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Growth and secretome analysis of possible synergistic interaction between green algae and cyanobacteria

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Synergistic coexistence of nitrogen fixing cyanobacteria such as *Anabaena variabilis*, *Nostoc muscorum* and *Westiellopsis prolifica* with green algae namely *Scenedesmus obliquus*, *Chlorella vulgaris* and *Botryococcus braunii* was studied under nitrogen deficient conditions. The effect of these interactions was investigated on growth, fixed nitrogen content, lipid content and their secretomes in individual cultures and cocultures. Based on the cocultivation studies, it was found that out of the nine interactions studied, *B. braunii*–*N. muscorum* synergism was best established. This interaction resulted in a maximum of 50% enhancement in nitrogen fixation in *B. braunii*–*N. muscorum* co-culture leading to 27% enhancement in lipid content (membrane and neutral lipid). In general, *B. braunii* co-cultures showed an enhancement in biomass content of up to 38%. Secretome analysis showed presence of new and modified secondary metabolites having roles in quorum sensing/quenching, interspecies signaling, N-fixation, carbon metabolism, lipid metabolism, antimicrobial activity. Compounds such as trichloroacetic acid and hexadecane were identified that are known to have roles in nitrogen assimilation and carbon metabolism, respectively, were present in some of the co-culture secretomes. The combination of *B. braunii*–*N. muscorum* led to the formation of new compounds such as triacontanol which have role in improvement of glucose-lipid metabolism and 9-octadecenamide that is known to be a phytohormone.

[**Key words:** Green algae; Cocultivation; Cyanobacteria; Fixed nitrogen; Lipid]

Oil reserves of the world are limited. However, the demand of oil is increasing with the rapid increase in population (1,2). Therefore, there is a need to find an alternative renewable source of fuels. Efforts are now aimed at the production of oil rich microalgae as microalgae utilize sunlight more efficiently and thus have higher photosynthetic efficiency than land plants (3–5).

Cyanobacteria have the ability to fix atmospheric nitrogen through special cells called heterocysts. Cyanobacteria and green algae are presently also cocultivated with microbes such as bacteria, fungi and more recently with algae because of their ability to produce some growth promoting substances that result in a symbiotic relationship (6). Earlier, many well-known studies have shown enhancement in growth, lipid, biomass and nitrogen fixation in a symbiotic relationship between nitrogen fixing bacteria and microalgae (7–10). Cyanobacteria provide several benefits to their hosts, e.g., metabolites fixed through photosynthesis, N-fixation, UV protection, defence against toxins (11). *Azolla–Anabaena azollae* is a well-known symbiotic association of cyanobacteria with a eukaryotic fern. Ray et al. (12) have studied the symbiotic association between two species and it was found that *Anabaena* cells were reduced in size however the activity of nitrogenase enzyme was enhanced. The physiological changes also indicated a possible genetic adaptation in the cyanobacteria.

Various studies of cocultivation between microalgae and cyanobacteria have also been performed strongly emphasizing the symbiotic association between the two organisms. Recently, Foster et al. (13) have reported nitrogen fixation and its 97% subsequent transfer by cyanobacteria to the diatoms in the open ocean. Cyanobacteria associated with diatoms was also observed to fix 60–70% more nitrogen over that require for its own use. This study was following another lesser known study carried out by Carpenter and Foster (14) between a pennate diatom *Climacodium frauenfeldianum* and a cyanobacteria. It was unequivocally assumed that the primary interaction between the two was due to the provision of nitrogen to diatom. Thompson et al. (8) recently reported a symbiotic association which involved the sharing of nitrogen fixed by a cyanobacteria and carbon fixed by a unicellular alga. The symbiotic association between cyanobacteria and alga showed an evolution in terms of a reduction in the cyanobacterial genome. Recently, Chen and Guo (15) have reported the inhibition effect of green algae on the cyanobacteria by interspecies interactions. Therien et al. (16) have studied the co-cultivation of acetate producing mutant strain *Synechococcus* sp. PCC 7002 with *Chlamydomonas reinhardtii*. It was demonstrated by these workers that blue green algae could support the growth of *C. reinhardtii* by supplying the acetate necessary for the lipid production.

In the present study, three green algal species namely *Chlorella vulgaris*, *Scenedesmus obliquus* and *Botryococcus braunii* were selected for co-culturing with cyanobacteria. Different combinations of green algal species and cyanobacteria were made for each

