Exploitation of siderophore producing bacteria *Acinetobacter soli* (MTCC-5918) for lipids and reactive oxygen species in *Chlorella variabilis* (ATCC- PTA 12198) through co-cultivation

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Research

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Abstract

Background

Increased iron uptake *via* siderophores triggers a series of physiological processes and generation of reactive oxygen species (ROS) which causes damage to proteins, lipids, carbohydrates, resulting into micro algal cell lysis. Moreover, there are reports mentioning oxidative stress is a mediator for increased lipid accumulation in microalgae. The main aim of this study is co-cultivation of the bacteria *Acinetobacter soli* (MTCC- 5918) and the microalgae *Chlorella variabilis* (ATCC-PTA 12198) under iron limiting conditions and the threshold value of iron that trigger oxidizing stress to microalgae. Further, the ROS generation in the microalgae *C. variabilis* was determined in terms of OH, SO₂ and H₂O₂ concentration in the cells while co-cultivation.

Results

The co-cultured biomass contains ($45.92 \pm 0.74\%$) lipid content which was about 21% higher than that of the axenically grown microalgae. Carbohydrate content also increased to 40% than that of the control culture. Oxidative stress is a mediator for increased lipid accumulation in microalgae. As growth inhibition triggered due to the generation of high ROS toxicity during iron deficiency an increase in concentration of OH and H₂O₂ content was observed. In iron sufficient medium ash content of co-cultivated microalgae showed 32% and in iron deficient medium showed 14.23% which shows 44% decrease of ash content. Our novel approach significantly outperforms the involvement of different reactive oxygen species (ROS) in induction and in regulation of chelator release from cells at adequate Fe supply, which is also affecting the growth, lipids, carbohydrates, proteins, pigments, etc.

Conclusions

The findings of the present study highlights that oxidative stress is a mediator for increased lipid accumulation in microalgae that simultaneously becomes an alternative strategy for the improvement of biofuel potential in *C. variabilis*. The study portrays the significance of co-cultivation of *A. soli* and *C. variabilis* induced oxidative stress (ROS generation) in microalgae caused due to higher uptake of iron *via* siderophore

Background

Iron is an essential element for all microorganisms as it is involved in multiple metabolic processes like respiration (contains ferredoxins, heme containing cytochromes) and key enzymatic reactions [1]. Besides these benefits, iron can also be hazardous to organisms because Fe²⁺ triggers Fenton/Haber-Weiss reaction that produces harmful reactive oxygen species (ROS) such as superoxide (O₂⁻), hydrogen peroxide (H₂O₂) and the highly destructive hydroxyl radical (OH) [2]. There are certain models that

showed that iron accumulation increased cell oxidants that triggered the early activation of redox-sensitivity with a stronger co-relation [3]. These ROS causes damage to [Fe–S] clusters, membrane lipid peroxidation, DNA damage, protein carbonylation, and Cys/Met-residue oxidation [4]. [5]Studied that the siderophore released by bacteria defends against oxidative stress and the protection was independent of iron availability. There are also studies where addition of iron stimulated the growth of *M.aeruginosa* and Chl-a production where 100 µM caused the dramatic oxidative stress in algal cells whereas in 10 µM it covered the cells. Adding to this microbial siderophores has also alleviated the metal induced oxidative stress in plants.

