Untargeted GC–MS reveals differential regulation of metabolic pathways in cyanobacterium *Anabaena* and its biofilms with *Trichoderma viride* and *Providencia* sp.

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**ABSTRACT**

Cyanobacteria and their biofilms are used as biofertilizing options to improve plant growth, soil fertility, and grain quality in various crops, however, the nature of metabolites involved in such interactions is less explored. The present investigation compared the metabolite profiles of cyanobacterial biofilms: *Anabaena torulosa* - *Trichoderma viride* (An-Tr) and *A. torulosa* - *Providencia* sp. (An-PW5) against the individual culture of *A. torulosa* (An) using untargeted gas chromatography-mass spectroscopy. Metabolites were identified using the NIST mass spectral library and the relative peak area of cultures analysed, after normalization with an internal standard, ribitol. An-Tr biofilm recorded approximately 66.85% sugars, with increased quantity and numbers of sugars and their conjugates, which included maltose, lactose, and D-mannitol, but decreased amino acids concentrations, attributable to the effect of Tr as partner. Heat map and cluster analysis illustrated that An-Tr biofilm possessed a unique cluster of metabolites. Partial least square-discriminate analysis (PLS-DA) and pathway analyses showed distinct modulation in terms of metabolites and underlying biochemical routes in the biofilms, with both the partners- PW5 and Tr eliciting a marked influence on the metabolite profiles of An, leading to novel cyanobacterial biofilms. In the An-PW5 biofilm, the ratios of sugars, lactose, mannitol, maltose, mannose, and amino acids serine, ornithine, leucine and 5-hydroxy indole acetic acid were significantly higher than An culture. Such metabolites are known to play an important role as chemoattractants, facilitating robust plant–microbe interactions. This represents a first-time study on the metabolite profiles of cyanobacterial biofilms, which provides valuable information related to their significance as inoculants in agriculture.

**Abbreviations**
PCC The Pasteur Culture Collection of Cyanobacteria
UV Ultra Violet light
EPS Extracellular polysaccharides
ITCC Indian Type Culture Collection
TMS Trimethylsilyl
MSTFA N-methyl N-(trimethylsilyl) trifluoroacetamide
PLSDA Partial least square discriminate analysis
VIP Variable importance in projection

1. Introduction

Cyanobacteria are oxygenic photoautotrophic gram-negative bacteria that can photosynthesize and assimilate CO₂ into a variety of biochemical compounds through a wide range of metabolic pathways (Fogg, 1956; Garcia-Pichel, 1998; Do Nascimento et al., 2019). Most of the metabolites produced by cyanobacteria are required for purposes other than growth and reproduction such as sensing, defense, and combating biotic and abiotic stresses (Alawiye and Babalola, 2021; Rastogi and Sinha, 2009). Light and carbon dioxide are two crucial abiotic factors for photosynthetic prokaryotes like cyanobacteria to thrive normally, any small change in these factors alters the intracellular metabolites instantaneously (Tomitani et al., 2006; Maruyama et al., 2019).

Metabolomics is a powerful tool to ascertain variations in metabolite levels in response to various environmental cues (Schwarz et al., 2013). It is well established that metabolites bridge the gap between genotypic and phenotype of an organism (Schrimpe-Rutledge et al., 2016) and metabolite profiles mirror the cellular activities, hence, they can provide
useful information regarding the beneficial effects of inoculants on soil and plant-related activities. Both intracellular and extracellular metabolomes have been investigated in several cyanobacteria, including *Anabaena variabilis* PCC 7937 (Singh et al., 2008), *Nostoc commune* (Ehling-Schulz et al., 1997), *Calothrix* sp. (Hartmann et al., 2015), *Synechocystis* sp. PCC 6803 (Werner et al., 2019), *Leptolyngbya* sp. PCC 7376 (Baran et al., 2013), and *Chlorogloeopsis fritschii* PCC 6912 (Portwich et al., 2000). The metabolome of *C. fritschii* has been studied in detail as it is of industrial significance (Balasundaram et al., 2012; Kultschar, 2020), particularly, *C. fritschii* PCC 6912 exposed to ultraviolet radiation (UV-A and UV-B) (Kultschar et al., 2019; 2021).