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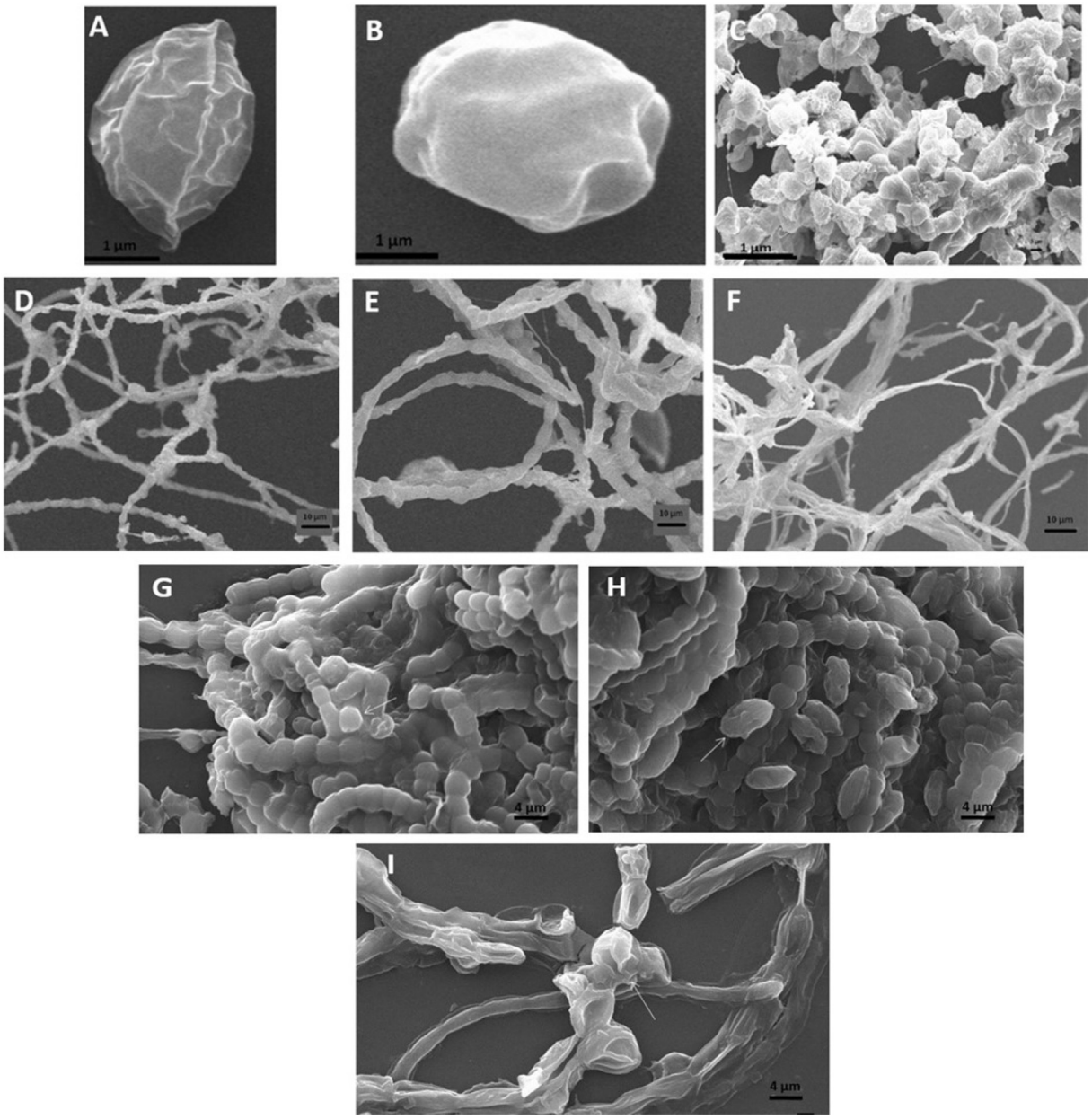

FIG. 1. The figure shows the SEM image of individual cultures *S. obliquus* (A), *C. vulgaris* (B), *B. braunii* (C), *A. variabilis* (D), *N. muscorum* (E) and *W. prolifica* (F) followed by images of *C. vulgaris*–*A. variabilis* (G), *S. obliquus*–*A. variabilis* (H) and *B. braunii*–*W. prolifica* (I) co-cultures that showed maximum nitrogen fixation with each of the green algae. The arrow shows the *S. obliquus*, *C. vulgaris* and *B. braunii* cells attached to their respective cyanobacterial filaments. The co-culture was lyophilized prior to coating with gold particles for viewing under SEM. The scale bar for individual green algal strains (*S. obliquus*, *C. vulgaris* and *B. braunii*) is 1 μm and that of individual cyanobacterial strains (*A. variabilis*, *N. muscorum* and *W. prolifica*) is 10 μm. Scale bar for the co-cultures is 4 μm. The white arrow in the co-cultures shows the green algal cells attached to the cyanobacterial filaments.

cocultivation experiment using the cyanobacteria strains such as *Anabaena variabilis*, *Nostoc muscorum* and *Westiellopsis prolifica* under nitrogen deficient conditions. As cyanobacteria are nitrogen fixers, nitrogen deficient conditions will allow more efficient N-fixation. The main objective is to understand if cyanobacteria fixes more nitrogen on cocultivation with green algae. The effect of cocultivation was studied on secretome, fixed nitrogen, lipid content and biomass production.

MATERIALS AND METHODS

Strains and growth conditions *S. obliquus* 276-1 and *C. vulgaris* 211-12 strains were procured from Experimental Phycology and Culture Collection of Algae (EPSAG), University of Goettingen, Germany. *B. braunii* UTEX LB572 strain was procured from University of Texas, TX, USA. *A. variabilis*, *N. muscorum* and *W. prolifica* were procured from National Facility for Conservation and Utilization of Blue Green Algae at Indian Institute of Agricultural Sciences, New Delhi, India. All the green algae and cyanobacteria cultures were maintained at pH 8 in Basal Medium/ES medium (recommended by EPSAG). The nitrogen deficient medium was prepared by omitting the KNO_3 component from the medium.

The cultures were grown under 2.5–2.7 K Lux of light intensity with 16:8 h light and dark regimes at 28°C temperature in a bioreactor. An inoculum (10%) of exponentially growing cells was used to inoculate the media for the individual cultures/control cultures of *S. obliquus*, *C. vulgaris* and *B. braunii*. The green algal inoculum was prewashed with nitrogen free medium to remove any traces of residual nitrogen. The cyanobacterial inoculum was grown in nitrogen free medium. The inoculum amount for the cocultivation was in the ratio of 1:1 on dry weight basis. The initial weight of the inoculum for all the individual and cocultures was 15–20 mg/L. The inoculum cells were prewashed with nitrogen deficient medium to remove all traces of nitrogen. Individual and co-cultures of *S. obliquus* and *C. vulgaris* were grown for 12 days and 15 days, respectively, with continuous shaking at 80–100 rpm. However, individual culture and co-cultures of *B. braunii* were grown