The strain *Chlorella variabilis* ATCC PTA 12198 is as an important feedstock for biofuel production owing to its higher biomass productivity, carbohydrate, lipid content, and also its capability to grow in several types of wastewater [7].It is important to choose microbial species like (bacteria, microalgae,etc.) with the aim of co-culture for growth promotion and lipid production and very recently, [8] developed sustainable process based on a mixed co-dominant culture of *Saccharomyces cerevisiae* and *Chlorella vulgaris*, in order to encourage the interaction of two different cultures, the medium design, culture conditions and inoculum ratio are very important which has been also discussed. In the present study *C. variabilis*(ATCC-PTA 12198) was co-cultivated with *Acinetobacter soli*(MTCC – 5918) [9]to check its growth and lipid enhancement. Specific quorum sensing signals are detected in *Acinetobacter* species that can interact with their host [10] microalgae and can be further evaluated to find out optimum growth period for higher biomass generation. Axenic cultures are important for high value products like nutraceuticals and pharmaceuticals, but for biofuel production axenic cultures are not essential, hence for improving the growth and lipid productivity co-cultivation strategies can be employed. Similar reports are present where *Mesorhizobium* and *Azospirillum* bacteria (*vice versa*) through nitrogen fixation and *Chlorella vulgaris* ATCC 13482 and *Pseudomonas* sp. interact each other to produce polysaccharises and exchange nutrients [11]. From our previous study [12] we found that *C. variabilis* was able to grow in the presence of *Idiomarina loihiensis* RS14 when inoculated in optimum conditions. But, with the increase of bacterial inoculum, microalgae growth started declining. Due to the extreme consumption of iron siderophore, it forms and destructs the ROS, a blanket term to collect relatively reduced O₂ containing molecules that possess peroxides (H₂O₂), superoxide (O₂⁻), and also free radicals (HO) [13]. There are many reports showing that increase in the metals can induce ROS and siderophores may increase the susceptibility of microorganisms to oxidative damage [14]. Iron influences in generating harmful oxygen radicals, hence, under oxidative stress conditions, iron management stimulates exposure to this compound.By favoring iron release, hydrolysis of the catechole iron complex assures its iron needs and also provides the cell with a molecule, exposed to hydroxyl groups. This molecule can scavenge radicals that reduces various oxidative stresses. Therefore, the present study addresses the question of whether the co-cultivated microalgae is under oxidative stress at higher concentration of siderophore production. Addition to this, experiments were conducted to check, whether lipid and carbohydrate contents are influenced by non-metal oxygen radical generator.

Results And Discussions

Effect of standard siderophore on ROS activity

Microalgae has the ability to activate several defence systems for scavenging the ROS that are generated in various cellular sections. During unfavourable conditions, the rate of ROS generation exceeds their scavenging rate, and causes oxidative damage to the cells [30]. This strategy was observed with respect to siderophore concentration. As siderophore has high affinity towards chelating iron, and iron is an important co-factor in ROS generation, hence different concentration of siderophore was taken to investigate the accumulation of 3 ROS (H_2O_2 , O_2 , and OH). We observed that with the increase in the concentration (50 μg) of siderophore, oxidative stress started increasing (Fig. 1a, 1b, 1c). At different concentration of siderophore, variation in accumulation of different ROS content was observed. OH content was increased to $(68.11 \pm 11.24 \mu\text{M/g FW})$ (Fig. 1a) at 50 μg siderophore concentration but The H_2O_2 content (Fig. 1c) decreased significantly ($P < 0.05$) nearly four folds $(20.15 \pm 0.28 \mu\text{M/g FW})$ than the control $(74.15 \pm 3.22 \mu\text{M/g FW})$. According to Fan et al. 2014 in *Chlorella pyrenoidosa*, OH and MDA content increased under iron deficiency. It can be further noted that pyochelin type siderophore could catalyse invitro through Haber-Weis reaction generating reactive oxygen species. Taking into account that certain siderophore in higher concentration also induced the microalgal inhibition which is contradictory to the reports by [31] that siderophore played as a protector against oxidative stress. As shown in Fig. 1b, SO_2 content significantly increased ($P < 0.05$) with the increased concentration of added siderophore from 10 μg $(13.31 \pm 0.25) < 30 \mu\text{g}$ $(91.58 \pm 2.16) < 50 \mu\text{g}$ (98.15 ± 2.85) . Unlike to this, H_2O_2 content decreased significantly ($P < 0.05$) with the increased concentration of siderophore i.e. (21.56).