Cyanobacteria are known to produce sugars, amino acids, auxins, vitamins, and phytohormones, which helps in stimulating plant growth (Ehling-Schulz et al., 1997), perhaps a synergy or additive effects among the functional attributes of 7376 (Baran et al., 2013), and *Synechocystis* sp. (Singh et al., 2008), *A. torulosa - Providencia* sp. (PW5) was firstly grown at 30 ± 2 °C for 24 h in a mechanical shaker (100 rpm) at 107 cfu ml⁻¹. The biofilms An-Tr and An-PW5 were developed as previously optimized by Prasanna et al. (2011) where Tr and PW5 were inoculated into one week actively grown An culture, such that 107 cfu ml⁻¹ and 106 cfu ml⁻¹ were obtained for An-Tr and An-PW5 biofilm preparation respectively, and grown under same conditions for 2 weeks after the inoculation of partners.

### 2.2. Experimental design and harvesting of cultures

The cultures were grown in triplicate in completely randomized design (CRD). The cyanobacterium, *A. torulosa*, An-Tr, and An-PW5 were harvested at 3 weeks after inoculation for An, and 2 weeks after inoculation of partners for biofilms respectively. The fungus- *Tricho-derma viride* ITCC2211 was grown in Potato Dextrose Broth (PDB), harvested after 7 days, and *Providencia* sp. was grown in Nutrient Broth (NB) and harvested after 24 h, corresponding to their respective log phase of growth. The harvested cultures were then flash frozen using liquid nitrogen, and stored at −80 °C until further analysis.

### 2.3. Chemical reagents

Methanol, water, and chloroform are of LC-MS grade from Honeywell; Internal standard ribitol (adonitol), methoxamine HCl, and pyridine bought from Sigma, and N-methyl N-(trimethylsilyl) trifluoroacetamide (MSTFA) from SRL.

### 2.4. Sample preparation for GC-MS analysis

Preparation of microbial samples for Gas Chromatography- Mass Spectrometry (GC-MS) analysis was performed as described previously by Kundu et al. (2018). About, 250 mg of liquid nitrogen frozen sample was taken in a 2 mL centrifuge tube and extracted with 480 µL pure methanol and 20 µL ribitol (adonitol – 0.2 mg mL⁻¹) was added as an internal standard. The contents were shaken vigorously for 2 min and then heated at 70 °C for 15 min followed by adding an equal volume of LC-MS grade water and mixed thoroughly. Chloroform (250 µL) was then added to and mixed vigorously. The mixture was then centrifuged at 2200 xg for 10 min at room temperature (aprx. 25 °C). After centrifugation, the upper phase was transferred to a new 2 mL centrifuge tube and dried in a speed vacuum rotator at 35 °C. The dried pellet was then redissolved in 40 µL methoxamine hydrochloride in pyridine (20 mg mL⁻¹) and incubated at 37 °C for 90 min. Derivatization of the sample was carried out by the addition of 60 µL N-methyl N-(trimethylsilyl) trifluoroacetamide (MSTFA) and incubated for 30 min at 37 °C. The derivatized sample of metabolites (100 µL) was then transferred to an insert containing a GC–MS glass vial and stored at 4 °C until GC-MS analysis.

### 2.5. Gas chromatography-mass spectrometry analysis of metabolites

The metabolite sample analysis was performed in Shimadzu GC–MS-QP2010™ coupled with an autosampler-auto injector (AOC-20sii) and an SH-Rxi.5Sil MS capillary column (30 m x 0.25 µm film thickness x 0.25 µm internal diameter; Restek Corporation, USA). A derivatized sample of 2 µL volume was injected into the inlet at 250 °C with a split ratio of 1:5. Purging of the standard septum was performed after sample injection at 3.5 ml min⁻¹ and carrier gas helium at 1 ml min⁻¹ attained a constant flow rate. Initially, the oven temperature was programmed at 80 °C for 2 min, ramped up to 250 °C at 5 °C min⁻¹, withheld for 2 min and further ramped to 300 °C at 10 °C min⁻¹, and withheld for 24 min. Ionization was done at 70 eV in an electron impact mode and the masses were scanned for full spectra from 40 to 600 m/z with a scan speed of 2000.
The solvent cut time was at 4 min.

2.6. Processing of GC–MS data and statistical analysis

All the compounds were identified based on retention time and different metabolites were differentiated after derivatization with different numbers of TMS (trimethylsilyl). Mass spectral analysis was performed through GC–MS solution software (Shimadzu®) and National Institute of Standards and Technology (NIST) mass spectral search program (version 14 s). Normalization of peak area was done by dividing the peak area of a metabolite by the peak area of internal standard (riboitol) and this unit less values were used for statistical analyses. The fold change of compounds was calculated using the normalized peak area. The standardized data was used for partial least square-discriminate analysis (PLS-DA); VIP >1 (variable importance in projection), and \( p < 0.05 \) was considered as significant. Pathway analysis of the metabolites was compared with the KEGG library of the nearest related member- *Synechococcus elongatus* PCC7942 for An, An-Tr, and An-PW5, *Saccharomyces cerevisiae* for Tr, and *Bacillus subtilis* for PWS respectively. All the statistical analyses were carried out in Metaanalysis 5.0 software (Xia and Wishart, 2011).