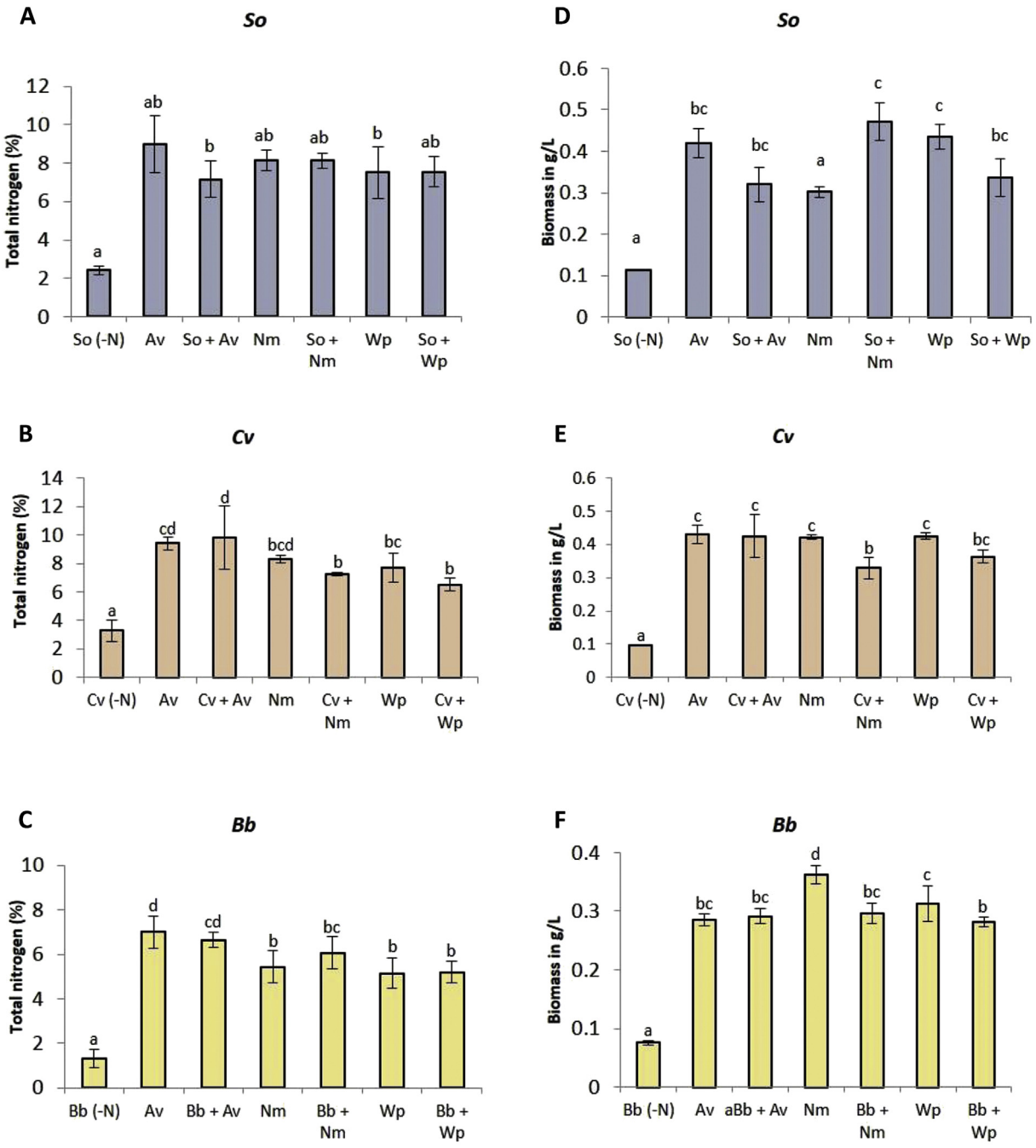

FIG. 2. (A–C) The total fixed nitrogen content in individual and co-cultures of *S. obliquus* (So) (A), *C. vulgaris* (Cv) (B) and *B. braunii* (Bb) (C). (D–F) The biomass content in individual and co-cultures of *S. obliquus* (So) (D), *C. vulgaris* (Cv) (E) and *B. braunii* (Bb) (F). Biomass content of *A. variabilis* (Av), *N. muscorum* (Nm) and *W. prolifica* (Wp) under the three conditions, i.e., 12 days, continuous shaking, 15 days, continuous shaking and 24 days, manual shaking twice a day was determined for comparison with their co-cultures. Data points represent the average values of the samples; ± standard deviation. –N stands for nitrogen deficient growth medium. All values are average of the three determinations. Means in a particular colour bar for each type of sample marked with different letters/digits differ significantly at $p < 0.05$.

for 24 days with gentle manual shaking twice a day. The harvesting time was optimized for each alga on the basis of their maximum growth rate, i.e., stationary phase of growth. Continuous shaking was not used in case of *B. braunii* individual and co-cultures as it disturbs colony formation and resulting in decreased lipid accumulation. The individual cyanobacterial cultures for each green alga were also cultivated and harvested according to the conditions mentioned for the corresponding green algae for the purpose of comparison.

Biomass estimation Biomass was estimated using the method given by Gautam et al. (17). A culture volume of 150 mL was centrifuged in a centrifuge tube to form a tight pellet. The tube containing the pellet was loosely capped and lyophilized for 24 h. The dried pellet was weighed immediately and stored in deep freezer (manufactured by Dairei Europe, Esbjerg, Ribe, Denmark) at −70°C for oil extraction.

Total fixed nitrogen estimation The culture was harvested on the 12th day in case of *S. obliquus*, 15th day in case of *C. vulgaris* of growth by centrifugation and on 24th day for *B. braunii*. The pellet was lyophilized and used for estimation of fixed nitrogen using Kjeldahl method (18). The lyophilized pellet was weighed and put in the Kjeldahl apparatus with the digestion mixture. The sample was digested at 350°C for about 2–3 h in the digester. The samples were diluted 100 times from which 0.1 mL sample was used for colour development. The absorbance was recorded at 650 nm and compared against standard curve for nitrogen prepared using the same method.

Total lipid estimation The total lipid content was estimated using the method reported by Gautam et al. (17). The algal pellet after the estimation of biomass was crushed in a mortar and pestle. The biomass was homogenized in hexane for 15 min. The homogenized biomass with the solvent was centrifuged in a falcon tube and the supernatant was collected in glass vials. The biomass was extracted in hexane thrice and all the supernatant was pooled in the glass vial. The hexane extracted biomass was further extracted with chloroform-methanol (C–M) (2:1) in the second step by homogenizing it in mortar and pestle. The homogenized biomass with the solvent was centrifuged and the supernatant was collected in another glass vial. The supernatant is evaporated using rotatory vacuum evaporator in a round bottom flask. The oil is then transferred to pre-weighed vials using a small amount of solvent. The lipid sample is further dried in oven set at 50°C. The hexane and C-M fractions were transferred to pre-weighed glass vials. The oil samples were completely dried in desiccator and weighed to obtain the weight of the oil fractions in grams. All estimations were performed on three independent replicates and are shown as the average of the triplicates.