Effect of extracted and purified siderophore on ROS activity

After observing results from standard siderophore, extracted and purified crude siderophore was added to the microalgal media with the previous described concentration to check the accumulation of ROS content. An increase in concentration of OH and H_2O_2 content was observed as compared to SO_2 content. The fluctuating levels of ROS in all iron-starved cultures with the added siderophore propose a variance in the degree of a cellular defense response. Apart from 10 μg , both in 30 μg and 50 μg , the OH content showed significantly ($P < 0.05$) similar trend i.e. (65.25 ± 0.11) and (66.11 ± 0.15) respectively. Moreover, we also observed similar results in SO_2 content, i.e. 30 μg and 50 μg siderophore concentration showed (95.58 ± 0.21) and (91.15 ± 0.19) of SO_2 with not much significant difference. In both Fig. 2a and 2b, we can observe that at 10 μg both SO_2 and OH content were lower even after 15 days. This might be because growth inhibition triggered due to the generation of high ROS toxicity during iron deficiency. Benderliev also observed similar results where H_2O_2 content increased in *Scenedesmus incrassatulus* under iron-limited conditions [32]. Morrissey also observed significant increase in H_2O_2 content under iron starvation [33].

Effect of co-cultivation of microalgae on ROS activity

To observe the involvement of siderophore producing bacteria *A. soli* towards the accumulation of ROS content in the co-cultivated green algae *C. variabilis*, growth media was prepared in both iron sufficient

and deficient conditions as described in the previous section. According to earlier reports increase of metal content increases oxidative stress [34]. This promoted to observe the oxidative stress response in microalgae during iron stress conditions by increasing the bacterial supplement that produces siderophores. Hence, to address this, co-cultured microalgae was compared with axenic microalgae leading to the effect of producing three ROS (H_2O_2 , O_2 , and OH). We observed significant increase of OH content ($71.11 \pm 8.12 \mu\text{M/g FW}$) in co-cultured microalgae with the increase in time compared to axenic microalgae i.e. ($40.21 \pm 3.12 \mu\text{M/g FW}$). Contrary to above experiments of direct involvement of siderophores to the microalgae, the co-cultured cells showed increase in all the ROS contents. The SO_2 content increased significantly ($P < 0.05$) to 56% from ($37.31 \pm 3.18 \mu\text{M/g FW}$) in axenic cells to ($101.15 \pm 13.12 \mu\text{M/g FW}$) in co-cultured cells. Similarly the H_2O_2 content also increased simultaneously to 49% from ($64.14 \pm 2.11 \mu\text{M/g FW}$) in axenic cells to ($131.56 \pm 23.18 \mu\text{M/g FW}$) in co-cultured cells. Yilancioglu also observed similar results in *D. salina* where both ROS production as well as lipid peroxidation was increased under co-cultured conditions [35]. We can observe in all the following Fig. 3a, 3b, and 3c that in co-cultured microalgae under iron deficient conditions the level of ROS increased significantly ($P < 0.05$) compared to axenic cultures. The release of chelators in presence of iron seems to be an adaptive strategy, both because free iron is practically insoluble at neutral and alkali pH and because of its potential toxicity. Another possible reason behind this might be the Fe^{3+} induced release of chelators enhancing the capacity of cells to scavenge and later to take up iron [36].

Effect of co-cultivation on microalgal biomass productivity

Convinced by the results in (5:1) volumetric ratio, *A. soli* with *C. variabilis* were co-cultivated in iron sufficient and deficient conditions at higher scale (500 mL) to check its potential role in the growth enhancement of micro algae. Under iron deficient conditions the DCW of axenic culture was ($202.2 \pm 2.02 \text{ mg mL}^{-1}$) and co-cultured was ($240.6 \pm 1.04 \text{ mg mL}^{-1}$) which shows there was not any significant increase in the biomass of co-cultured microalgae. In iron sufficient conditions DCW of axenic culture was ($320.8 \pm 1.36 \text{ mg mL}^{-1}$) and co-cultured was ($138.1 \pm 3.24 \text{ mg mL}^{-1}$) that shows a reduction of (61.7%) of DCW of co-cultured compared to axenic microalgae (Fig. 4). It can be interpreted due to the iron overload in the media the presence of both iron source and secretion of siderophores in the growth media becomes toxic [37].