3. Results

3.1. Metabolite identification and variations

The intracellular metabolite profiling of cyanobacterial biofilms, An-Tr, and An-PW5 was undertaken using 3 weeks old cultures, and compared with *A. torulosa* as individual culture. Tr and PW5 were also analyzed for cross-analysis with biofilms. A total of 124 and 141 peaks were detected in An-Tr and An-PW5 biofilms respectively, and these peaks were identified to 84 and 92 metabolites respectively in the NIST library (Fig. 1A). Significant changes in concentrations of metabolites were recorded in all cultures, An, Tr, PW5, An-Tr, and An-PW5. The metabolites identified were categorized under five major chemical classes: sugars and their conjugates, amino acids, peptides, and their derivatives; fatty acids and their conjugates; nucleoside, nucleotide, and analogs; inorganic acids; and other organic acids. Sugars and their conjugates were found to be higher in An-Tr biofilm (66.85%), with a 1.55- and 1.03- fold increase compared to An (26.20%), and Tr (32.99%) respectively. Similarly, fatty acids and their conjugates were higher in An-Tr (4.34%), with values which were 1.09- and 1.97- fold greater; while nucleotide, nucleoside and analogues were higher by 0.62- and 2.72- fold over An, and Tr respectively. Albeit, amino acids, peptides and their derivatives were lower in An-Tr biofilm by 3.93- and 0.30- fold compared to An, and Tr respectively. An-PW5 biofilm showed lower values in terms of sugar and their conjugates (1.49- and 0.05- fold), amino acids, peptides, and their derivatives (3.32- and 0.11- fold), over An and PW5 respectively. A fold change of 0.36 increase over An, and 3.32 decrease over PW5 was observed in fatty acids and their conjugates of An-PW5. With respect to nucleotide, nucleoside, and analogues, An-PW5 recorded highest values of 3.55% which was 0.19- and 9.88- fold higher than An and PW5 respectively. The percent distribution and the number of metabolites in each of the chemical classes have been given for each culture and the biofilms in Fig. 1B, C, Supplementary Table 1, and Supplementary Fig. 1.

3.2. Tr and PW5 induced changes in metabolites in the cyanobacterial biofilms

Among the 27 metabolites involved in the carbon and nitrogen cycle in cyanobacteria, changes in ten major pathways were selected and compared with individual cultures and biofilms (Fig. 2). Changes in the metabolite levels were found by comparing the cyanobacterial biofilms and their respective partners. In An-Tr biofilm, the addition of Tr increased maltose, lactose, and citric acid significantly (\( p \leq 0.05 \)), while glucose and fructose were reduced. All amino acids including alanine, isoleucine, threonine, aspartate, methionine, glutamate, glutamine, and proline were reduced significantly except 5-oxoproline, which increased significantly by Tr inoculation. Stearic and palmitic acid also increased significantly in the An-Tr biofilm. Whereas in An-PW5 biofilm, sugars (mannose and lactose), amino acids (leucine, glutamine, and ornithine) increased significantly (\( p \leq 0.05 \)), while fatty acids, stearic, palmitic, and myristic acids were lowered as a result of PWS inoculation. Oxalic acid was distinctly present in An-Tr and PWS.

The metabolites having the greater variable importance in the projection value (VIP > 1) were considered as relevant for further analyses. Based on VIP > 1 and fold change (FC > 2), 114 metabolites in An-Tr and 79 in An-PW5 were found, accompanied by significant changes (\( p < 0.05 \), student’s T-test) after coculturing with Tr and PW5 partners respectively. Sugars, amino acids, and organic acids were identified as the most influenced metabolites and were tabulated (Table 1). Compared to An alone, 37 metabolites increased, 77 decreased, and 20 showed no change in the levels of An-Tr biofilm after co-culturing with Tr. In An-PW5, 36 increased, 43 decreased, and 43 showed no change in the levels of the metabolites after co-culturing with PW5.
3.3. Partial least squares- discriminant analyses (PLS-DA) of cyanobacterial biofilms