Extraction and gas chromatography–mass spectometry analysis of secondary metabolites The spent media of the individual green algal cultures, individual cyanobacterial cultures and cocultures of green algae and cyanobacteria was extracted with ethyl acetate. The spent media was mixed with ethyl acetate in the ratio of 1:1 (19). The mixture was shaken vigorously for 5 min in a separating funnel and left undisturbed to allow separation of the ethyl acetate layer. The spent media was extracted thrice using fresh ethyl acetate solvent. The ethyl acetate layer of all the extractions was pooled and the solvent was evaporated using rotatory vacuum evaporator. The extract was stored at 4°C till analysis and dissolved in HPLC grade chloroform just before analysis. An injection volume of 2 μL was used for the analysis using gas chromatography-mass spectrometry (GC–MS) (GCMS-QP2010 Plus, Shimadzu, Kyoto, Japan) with Restex column. The oven temperature was kept at 80°C for 3 min and then heated up to 250°C at the rate of 8°C/min with the holding time of 5 min and further heated up to 320°C at the rate of 15°C/min for 11 min. The peaks were identified based on the best match using NIST and WILEY libraries. The secondary metabolites produced in the individual and cocultures were compared to identify new secondary metabolites produced as a result of cocultivation.

Calculations All the above parameters were determined for a fixed amount of culture/biomass in the individual and cocultivation cultures. The relative percentage increase or decrease in a parameter was calculated using the calculations given below.

$$\text{Percent relative increase/decrease, } R = \frac{\left[\{P_{GA+CY}\} - \frac{(P_{GA}+P_{CY})}{2}\right]}{\frac{(P_{GA}+P_{CY})}{2}} \times 100 \qquad (1)$$

where P_{GA}, P_{CY} and P_{GA+CY} are the values of a parameter (biomass/lipid/nitrogen) in green algal individual culture, cyanobacterial individual culture and green algal-cyanobacterial co-culture, respectively. The percentage relative increase/decrease for biomass content, nitrogen content and lipid content in the coculture as compared to individual cultures is calculated and reported using Eq. 1.

Statistical analysis All the estimations were performed in triplicates and are expressed in the form of the average value ±standard deviation. The significant difference between the mean values of the co-cultures with their respective controls were assessed by one way analysis of variance (ANOVA). Post hoc (Tukey) test with Duncan's test algorithms was carried out using SPSS 16.0 software to determine whether there was any significant difference at the level of $p < 0.05$.

RESULTS AND DISCUSSION

The three green algal strains were cocultivated with various cyanobacterial strains by selecting one of these separately for each experiment as described earlier in the text. Fig. 1 shows the scanning electron microscopy (SEM) images of *S. obliquus*, *C. vulgaris*, *B. braunii*, *A. variabilis*, *N. muscorum* and *W. prolifica* in individual cultures. SEM image of the three co-cultures which showed the maximum nitrogen fixation in cocultivation with each of the green alga is also shown.

Effect on biomass content The biomass contents were estimated after the completion of the growth period optimized for each of the individual culture and co-culture. *S. obliquus* accumulated 0.11 g/L of biomass which was the highest among all green algae under nitrogen deficient conditions followed by 0.09 g/L in *C. vulgaris* and 0.07 g/L in *B. braunii*. Individual cultures and co-cultures of *S. obliquus* were cultivated for 12 days with continuous shaking conditions. The biomass accumulation in individual culture of cyanobacteria produced variable amounts of biomass under the three different growth periods used for the three green algae. Biomass accumulation in cyanobacteria under condition favouring growth of *B. braunii* was relatively low as compared to that under continuous shaking conditions. Cyanobacteria produced maximum biomass after 15 days of growth under continuous shaking favourable for growth of *C. vulgaris*. In case of cocultivation with *S. obliquus*, *S. obliquus–N. muscorum* produced 0.47 g/L of biomass that showed significant maximum increase as compared to all the co-cultures (Fig. 2D–F). Cocultivation of *S. obliquus* with *A. variabilis*, *N. muscorum* and *W. prolifica* produced 52.4%, 135% and 50% increase, respectively. *C. vulgaris* co-cultures produced maximum biomass of 0.42 g/L with *C. vulgaris–A. variabilis* followed by 0.36 g/L and 0.32 g/L in *C. vulgaris–W. prolifica* and *C. vulgaris–N. muscorum*, respectively. The increase in biomass production of *C. vulgaris* cocultivation with *A. variabilis*, *N. muscorum* and *W. prolifica* as found to be in 61.5%, 28% and 44%, respectively. However, significant increase was obtained only in case of *C. vulgaris–W. prolifica* co-culture. Cocultivation of *B. braunii* with cyanobacteria yielded maximum biomass of 0.29 g/L with both *A. variabilis* and *N. muscorum*. Cocultivation of *B. braunii* with *A. variabilis*, *N. muscorum* and *W. prolifica* resulted in the increased biomass production by 70.6%, 38% and 55.6%, respectively, as compared to individual culture. Increase in biomass was significant in *B. braunii–A. variabilis* and *B. braunii–N. muscorum* co-cultures. There are not any well-known studies of green algal-cyanobacterial symbiosis to support these results, however, a lot of literature is available on microalgal and nitrogen fixing bacterial symbiotic association showing the enhancement of biomass in co-cultures (20–22). Hernandez et al. (20) have reported that *C. vulgaris* shows stimulated growth in the symbiotic association with nitrogen fixing bacterium *Bacillus pumilus*. It was interestingly concluded that the increase in the growth was not due to enhanced uptake of nutrients from the medium but by using the nitrogen fixed by the bacterium. Enhancement in growth and fatty acid content was also reported in case of *B. braunii* in the presence of novel bacterium, *Rhizobium* sp. (23). In another cocultivation study by Xue et al. (24), *C. vulgaris* and symbiotic bacterium, *Stenotrophomona smaltophilia* showed enhancement in biomass specific growth rate and productivity by 21.9%, 20.4% and 18%. Significant increase in lipid productivity by 8.2–33.83% was also recorded wherein saturated fatty acids and oleic acid contents showed maximum improvement. In a study of symbiotic association between green algae and nitrogen fixing bacteria, *C. vulgaris* co-cultured with *Azospirillum brasiliense* showed significant increase in starch and carbohydrate content of *C. vulgaris* as compared to immobilized algal cells alone (25).