Effect of co-cultivation of microalgae on ash and pigment contents

Ash and pigments contents were measured for comparative studies between effects on co-cultivation and axenic culture of microalgae. Examination of ash content obtained indicated significant differences between axenic and co-cultivated microalgae. In iron sufficient medium ash content of co-cultivated microalgae showed 32% and in iron deficient medium showed 14.23% which shows 44% decrease of ash content. Concomitantly, there was only 3.87% of increase in ash content in co-cultivated microalgae compared to axenic in iron deficient medium. The increase of ash content in co-cultivated microalgae is due to the association of bacteria with microalgae. Similar results were observed in [22] where high ash

content of algae was observed when algae was contaminated with diatoms or sandy particles. During initial test at different inoculum ratio chlorophyll- a content was similar in algal cells over a period of 15 days at (5:1) ratio in both axenic and co-cultured (data not shown). But during the scaled up test, when chlorophyll content was compared to algae & bacteria in iron deficient medium with only algae in iron sufficient conditions, both showed similar results (Table 2). It was observed that highest chlorophyll-a ($13.06 \mu\text{g mL}^{-1}$), chlorophyll-b ($7.24 \mu\text{g mL}^{-1}$) were found in co-cultivated culture under iron starvation, which was significantly higher ($P < 0.05$) than the axenic culture under iron deficient conditions. It was again similar with the axenic and co-cultured under iron sufficient conditions. Effect of siderophore on chlorophyll was previously observed by [38] and found higher pigment concentration than the control ones

Table 1
 Estimation of Ash content of axenic and co-culture *C. variabilis* in iron sufficient and deficient conditions. YA and YAB are algae and algae with bacteria in the presence of iron. NA and NAB are algae and algae with bacteria in the absence of iron. The results are presented as the average $\pm$ standard deviation (SD). Means with a common letter are not significantly different ($p > 0.05$)

Treatments	Ash Content (%)
YA	2.97 ^{ab}
YAB	32 ^b
NA	0.55 ^a
NAB	14.23 ^a

Table 2

Estimation of pigment composition of axenic and co-culture *C. variabilis* in iron sufficient and deficient conditions. YA and YAB are algae and algae with bacteria in the presence of iron. NA and NAB are algae and algae with bacteria in the absence of iron. The results are presented as the average $\pm$ standard deviation (SD). Means with a common letter are not significantly different ($p > 0.05$)

Treatments	Chl a ($\mu\text{g/mL}$)	Chl b ($\mu\text{g/mL}$)	Chl (a + b) ($\mu\text{g/mL}$)	Chl (a / b) ($\mu\text{g/mL}$)	Caro ($\mu\text{g/mL}$)	Caro/Chlo(a + b) ($\mu\text{g/mL}$)
YA	12.01 $\pm$ 0.32 ^a	7.02 $\pm$ 0.29 ^a	19.03 $\pm$ 0.61 ^a	1.71 $\pm$ 1.10 ^a	14.01 $\pm$ 1.23 ^a	0.73 $\pm$ 2.01 ^a
YAB	5.23 $\pm$ 0.24 ^a	2.56 $\pm$ 0.41 ^b	7.79 $\pm$ 0.65 ^{ab}	2.04 $\pm$ 0.58 ^a	8.02 $\pm$ 2.15 ^a	1.02 $\pm$ 3.30 ^a
NA	7.12 $\pm$ 0.36 ^a	3.23 $\pm$ 0.36 ^b	10.35 $\pm$ 0.72 ^{ab}	2.20 $\pm$ 1.00 ^a	10.01 $\pm$ 0.06 ^a	0.96 $\pm$ 0.08 ^a
NAB	13.06 $\pm$ 0.25 ^a	7.24 $\pm$ 0.54 ^a	20.30 $\pm$ 0.79 ^a	1.80 $\pm$ 0.46 ^a	9.02 $\pm$ 0.14 ^a	0.44 $\pm$ 0.17 ^a

Effect on quantum yield of photosystem II

During nutrient limitation in microalgae, ROS are formed due to the impairing of electron transport chain wherein the electron flows from the photosystems to it. It is illustrated by the decrease in F_v/F_m due to nutrient limitation [39] indicating PS-II being negatively influenced [40]. In the present study, F_v/F_m increased significantly ($P < 0.05$) in co-cultured microalgae (0.596 ± 0.019) compared to axenic microalgae (0.321 ± 0.021) in iron deficient conditions (Table 3). In iron sufficient conditions cultures exhibited F_v/F_m of (0.498 ± 0.014), which was nearly similar to that of the co-cultured microalgae. These results are accordance with that of the pigments contents confirming that these co-cultured organisms during iron starvation significantly affects the photosynthetic apparatus of *C. variabilis*.