Analyses of the data used for quantification of all cultures was performed by comparing with the internal standard ribitol and represented as relative peak areas (normalized values). The PLS-DA, heat map and cluster analyses of metabolite illustrating the differences between A. torulosa and their biofilms An-Tr and An-PW5 as shown, are based on these normalized values. In PLS-DA, the top 15 metabolite hits having a VIP score greater than 1 for both An-Tr and An-PW5 biofilms is also given (Fig. 3A, B). In An-Tr biofilm, only 3 sugars (maltose, d-mannitol, lactose) were found to be higher than An culture, whereas 1 sugar (D-glucose) and 11 amino acids such as L-leucine, L-alanine, L-glutamic acid, glycine, L-valine, L-lysine, L-isoleucine, L-aspartic acid, L-threonine, L-tyrosine and L-proline were higher in An culture alone, with 15 top hit metabolites. The metabolites hits in An-PW5 were found to be different than An-Tr biofilm, with one additional sugar- mannose, besides the common ones- L-lactose, D-mannitol, maltose, and 4 amino acids (L-serine, L-ornithine, L-leucine, 5-hydroxyl indoleacetic acid) being higher in An-PW5 biofilm, and 3 sugars (D-glucose, D-galactose, sucrose) and 4 amino acids (L-glutamic acid, L-5-oxoproline, L-alanine) were higher in An culture alone. The heatmap of all the cultures is given with only the metabolites exhibiting significant changes and of importance in the cyanobacterial metabolism (Fig. 3C).

3.4. Pathway analysis

Based on the specific hypergeometric test, the pathway enrichment and metabolite topology analyses of cyanobacterial biofilms and their partners were performed and mapped into the biological pathways using the KEGG database. The database was assigned to the number of pathways for each culture, An (41), Tr (40), PW5 (41), An-Tr (39), and An-PW5 (42) respectively. Comparing An and cyanobacterial biofilms, An-Tr and An-PW5, the difference in metabolites enriched in several metabolic pathways were tabulated, and statistically significant and most enriched 15 pathways of An, An-Tr, and An-PW5 compared (Fig. 4). Among the enriched pathways, An-Tr biofilm showed reduced hits over An in all pathways including aminoacyl-tRNA biosynthesis, valine, leucine and isoleucine degradation, glycine, serine and threonine metabolism, arginine biosynthesis, alanine, aspartate and glutamate metabolism, and glyoxylate and dicarboxylate metabolism. Glutathione metabolism showed a down regulation vis-à-vis both the partners-An and Tr, while nitrogen metabolism was represented minimally (as compared to An) in the biofilm. Methane metabolism was a new introduction in the An-Tr biofilm (Table 2). Albeit, An-PW5 showed an equal number of enriched pathways hits for most of the pathways over An, and also specifically enriched in 5 pathways including alanine, aspartate and glutamate metabolism, glutathione metabolism, cyanoamino acid metabolism, glycolysis/ gluconeogenesis, and amino sugar and nucleotide sugar metabolism (Table 2).

4. Discussion

Biofilms represent conglomerations or assemblages of either known or mixed species and their interactions can often result in differential regulation of metabolites, as compared to each of these species growing alone (Beveridge et al., 1997; Fleming et al., 2016). Phototrophic biofilms are considered of immense significance both in agriculture and environmental management, due to their ecological roles in nutrient sequestration, mobilization and remediation (Roeselers et al., 2008; Bharti et al., 2017). Metabolites are the ‘response factors’ that bring about phenotypic changes to counteract the genetic and environmental perturbations, either biotic or abiotic (Hu et al., 2016). In this present study, untargeted metabolite profiling using gas chromatography- mass spectroscopy showed significant metabolite changes in the An versus cyanobacterial biofilms, An-Tr and An-PW5 which was supported by heat map and cluster analysis, delineating a separate group based on the known metabolites identified. An-Tr showed highest concentrations in terms of sugars and their conjugates of 66.85%, compared to An (26.20%), and Tr (32.99%), however, the number of sugars decreased from 41 (Tr) to 31 (An-Tr). Even though, An-PW5 exhibited lower percent sugars, the numbers were almost same in all three, An (25), PW5 (22), An-PW5 (24).

Untargeted comparative metabolite profiling was preferred over...
targeted metabolomics to identify multiple pathways in the targeted organism (this study- *A. torulosa*) that are affected by inoculation and coculturing with fungal -Tr, and bacterial -PW5 partners, as against other abiotic/biotic stresses reported in earlier studies (Jin et al., 2022).