Effect on total fixed nitrogen content A comparison in the nitrogen fixing capacity of the individual cyanobacterial culture systems with the cocultivated algal culture system was performed. Interestingly, all the mixed algal culture systems exhibited enhanced nitrogen fixation as compared to their respective individual cultures. In case of mixed cyanobacterial cultures with *S. obliquus*, it was seen that maximum increase in fixed nitrogen was found with *N. muscorum* (34.8%) and *W. prolifica* (34.4%). However, in case of *S. obliquus–A. variabilis* culture the fixed nitrogen increased by 16%. Mixed cultures with *C. vulgaris* showed a different trend, wherein the maximum increase in fixed nitrogen was found in *C. vulgaris–A. variabilis* culture (37%). The increase in fixed nitrogen in *C. vulgaris–N. muscorum* and *C. vulgaris–W. prolifica* was found to be 17% and 13%, respectively (Fig. 2A–C). The nitrogen fixation capacity of cyanobacteria on cocultivation with *B. braunii* resulted in a completely different trend. The maximum increase in the amount of nitrogen fixation was found to be 50% in case of cocultivation with *N. muscorum*. The increase in fixed nitrogen in case of *A. variabilis* and *W. prolifica* was almost same at 38%. It can thus be understood that in case of nitrogen fixation *N. muscorum* and *W. prolifica* show a better association with *S. obliquus*, however *C. vulgaris* exhibits a better

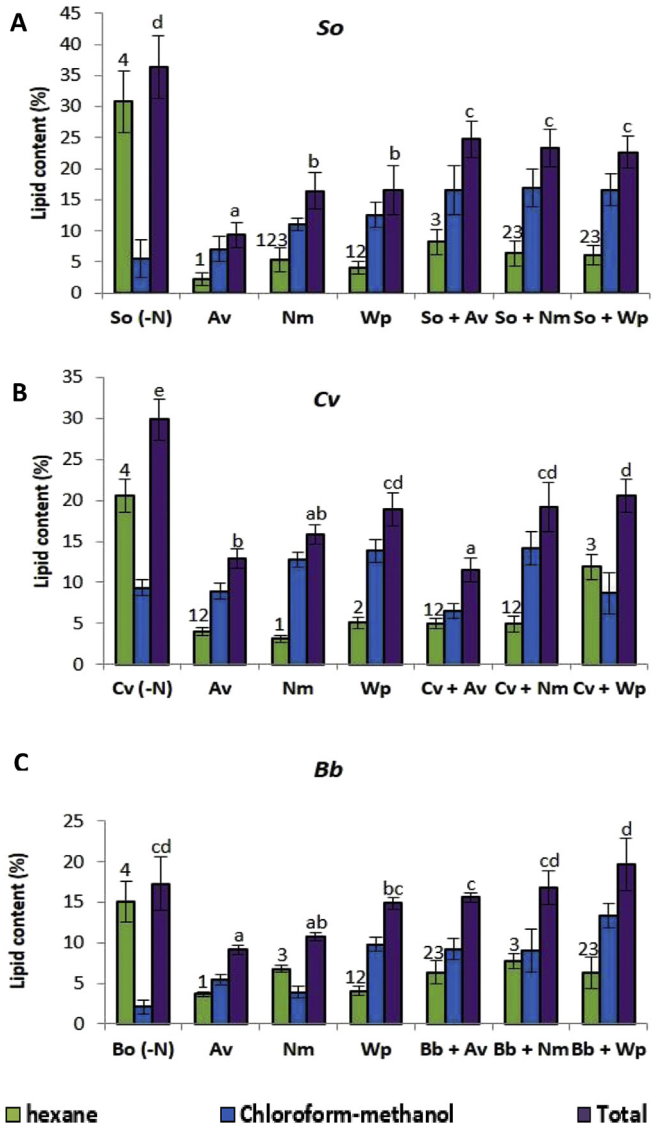

FIG. 3. The percentage of lipid content in individual cultures and co-cultures of *S. obliquus* (So) (A), *C. vulgaris* (Cv) (B) and *B. braunii* (Bb) (C). *S. obliquus*, *C. vulgaris* and *B. braunii* biomass were harvested on 12th day, 15th day and 24th day, respectively. The lipid content was extracted from ≈100 mg of lyophilized algal biomass. Data points represent the average values of the samples ±standard deviation. All values are average of three determinations. Means in a particular colour bar for each type of sample marked with different letters/digits differ significantly at $p < 0.05$.

association with *A. variabilis*. The maximum significant increase in fixed nitrogen content out of all the individual and cocultures was observed in case of *B. braunii* with *N. muscorum*. This could be interesting as *B. braunii* is a promising hydrocarbon yielding green alga having a slow growth rate. Availability of naturally fixed nitrogen through cyanobacteria may help in boosting its growth without adding external nitrogenous nutrients, thus saving the cost by avoiding a nitrogen nutrient material in the culture. Present studies have shown that cyanobacteria fixes more nitrogen in the presence of green algae (when no external nitrogen source is supplied). Interaction between the green algae and cyanobacteria seems to be cometabolic and synergistic. There is plenty of literature on cocultivation especially by Foster et al. (13) and Carpenter and Foster (14), clearly emphasizing the enhancement of nitrogen fixation in the present of microalgae. Foster et al. (13) have demonstrated 60—70% more nitrogen fixation by cyanobacteria *Richelia intracellularis* and *Calothrix rhizosoleniae*, and 97% of it being transferred to a diatom. Thompson et al. (9) described an evolutionary reduction in the cyanobacterial genome as a result of symbiotic association which involved the sharing of nitrogen fixed by a cyanobacteria and carbon fixed by a unicellular alga. Apart from algal-nitrogen fixing cyanobacterial cocultivations, there are numerous well studied algal-nitrogen fixing bacterial cocultivations which support similar results. Gyurjan et al. (26) showed the symbiotic growth of *C. reinhardtii* and nitrogen fixing cyanobacteria, *Azotobacter* in nitrogen and carbon free media. The energy supply for nitrogen fixation was corroborated by active photosynthesis of algal cells.

