic nitrogen sources, particularly ammonium.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00248-021-01832-6.

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Declarations

Conflict of Interest The authors declare no conflict of interest.

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