Various studies have been undertaken to understand the influence of microbial metabolites in improving plant growth. Combes-Meynet et al. (2011) showed that 2,4-Diacetylphloroglucinol (DAPG), a secondary metabolite produced by *Pseudomonas fluorescens* F114 strain shows phytostimulatory effects on wheat, as supported by the upregulation of the phytostimulation genes (*ppdC, flgE, nirK, and nifX-nifB*) of *Azospirillum brasilense* Sp245-Rif on wheat rhizoplane. The metabolites released by plant growth promoting rhizobacteria, *Azospirillum, Alcaligenes, Burkholderia, Pseudomonas, Streptomyces*, and *Rhizobium* also help the plant to withstand adverse environmental conditions, including salinity (Bharti et al., 2016; Liu et al., 2017).

In this study, based on the enrichment pathway analysis, it is evident that the inoculation of Tr and PW5 to the cyanobacterial biofilms altered the metabolic pathways, including those involved in amino acid and carbohydrate metabolism. These can facilitate better translocation, uptake of nutrients and help to improve plant vigor, and prove better in terms of their biofertilization and plant growth promotion potential as inoculants. It is well established that in phototrophic partner is involved in intercellular signaling, aggregation, carbohydrate and amino acid metabolism (Bharti et al., 2017), and studies on cyanobacterial

### Table 1

| Metabolites | An-Tr | VIP | log2(An-Tr/An) | An-PW5 | VIP | log2(An-PW5/An) |
|-------------|-------|-----|---------------|--------|-----|----------------|
| Sugars      |       |     |               |        |     |                |
| D-(+)-Galactopyranose | 1.10  | 2.50 | 1.09 2.14    |        |     |                |
| D- (+)-Galactose   | 1.10  | -2.37 | 1.10 -2.37 |        |     |                |
| D-Glucose         | 1.10  | -3.14 | 1.10 -3.38 |        |     |                |
| D-Mannitol        | 1.10  | 5.84  | 1.10 3.66    |        |     |                |
| D-Mannose         | 1.09  | -2.46  | 1.10 2.08  |        |     |                |
| D-Trehalose       | 1.04  | -2.69  | 1.03 -2.69  |        |     |                |
| Maltose           | 1.10  | 5.03  | 1.10 2.81    |        |     |                |
| Sucrose           | 1.10  | -5.58  | 1.10 -4.31  |        |     |                |
| Amino acids      |       |     |               |        |     |                |
| Ala-beta-Ala     | 1.10  | -2.39  | c           |        |     |                |
| Glycine          | 1.10  | -3.73  | c           |        |     |                |
| Homoserine       | 1.10  | -2.34  | c           |        |     |                |
| L-5-Oxoproline   | 1.10  | d      | 1.10 -5.35  |        |     |                |
| L-Alanine        | 1.10  | -4.27  | c           |        |     |                |
| L-Aspartic acid  | 1.10  | -2.93  | c           |        |     |                |
| L-Glutamic acid  | 1.10  | -1.98  | c           |        |     |                |
| L-Glutamine      | 1.10  | -2.40  | c           |        |     |                |
| L-Isoleucine     | 1.10  | -3.59  | c           |        |     |                |
| L-Leucine        | 1.10  | -2.33  | c           |        |     |                |
| L-Lysine         | 1.09  | -2.98  | c           |        |     |                |
| L-Ornithine      | 1.10  | -2.40  | c           |        |     |                |
| L-Phenylalanine  | 1.10  | -2.35  | c           |        |     |                |
| L-Proline        | 1.10  | -4.26  | c           |        |     |                |
| L-Serine         | 1.10  | -2.15  | c           |        |     |                |
| L-Threonine      | 1.10  | -3.45  | c           |        |     |                |
| L-Tryptophan     | 1.10  | -2.40  | c           |        |     |                |
| L-Tyrosine       | 1.10  | -2.59  | c           |        |     |                |
| L-Valine         | 1.10  | -4.49  | c           |        |     |                |
| Organic acids   |       |     |               |        |     |                |
| 2-Butanediolic acid | 1.04  | 2.70  | 1.08 -1.84  |        |     |                |
| 2-Ketoisocapric acid | 1.10  | -2.89  | 1.36        |        |     |                |
| 2-Pentanediolic acid | 1.10  | -2.43  | 1.10 -2.43  |        |     |                |
| 3-Hydroxybutyric acid | 1.09  | -2.32  | 1.10 1.03  |        |     |                |
| 4-Aminobutanonic acid | 1.08  | -2.52  | 1.10 1.45  |        |     |                |
| S-Aminovaleric acid | 1.08  | -1.59  | c           |        |     |                |
| S-Hydroxysalicylic acid | 1.08  | -1.83  | 1.10 1.37  |        |     |                |
| D-Gluconic acid  | 1.07  | 2.59  | 1.08 d      |        |     |                |
| Oxalic acid      | 1.10  | 2.20  | c           |        |     |                |