Table 3
 Estimation quantum yield of photosystem II of axenic and co-culture *C. variabilis* in iron sufficient and deficient conditions. YA and YAB are algae and algae with bacteria in the presence of iron. NA and NAB are algae and algae with bacteria in the absence of iron. The results are presented as the average $\pm$ standard deviation (SD). Means with a common letter are significantly different ($p > 0.05$)

Treatments	Fv/Fm
YA	0.392 ± 0.014^a
YAB	0.498 ± 0.022^b
NA	0.321 ± 0.021^a
NAB	0.596 ± 0.019^a

Effect of co-cultivation on microalgal total lipid, carbohydrate and proteins

Carbon fixation through photosynthesis is used for the production of major macromolecules like proteins, carbohydrates and lipids. In the current study, the highest protein content of ($44.58 \pm 0.87\%$) was observed in the control (iron sufficient condition) in axenic microalgae (Fig. 5) but, reduced significantly ($P < 0.05$) to ($29.46 \pm 0.92\%$) in co-cultured microalgae in iron sufficient conditions. In the present study, lipid content of *C. variabilis* was significantly higher ($P < 0.05$) in co-cultured microalgae under iron deficient treatments when compared to control culture (Fig. 5). It was highest ($45.92 \pm 0.74\%$) in co-cultured microalgae which was about 21% higher than the axenic microalgae ($25.31 \pm 1.20\%$). Under iron deficiency, a significant trigger in the lipid accumulation by various microalgae like *Chlorella vulgaris*[41], *Dunaliella salina*[35], *Chlorella sorokiniana*[42], *Nannochloropsis oceanica*[43] are reported previously. In our results, although there were no significant changes in the micro-algal growth, but, there was significant ($P < 0.05$) increase in lipid, carbohydrate and the protein content of the cells (Fig. 5) supporting the above perception. Microalgal carbohydrates are the potential resource for production of bioethanol, due to its various benefits like easy hydrolysis, easy conversion to fermentable sugars, and no harsh pretreatment [44]. When compared to lipid accumulation, similar results were obtained in carbohydrate content, where *C. variabilis* also increased in the co-cultured microalgae under iron deficient conditions (Fig. 5). It was maximum ($41.80 \pm 5.03\%$) in co-cultured microalgae, which was about 40% higher than that of the control culture ($30.21 \pm 2.12\%$). From the biochemical composition of *C. variabilis* it could be inferred that co-cultured microalgae under iron starvation can be preferred for producing higher biomass, lipid and carbohydrate contents.

Effect of co-cultivation on lipid fragments in microalgae

As microalgal lipids are fractionalised into three major components NLs, GLs, and PLs, hence to observe the effects of co-cultured on the lipid composition of *C. variabilis* under iron deficient medium, total lipids were further fractionated by a column chromatography. Figure 6 shows that PLs of the cells decreased significantly ($P < 0.05$) in co-cultured microalgae compared to axenic microalgae, i.e., ($21.62 \pm 0.80\%$) in axenic microalgae to ($9.90 \pm 0.90\%$) in co-cultured microalgae. The accumulation of NLs was almost similar in all the treated cells ($70-80 \pm 0.40\%$) except in axenic microalgae in iron deficient condition i.e. ($60.12 \pm 0.51\%$). The GLs decreased significantly ($P < 0.05$) from ($19.89 \pm 2.69\%$) in control to ($14.39 \pm 0.62\%$) in iron deficient conditions. Under iron deficiency the co-cultured showed increase in neutral lipid i.e. ($77.05 \pm 0.21\%$) compared to axenic microalgae ($60.12 \pm 0.11\%$). Our results are accordance to the previous observations that suggests that in exponential phase the availability of optimal iron increases both biomass and lipid production [45].