Effect on total lipid content Lipid content of the individual and cocultivated cultures was determined in the form of hexane extracted fraction, C-M extracted fraction as well total lipid content by adding these two extractions. It was found that *S. obliquus* under nitrogen deficient conditions produced maximum 32% of total lipids as 0.32 g/g of biomass (Fig. 3A). The hexane extracted fraction produced was 27% contributed mainly to the total lipid content of *S. obliquus* whereas the C-M extracted fraction was only 6%. Under 12 days of growth under continuous shaking conditions, *N. muscorum* and *W. prolifica* produced about 16% total lipids as compared to *A. variabilis* that produced only about 9% (Fig. 3A). The hexane extracted lipid content of the three cyanobacterial strains was found to be maximum in case of *N. muscorum* followed by *W. prolifica* and *A. variabilis*. The C-M extracted fraction in case of *W. prolifica* was found to be 13% followed by that of *N. muscorum* and *A. variabilis* at 11% and 7%, respectively. All the co-cultures produced almost similar amounts of C-M extracted fraction. *S. obliquus—A. variabilis* co-culture produced 8% followed by *S. obliquus—N. muscorum* (6%) and *S. obliquus—W. prolifica* (6%). A comparison was made to study if there was any significant increase in the lipid content under cocultivation conditions, and it was found that the C-M extracted fraction increased from 6% in individual culture to 17% in the co-culture, which shows a 161% increase in the case of co-culture. The total lipid content also increased from 23% in individual culture to 25% in case of co-culture, which is 8% increase in co-culture.

In case of *C. vulgaris*, under nitrogen deficient conditions, hexane and C-M extracted fractions were 21% and 9%, respectively, which showed a total lipid content of 30%. The individual cyanobacterial cultures produced 5%, 4%, and 3% hexane extracted fractions in *W. prolifica*, *A. variabilis* and *N. muscorum*, respectively (Fig. 3B). The amounts of C-M extracted fraction were quite similar in *W. prolifica* and *N. muscorum* at 14% and 13%, respectively, followed by 9% in *A. variabilis*. Therefore, the total lipid content was maximum in case of *W. prolifica* (19%) followed by *N. muscorum* (16%) and *A. variabilis* (13%) (Fig. 3B). In case of its cocultivation with cyanobacteria, *C. vulgaris—W. prolifica* produced maximum amount of hexane extracted fraction (12%) where *C. vulgaris—A. variabilis* and *C. vulgaris—N. muscorum* produced only around 5% of hexane extracted fraction. However, the maximum amount of C-M extracted fraction was produced in *C. vulgaris—N. muscorum* (14%) followed by *C. vulgaris—W. prolifica* (9%) and *C. vulgaris—A. variabilis* (7%). Hence, the maximum amount of total lipids were produced in *C. vulgaris—W. prolifica* at 21% which was quite similar to that in *C. vulgaris—N. muscorum* (19%) followed by *C. vulgaris—A. variabilis* at 12%. Comparison of lipid production in individual culture and co-culture showed that in all the co-culture the lipid content could not surpass the amount produced in individual culture.

However, *B. braunii* on cocultivation with *N. muscorum* and *W. prolifica* resulted in significant increase in the total lipid content. Under nitrogen deficient medium (*B. braunii*) produced of hexane fraction (15%), C-M extracted fraction (2%) and total lipid content (17%), respectively. The individual cyanobacterial cultures under 24

days of growth with gentle manual shaking twice a day produced 4%, 7% and 4% of hexane extracted fraction in *A. variabilis*, *N. muscorum* and *W. prolifica*, respectively (Fig. 3C). The C-M extracted fraction amount was quite variable in all the three cyanobacterial strains. Maximum amount of C-M extracted fraction was produced in *W. prolifica* (10%) followed by *A. variabilis* (6%) and *N. muscorum* (4%). *W. prolifica* produced 14% of total lipid followed by 11% in *N. muscorum* and 9% in *A. variabilis* (Fig. 3C). The co-culture of *B. braunii* produced maximum lipid content with *W. prolifica* with 6%, 13% and 20% of hexane fraction, C-M extracted fraction and total lipid content, respectively. *B. braunii*—*N. muscorum* produced maximum amount of hexane extracted fraction (8%) however, the C-M extracted fraction and total lipid content was 10% and 18%, respectively. *B. braunii*—*A. variabilis* produced least amount of lipids and hence the total lipid content produced was 16%. The results showed that maximum significant increase in lipid content and its different fraction out of all the green algae is observed in *B. braunii*. In all the cocultivations with *B. braunii*, both C-M fraction and total lipid content showed an increase except in the hexane extracted fraction. *B. braunii*—*A. variabilis* co-culture showed an increase of 144% and 20% in C-M extracted fraction and total lipid content, respectively. Similar increase were observed in case of *B. braunii*—*N. muscorum* and *B. braunii*—*W. prolifica* co-cultures where the C-M and total lipid content showed a significant increase of 222%, 124% and 27%, 28%, respectively. Fig. 3 shows the lipid production by different individual culture and co-culture in grams per gram of biomass produced. As a result, it could be observed that the cocultivation technique led to the enhancement of the C-M extractable fraction in the co-culture as compared to the individual culture. Similar results were obtained in the co-culture of two microalgae, *Chlorella* sp. U4341 and *Monoraphidium* sp. FXY-10 which resulted in enhanced lipid production. It was concluded that these results were essentially because of symbiotic association of the two algae under nitrogen deficient conditions (7). Another study of cocultivation of *C. vulgaris*/*Chlorella sorokiniana* with *Azospirillum brasilense* resulted in enhanced lipid productivity as well as chlorophyll, β-carotene and lutein contents (27—29).