**a,b** Fold changes in the metabolite concentration of An-Tr and An-PW5 against *A. torulosa* (An).

**c** VIP (Variable Importance in the Projection) score less than 1.

**d** Fold change threshold value less than 2.

![Fig. 3. Partial least square-discriminate analysis (PLS-DA) showing top 15 hits for A. An-Tr, and B. An-PW5 biofilms respectively. C Heat map and cluster analysis of all cultures, An, Tr, PW5, An-Tr, and An-PW5.](image-url)
metabolome have gained attention because of their biotechnological applications in discovering unknown novel compounds (Ferreira et al., 2021; Shahid et al., 2022), and dissecting metabolic fluxes in such biofilms. Development of biofilms is known to lead to several orchestrated changes in gene expression, regulation in the metabolic pathways, leading to the production of metabolites differentially in both space and time (Velmourougane et al., 2017a).

Cyanobacteria release several metabolites rich in carbon, and nitrogen which stimulate the growth of plants and modulate the C–N status of soil, as documented across several crops (Prasanna et al., 2014; Bharti et al., 2021, 2021b; Kokila et al., 2022). Their application as cyanobacterial biofilms based biofertilizers in agriculture has been evaluated in various crops such as rice, wheat, maize, flowers and vegetables towards improving soil fertility and macro- and micro-nutrient enrichment in produce, particularly biofortification to improve iron and zinc content in grains/produce (Abuye and Achamo, 2016; Shahane et al., 2020a; Sharma et al., 2021).

Metabolomic studies in cyanobacteria illustrating the total metabolite pool and its change, by co-culturing helps in the greater understanding of cellular metabolite functions (Kultschar et al., 2019; Kato et al., 2022). A. torulosa (An) used in this study is a promising cyanobacterium which can utilize sugars, amino acids such as ribose, citrate, phenylalanine and development of biofilms with agriculturally useful bacteria such as Azotobacter chroococcum, Mesorhizobium ciceri and Pseudomonas striata led to utilization of new saccharides (Prasanna et al., 2011). This study showed lower D-glucose, D-mannose, galactose, glucopyranose and fructose concentration in An-Tr biofilm indicating reduction in the main CO$_2$ fixation and perhaps use of alternate pathways, glyoxylate and dicarboxylate pathway for the synthesis of ATP and NADPH$_2$ for sustained metabolism. The glyoxylate shunt is well known for serving as an alternate route within TCA in aerobic bacteria, which bypasses the NADH producing steps, with the primary function to circumvent the carbon dioxide (CO$_2$) production step within the TCA cycle. This helps to drive the metabolism of fatty-acids or two carbon-compounds, e.g. acetate towards the production of oxaloacetate, and thereby serve as a precursor for gluconeogenesis. This is also evidenced with significantly high production of oxalic acid, which is a product of the glyoxalate shunt, in An-Tr biofilm. Interestingly, Koedooder et al.

Fig. 4. Pathway enrichment analyses of cyanobacterial biofilms- A. An-Tr B. An-PW5, and C. An culture alone.
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chlorophyll content (4.62 µg ml⁻¹) in 2021. In addition, oxalic acid also known to play an important role in analyses to decipher their significance in our previous study on Fe biofortification in maize kernels (Nishanth et al., 2021a; Sharma et al., 2019). Previous studies on An-Tr biofilm showed an increase in the regulation of bacterial-fungal interactions as reported by Deveau et al. (2018). Previous studies on An-Tr biofilm showed an increase in the regulation of glyoxylate shunt, being of ubiquitous nature, can serve as an important acclimation strategy in bacteria growing under Fe-limitation. This aspect needs more in-depth examination, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Velmourougane et al. (Velmo, 2017b) have shown that in association with An, increased carbon fixation through alternative pathways (such as the glyoxylate shunt) were utilized to generate ATP and NADPH₂ as 3-glucose and 3-fructose were lower. The modulation in the metabolite profiles towards amino acids, as compared to PW5 revealed greater influence of An, while in terms of fatty acids such as stearic and palmitic acids along with malic acid depicts its influence on PW5; overall, a beneficial balance among the partners’ functional attributes. To the best of our knowledge, this is the first study that deals with the modulation of metabolites in cyanobacteria by the introduction of an agriculturally beneficial bacterium or fungus as partner during biofilm formation. This study can serve as a foundation for focused metabolomics of cyanobacterial biofilm research towards their wider industrial applications (Almendinger et al., 2021) and utilization as an inoculant, across crops and ecologies (Alvarez et al., 2021).