Conclusion

In the present study, we investigated microalgal growth and the biofuel potential of green microalga *C. variabilis* (ATCC-PTA 12198) with *A. soli* (MTCC-5918) through co-cultivation strategy. Different efforts were made to link the lipid and carbohydrate content with the oxidative stress by the addition of siderophore under iron deficiency. The results of the present study showed the interaction of siderophore-induced oxidative stress with the signalling in the growth and development of the *C. variabilis*. Lipid and carbohydrate content were highest in co-cultured microalgae. In the cell, O_2 is either directly catalysed to H_2O_2 , by the action of SOD which produces OH through the Fenton reaction, or OH via Haber–Weiss cycle. OH levels were similar in all three iron-starved co-cultured cultures, but there was a significant difference in the accumulation of H_2O_2 and O_2 . Though, there was not much increase in the microalgal growth in co-cultured microalgae but, the lipid and carbohydrate content increased. Future research could be focused on how the oxidative stress is triggered by iron through siderophore affecting the biochemical changes on microalgae. Hence, this study provides new approach to improve the biofuel potential of the promising strain of the *C. variabilis* through co-cultivation strategy presenting a comprehensive picture of oxidative stress of the cells instead of cultivating the isolated strains for the purpose of biofuel as it requires many challenges to be faced while shifting from high value low volume products during scale up for commercial production.

Methods

Experimental conditions

Tests conducted in this section were aimed to observe the production of ROS in microalgae due to the increase in the production of siderophore. Initial experiments were conducted in microalgae *Chlorella variabilis* under iron limiting conditions by inoculating standard siderophore as preliminary test and then

siderophore producing bacteria *Acinetobacter soli* to scale it up to 1L. The concentration of siderophore was increased in all the experiments to check the level of ROS production.

Micro-algal growth conditions

The axenic micro-algal culture was grown in Zarrouk's medium [15] in both iron sufficient and iron deficient conditions. In iron deficient it was grown with the exception that FeSO_4 and EDTA was omitted following the cultivation recommendations for this algal strain, and the medium was referred to as Fe deficient media. In a Percival chamber microalgae was grown in static cultures at 18 °C under a light/dark cycle of 12/12 h. Light intensity was $150 \mu\text{moles photons m}^{-2} \text{ s}^{-1}$. A mixture of penicillin and streptomycin at a final concentration of (0.5 mg/mL and 1 mg/mL) was used to maintain axenic cultures. Cultures were maintained axenic respectively. The cultures treated with antibiotics were used to inoculate antibiotic free cultures before preliminary experimental procedure. Absence of bacteria in axenic micro-algal culture was monitored periodically both by spread-plate technique using Nutrient agar [16] and under the microscope. The initial microalgal concentration was the similar for all growth conditions by (4×10^9 cells/ mL) or 0.25 OD (optical density) reading at 750 nm. Micro-algal cell count was monitored initially using a hemocytometer.

Bacterial growth conditions

The bacterial strain of *Acinetobacter soli* was grown in liquid medium aerobically in chemically defined low iron media (CDLIM) constituted of 2 g K_2SO_4 , 3 g K_2HPO_4 , 1 g NaCl, 5 g NH_4Cl , 2 mg thiamine, 100 mg CaCl_2 , 0.005 mg CuSO_4 , 0 mg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0035 mg MnSO_4 , 2.5% glycerol stock at 28°C and 120 rpm for 120 h that was specially designed for production of siderophores [17]. Cultures were incubated at 30 °C shaking at 120 rpm.

Extraction and purification of siderophore

Bacterial isolate was cultured in Nutrient broth (Himedia) medium at 30 °C, 180 rpm for 24 h. The cultures were harvested by centrifugation at $10,000 \times g$ for 15 min at 4° C. The cell free supernatant was acidified to pH 2.5 and added with Amberlite XAD-2 resin, (ca. 100 g L^{-1} , Himedia) to absorb the siderophore [18]. The resin was poured into column and washed several bed volume of deionised water to remove salt followed by the suspension was shaken for 12 h. Methanol (100%) was then used for final elution further which concentrated by rotary evaporation (Buchi-R215) to yield the crude extract [19]. Crude siderophore extracts was purified on a size exclusion column (Sephadex G-10, GE Healthcare). The recovered fractions containing siderophore were identified by CAS assay [20] pooled and then subjected to reversed-phase high-performance liquid chromatography (RP-HPLC) for further purification. Purified siderophore was lyophilized and stored at -20 °C.