Effect on secretome composition The comparison of secretome was essentially done by comparing the number of compounds identified in each individual and co-culture. Maximum number of compounds were identified in *B. braunii* followed by *C. vulgaris* and *S. obliquus*. *B. braunii* was cultivated for 24 days unlike other green algae which could be the reason for the presence of more number of secondary metabolites. Grossly, it was found that compounds such as flavanoids, polyphenols, terpenes, quinines, pyrones, alkanes, aldehydes, esters, acids and alcohols are produced in both individual and c-ocultures of green algae and cyanobacteria. The secondary metabolites so formed in the different types of interactions studied presently were mostly belonging to the category of semiochemicals/allelochemicals. It was also observed that compounds present in the cyanobacterial cultures were highly complex in nature with multiple functional groups and unsaturations unlike in the green algal cultures. The secondary metabolites from individual cultures of *S. obliquus*, *C. vulgaris* and the three cyanobacteria were extracted and compared to those produced in their respective co-cultures. The comparative GC—MS chromatographic analysis of secondary metabolites produced in the individual and co-cultures is given in Tables S1—S9.

The secretome of co-cultures were modified depending on the cyanobacterial strain it was co-cultured with indicating that this response is species specific. Table 1 shows the number of new compounds formed and number of compounds that became absent in the secretomes of the nine combinations. It was also observed that most of the cyanobacterial compound were found absent in the co-culture secretomes. This signifies that the dominance of green algal strains in the co-cultures during cometabolic interactions. Green algal metabolites species are utilizing the cyanobacteria metabolites during interactions. It was also found that the number of newly formed compounds were much lesser in comparison to the number of compounds that became absent or disappeared in the co-cultures. This could be because of the phenomenon of quorum quenching, where the green algae disrupts the quorum sensing in cyanobacteria. The compounds that got quenched were mostly belonging to the class of alkanes, esters, acids, and ketones. However, in *B.braunii*—*N. muscorum* co-culture, this dominance was not predominant as about 50% of the individual *B.braunii* and *N. muscorum* culture compounds were absent in the co-culture (Table 2). Such a cocultivation study was previously carried out by Dunkar et al. (30), who reported the contrasting effects of the cyanobacterium *Microcystis aeruginosa* on the growth and physiology of two green algae, *Oocystis marsonii* and *S. obliquus*. In one of the green alga, the cyanobacterium showed inhibitory effects whereas in *S. obliquus*, it did not show any effect.

As revealed by GC—MS analysis, the compounds such as hexanol, heptanone, tetradecane, decanone, octanol, decanol, pentadecane, hexadecane, heptadecane and palmitic acid were found to be present in all the cocultivations studied. Interestingly, these were also reportedly present in study carried out on symbiotic relationship between *Anabaena*—*Azolla* by Pereira et al. (31). However, some of the compounds were present in the form of minor modifications showing the transformations at the functional groups, such as, methyl or methylene. The cocultivation of green algae with cyanobacteria also led to increase in nitrogen fixation by cyanobacteria (13—50%) in the presence of green algae. Detection of trichloroacetic acid (0.2—1%) in all the co-cultures especially with *S. obliquus*—*A. variabilis* and *C. vulgaris*—*A. variabilis* co-culture showed that this would have helped in increasing the incorporation of nitrogen in amino acids and proteins (32). Dodecane present in *S. obliquus*—*A. variabilis* co-culture is also known to be used as substrate for nitrogen fixation by *Azospirillum* sp. (33). Further, the presence of certain chemicals such as hexadecane which is involved in regulation of central carbon metabolism and beta-oxidation of fatty acids, may be involved in the enhancement of lipid production and increase biomass growth during co-culture conditions (34). Plant growth

TABLE 1. Number of secondary metabolites produced in the various cocultures, the new compounds formed, and the compounds that became absent in the co-culture.

	Number of compounds in secretome		
	Compounds in individual/ co-culture secretome	New compounds in co-culture secretome	Compounds absent in co-culture secretome
Individual cultures			
S. obliquus	47	–	–
C. vulgaris	53	–	–
B. braunii	72	–	–
A. variabilis	28 (35[a])	–	–
N. muscorum	15 (70[a])	–	–
W. prolifica	26 (68[a])	–	–
Co-cultures			
S. obliquus—*A. variabilis*	50	20	55
S. obliquus—*N. muscorum*	50	11	27
S. obliquus—*W. prolifica*	52	21	38
C. vulgaris—*A. variabilis*	54	10	45
C. vulgaris—*N. muscorum*	55	6	29
C. vulgaris—*W. prolifica*	40	17	41
B. braunii—*A. variabilis*	63	28	70
B. braunii—*N. muscorum*	69	28	106
B. braunii—*W. prolifica*	84	45	109

[a] With growth conditions of *B. braunii* (24 days with manual shaking).

TABLE 2. Type of interaction on the basis of the secondary metabolites, lipid and fixed nitrogen in the co-cultures.

Co-cultures		Green algal secondary metabolites absent in co-cultures (%)	Cyanobacterial secondary metabolites absent in co-cultures (%)	Increase in amount of total lipids	Increase in amount of fixed nitrogen	Type of interaction (positive/negative)
Green algae	Cyanobacteria					
S. obliquus	*A. variabilis*	47	86	−	+	Negative
	N. muscorum	23	80	−	++	Negative (strong green algal dominance)
	W. prolifica	34	85	−	++	Negative
C. vulgaris	*A. variabilis*	18	83	−	++	Negative (strong green algal dominance)
	N. muscorum	53	87	−	+	Negative
	W. prolifica	60	93	−	+	Negative
B. braunii	*A. variabilis*	54	89	+	++	Positive
	N. muscorum	**56**	**54**	++	+++	**Positive (symbiotic)**
	W. prolifica	53	79	+	++	Positive

+ indicates increase and − indicates decrease. Number of + and − signs indicate the extent of increase or decrease. Bold letters highlight the green-algal cyanobacterial co-culture with maximum enhancement in lipid content and fixed nitrogen content.