5. Conclusion

In summary, an untargeted GC-MS workflow demonstrated the dynamic metabolite changes in laboratory developed cyanobacterial biofilms-An-Tr and An-PW5, which are potential biofertilizers and plant-growth promoting agents. Addition of Tr and PW5 to the cyanobacterial partner showed a significant reduction in sugars like 3-glucose, 3-fructose in both the biofilms. An-Tr biofilm decreased the concentration of most amino acids, except 5-oxoproline. Whereas An-PW5 improved in three amino acids such as serine, ornithine and leucine. Heat map and cluster analysis also showed clear-cut changes in metabolites among cyanobacterial biofilms and their individual partners. The enrichment pathway analysis demonstrated only a few significant changes in the pathway hits in An-Tr against An, illustrative of mutual distinction of functions among the partners. An-PW5 showed a change in pathways including alanine, aspartate and glutamate metabolism, glutathione metabolism, cyanobacterial metabolism, glycolysis/glucogenesis, and amino sugar and nucleotide sugar metabolism. Novel information regarding the metabolic machinery of biofilms vis a vis partners was generated which can help to undertake further analyses using targeted approaches and developing function-based inoculants.

Declarations

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Table 2

Pathway analysis illustrating the differences in the enriched metabolites in the profiles of An, An-Tr and An-PW5.

| Pathway                                      | An-Tr | An   | An-PW5 |
|----------------------------------------------|-------|------|-------|
| Aminoacyl-tRNA biosynthesis                  | 10    | 14   | 14    |
| Glycolysis / Gluconeogenesis                 | 0.00021 | 7.18E-06 | 7.18E-06 |
| Pentose phosphate pathway                    | 3     | 4    | 4     |
| Adi value                                    | 0.02608 | 0.011059 | 0.011059 |
| Arginine biosynthesis                        | 3     | 4    | 4     |
| Adi value                                    | 0.23732 | 0.03359 | 0.03359 |
| Alanine, aspartate and glutamate metabolism  | 3     | 4    | 5     |
| Adi value                                    | 0.06031 | 0.03539 | 0.00632 |
| Glutathione metabolism                       | 3     | 4    | 5     |
| Adi value                                    | 0.12163 | 0.08585 | 0.02318 |
| D-Glutamine and α-glutamate metabolism       | 1     | 2    | 2     |
| Adi value                                    | 0.36241 | 0.11634 | 0.11634 |
| Nitrogen metabolism                          | 1     | 2    | 2     |
| Adi value                                    | 0.47490 | 0.21118 | 0.21118 |
| Citrate cycle (TCA cycle)                    | 1     | 2    | 1     |
| Adi value                                    | 0.64464 | 0.40892 | 0.76761 |
| Cyanoamine acid metabolism                   | 2     | 2    | 3     |
| Adi value                                    | 0.37874 | 0.55508 | 0.26958 |
| Amino sugar and nucleotide sugar metabolism  | 1     | 1    | 2     |
| Adi value                                    | 0.70799 | 0.82384 | 0.82384 |
| Fatty acid biosynthesis                      | 1     | 1    | 1     |
| Adi value                                    | 0.85878 | 0.93679 | 0.75033 |

(2018) were able to show that the regulation of glyoxylate shunt, being of ubiquitous nature, can serve as an important acclimation strategy in bacteria growing under Fe-limitation. This aspect needs more in-depth analyses to decipher their significance in our previous study on Fe biofortification in maize kernels (Nishanth et al., 2021a; Sharma et al., 2021). In addition, oxalic acid also known to play an important role in the regulation of bacterial-fungal interactions as reported by Deveau et al. (2018). Previous studies on An-Tr biofilm showed an increase in chlorophyll content (4.62 µg ml⁻¹ culture) after 4 weeks of coculturing, and other parameters including indole acetic acid, exopolysaccharides, and glomalin related proteins, by 25%, 26%, and 62% respectively over An alone (Sharma et al., 2020). Similarly, An-PW5 also showed an improvement on growth related parameters compared to An alone (data unpublished).

T. viride (Tr) is a free-living fungus, with application as a source of enzymes, besides use in crop protection and disease management strategies globally (Harman et al., 2004). Their beneficial role in plant growth and development is attributed to their ability to elicit resistance through induction of defense responses, thereby improving crop productivity. Plant colonization is often mediated through small effector molecules, including proteins, secondary metabolites, and small RNAs, and transcriptomic profiling of biofilm formation with Azotobacter sp. illustrated significant changes in the CHO metabolism, particularly, upregulation of glucose-6-phosphate dehydrogenase (Velmourougane et al., 2019). In the present investigation, An-Tr recorded higher maltose, lactose, galactopyranose and citric acid, as compared to An alone; additionally changes in the carbohydrate, protein, uronic acid, acetyl levels in the EPS, as observed during biofilm formation are known to influence its biological activity (Velmourougane et al., 2017b).

Providencia sp. (PW5) is a bacterial strain with multifarious PGP traits, including catalase activity, indole utilization, phosphate solubilization, nitrogen fixation, IAA production and biocontrol-related attributes (Rana et al., 2011). It is known to exhibit citrate utilization, hydrolyosis of gelatin, casein and starch, besides nitrate reduction and in the present study, stearic and myristic acid were significantly higher in PW5. The An-PW5 biofilm exhibited increased α-mannose, α-glucopyranose, lactose, α-galactopyranose, oxalic acid and malic acid which suggests that in association with An, increased carbon fixation through alternative pathways (such as the glyoxylate shunt) were utilized to generate ATP and NADPH₂ as 3-glucose and 3-fructose were lower.
Availability of data and material
Metabolite profiles and corresponding metadata are available from the Metabolights repository (Haug et al., 2020) under the accession number MTBLS3528 (https://www.ebi.ac.uk/metabolights/MTBLS3528).

Code availability
None

CRediT authorship contribution statement

Sekar Nishanth: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft. Radha Prasanna: Methodology, Resources, Supervision, Formal analysis, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Radha Prasanna reports financial support provided by Indian Council of Agricultural Research (ICAR) through the Network Project on Microorganisms “Application of Microorganisms in Agricultural and Allied Sectors” (AMMA).

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Data Availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.crmicr.2022.100174.

References

Abuye, F., Achamo, B., 2016. Potential use of cyanobacterial bio-fertilizer on growth of tomato yield components and nutritional quality on grown soils contrasting pH. J. Biol. Ed. 6, 54–62.
Adak, A., Prasanna, R., Biswas, S., Bidyarani, N., Verma, S., Pal, M., Shivay, Y.S., Nain, L., 2016. Micronutrient enrichment mediated by plant-microbe interactions and rice cultivation practices. J. Plant Nutr. 39, 1216–1232. https://doi.org/10.1080/01904167.2016.1134872.
Alawiye, T.T., Babalola, O.O., 2021. Metabolomics: current application and prospects in crop production. Biologia 76, 227–239. https://doi.org/10.1007/s11756-020-00574-z.
Almendinger, M., Saiffrank, F., Rohn, S., Kurth, E., Pleissner, D., 2021. Characterization of selected microalgae and cyanobacteria as sources of compounds with antioxidant capacity. Algal Res 53, 102168.
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Kokila, V., Prasanna, V., Kumar, A., Nishanth, S., Shukla, J., Gulia, U., Nain, L., Shivay, Y. S., Singh, A.K., 2022. Cyanobacterial inoculation in elevated CO2 environment stimulates C enrichment and plant growth of tomato. Environ. Technol. Innov. 26, 102234. https://doi.org/10.1016/j.eti.2021.102234.

Kultschar, B., 2020. Metabolite Profiling of a robust cyanobacterium for industrial biotechnology (Doctoral dissertation, Swansea University). https://doi.org/10.23889/Slitheth.1905.

Kultschar, B., Dudley, E., Wilson, S., Llewellyn, C.A., 2021. Response of key metabolites to FZB42 in elevated CO2 environment: metabolomics of the alkaliphilic cyanobacterium Plectonema boryanum elucidated. Front. Microbiol. 12, 646. https://doi.org/10.3389/fmicb.2021.646.

Kultschar, B., Dudley, E., Wilson, S., Llewellyn, C.A., 2021. Response of key metabolites to FZB42 in elevated CO2 environment: metabolomics of the alkaliphilic cyanobacterium Plectonema boryanum elucidated. Front. Microbiol. 12, 646. https://doi.org/10.3389/fmicb.2021.646.

Kultschar, B., Dudley, E., Wilson, S., Llewellyn, C.A., 2021. Response of key metabolites to FZB42 in elevated CO2 environment: metabolomics of the alkaliphilic cyanobacterium Plectonema boryanum elucidated. Front. Microbiol. 12, 646. https://doi.org/10.3389/fmicb.2021.646.

Kultschar, B., Dudley, E., Wilson, S., Llewellyn, C.A., 2021. Response of key metabolites to FZB42 in elevated CO2 environment: metabolomics of the alkaliphilic cyanobacterium Plectonema boryanum elucidated. Front. Microbiol. 12, 646. https://doi.org/10.3389/fmicb.2021.646.