TABLE 3. New secondary metabolites produced during the interaction of *B. braunii*–*N. muscorum*.

Chemical compounds	Amount (%)	Molecular mass	Molecular formula	Significance	References
1,30-Triacontanediol	0.11	454.8	$C_{30}H_{62}O_2$	Improves glucose lipid metabolism, antimicrobial, anti-inflammatory and analgesic	36,37
1H-Pyrazolo [4,3-E]1,2,4-triazine, 3-methoxy-	0.23	165.1	$C_5H_5N_5O$	Anti-inflammatory, anticoagulant, antibiotic	38
Hexadecane, 2-methyl-	0.34	240.4	$C_{17}H_{36}$	Antimicrobial	39
2-Propenoic acid, 3-(4-methoxyphenyl)-, 2-ethylhexyl ester	0.28	290.4	$C_{18}H_{26}O_3$	Antibacterial, antifungal and healing properties	40
3-Chloropropionic acid, octadecyl ester	0.1	346.9	$C_{21}H_{41}ClO_2$	Antibacterial, herbicidal,	41
4-hydroxy-4-isopropyl-1,5,5-trimethyl-2-hexynyl acetate	0.1	–	$C_{14}H_{24}O_3$	Defense response, phytohormone	42
5-Isopropenyl-3,6-dimethyl-6-vinyl-4,5,6,7-tetrahydro-1-benzofuran	0.62	216.3	$C_{15}H_{20}O$	Antimicrobial, antifungal, anti-inflammatory, antitumour activity	43
9-Hexadecenoic acid, eicosyl ester	0.29	534.9	$C_{36}H_{70}O_2$	Antimicrobial	44
9-Hexadecenoic acid, 9-octadecenyl ester	0.11	504.8	$C_{34}H_{64}O_2$	Antimicrobial	45
9-Octadecenamide	4.86	281	$C_{18}H_{35}NO$	Antimicrobial, anti-inflammatory, antioxidant and antifouling activities, phytohormone, sleep inducing lipid, produced in defense reactions in plants	46–48
Bis(dodecanamido)methane	1.92	410.6	$C_{45}H_{90}N_2O_2$	–	
Butane, 1-(2,2-dichloro-3,3-dimethylcyclopr)	0.22	691.2	$C_9H_{16}Cl_2$	–	
Cholesterol	0.34	386.6	$C_{27}H_{46}O$	Constituent of cell membrane, precursor for most steroid hormones	49
Cyclohexanone, 3,3,5-trimethyl-5-phenyl-	0.16	140.2	$C_{15}H_{20}O$	–	
Docosane	6.25	310.6	$C_{22}H_{46}$	Antimicrobial, antiparasitic, kairomone	50
Docosyl pentafluoropropionate	1.58	472.6	$C_{25}H_{45}F_5O_2$	–	
Hexanoic acid, 2-ethyl-, hexadecyl ester	0.75	368.6	$C_{24}H_{48}O_2$	Membrane stabilizer, energy storage molecule, nutrient	51
Nonadecyl acetate	0.31	326.5	$C_{21}H_{42}O_2$	–	
Octadecanoic acid, methyl ester	0.19	298.5	$C_{19}H_{38}O_2$	Antimicrobial	52
Pentacyclo-octacosa-dodecaene-tetrol	6.76	648.9	$C_{44}H_{56}O_4$	–	
Pentadecane, 8-hexyl-	1.36	296.5	$C_{21}H_{44}$	Antimicrobial, antioxidant	53
Propanoic acid, 2-methyl-3-[4-t-butyl]phenyl	0.06	220.3	$C_{14}H_{20}O_2$	–	
Silane, trichlorooctadecyl-	0.89	386.1	$C_{18}H_{37}Cl_3Si$	Antimicrobial	54
Squalene	0.25	410.7	$C_{30}H_{50}$	Antimicrobial, anticancer, antioxidant, drug carrier, detoxifier, skin hydrating, and emollient activities	55,56
Tetracosane, 2,6,10,15,19,23-hexamethyl-	0.19	422.8	$C_{30}H_{62}$	Antimicrobial	41
Triacontyl acetate	0.81	480.8	$C_{32}H_{64}O_2$	–	

promoters such as cyclohexane were also identified that may have resulted enhancement of growth rate of green algae (35).

The GC–MS chromatographic analysis of secretomes of all nine combinations was performed however, the result is shown only for *B. braunii*–*N. muscorum* as it showed the best result with respect to enhancement in biomass and fixed nitrogen content. In case of *B. braunii*–*N. muscorum*, a molecule namely 1,30-triacontanediol was identified which is known for its role in improving glucose-lipid metabolism. Further, 9-octadecenamide which was present at the concentration of 4.86% is known to be a plant hormone and also has role in plant defense. Table 3 gives the list of new metabolites identified in the secretome as a result of the cocultivation. The new secondary metabolites formed as a result of other cocultivations is given in Tables S10–S17. The increase in biomass and lipid content supports the action of these metabolites and helped in establishing a strong synergism between *B. braunii* and *N. muscorum*.

It was concluded that interactions between green algae and cyanobacteria are species specific. The secretome analysis is important to understand the type of interaction present between algae and cyanobacteria. The combination of *B. braunii* with *N. muscorum* was found to be the best as it resulted in accumulation of maximum lipid and fixed nitrogen content. The secretome also did not show dominance of any of the two species in co-culture conditions. Thus, the present studies have huge scope for further studies on enhancement of lipid accumulation in algae for production of biofuels.

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