Estimation of standard siderophore on ROS activity

C. variabilis grown in ZM was transferred in flask washed with HCl that contained fresh media supplemented with standard siderophore. Both positive and negative control was prepared according to

our previous work [12]. Purified siderophore ranging 10 µg, 30 µg, 50 µg, was added in iron deficient medium. All the experiments were performed in triplicates.

Estimation of extracted and purified siderophore on ROS activity

The crude siderophore, after purification as described in the (sec 2.1.3) was added in to the media containing microalgae. The concentration of siderophore kept same as described in the (sec 2.1.4).

Estimation of biomass productivity

Biomass productivity was observed according to following equation.

$$P = (X_2 - X_1) / (t_2 - t_1),$$

Where, X_2 and X_1 are the dry cell weight concentration (mg/L) at time t_2 and t_1 , respectively BP was calculated in terms of (mg/L/day). The optical density at 750 nm was estimated for the growth of microalgae [12].

Estimation of quantum yield of photosystem II

The maximum quantum yield (F_v/F_m) of photosystem II (PS II) was measured using a fluorometry i.e. Pulse Amplitude-Modulated (PAM) by (AquaPen-C AP-C100, Photon System Instruments). Cultures were diluted appropriately to an absorbance (OD at 750 nm). Fluorescence of the samples was measured after the cells were adapted to 30 min darkness. This dark-adapted fluorescence with minimal level (F_0) and the fluorescence with maximal level measured after high intensity of short light pulse (F_m) were used to calculate F_v/F_m according to the following equation:

$$F_v/F_m = (F_m - F_0)/F_m \text{ [21].}$$

Estimation of ash content and pigments of microalgae

The ash content in microalgae is measured gravimetrically by a process called dry ashing or dry oxidation i.e burning samples in a muffle furnace at a high temperature for a specified duration. Ashing temperature was adjusted to 600 °C for at least 30 min [22]. Pigment content was estimated in the pellet after centrifugation of 2.0 mL of culture at 10,000 rpm for 15 min + 2.0 mL of 100% methanol, mixed well and incubated at 45°C for 24 h in the dark. The total pigment content was calculated according to following equations:

$$\text{Chlorophyll a; Chl-a } (\mu\text{g/mL}) = 16.72 (A_{665.2}) - 9.16 (A_{652.4})$$

$$\text{Chlorophyll b; Chl-b } (\mu\text{g/mL}) = 34.09 (A_{652.4}) - 15.28 (A_{665.2})$$

$$\text{Carotenoids } (\mu\text{g/mL}) = [1000 (A_{470}) - 1.63 (\text{Chl-a}) - 104.9 (\text{Chl-b})]/221 \text{ [12]}$$

Estimation of lipid and crude protein contents

Lipids were extracted by the method of (Bligh and Dyer 1959) (a mixture of chloroform and methanol) and quantified gravimetrically with separation through column chromatography [23]. Biomass was vortexed thoroughly, ultrasonicated at room temperature in chloroform:methanol (1:2, v/v), centrifuged and measured gravimetrically. Lipids are mainly phospholipids (PL), glycolipids (GL) and neutral lipids (NL). All the above fractions are fractionated by sequential elution by chloroform:acetic acid (9:1, v/v) for neutral lipid, acetone:methanol (9:1, v/v) for glycolipids and 100% methanol for phospholipids after drying at 60° C [24]. Crude protein was estimated according to [25].

Estimation of carbohydrate

Carbohydrate was determined by the addition of 100 mg dried biomass with 10 mL of 2% H₂SO₄ and then hydrolysed at 121°C for 20 min. Further CaCO₃ was used to neutralize and diluted to 100 mL distilled water. The total sugar content in supernatant was estimated after centrifugation at 10,000 rpm for 5 min [26].

Estimation of ROS activity

The H₂O₂ content of microalgal cells was measured by harvesting the culture by centrifugation and the cell pellet was homogenized in 0.1% w/v TCA solution. The obtained homogenate was centrifuged at 15,000 × g for 20 min. 0.5 mL supernatant of obtained aliquot was mixed with 0.5 mL of phosphate buffer (10 mM and pH 7.0) and 1 mL of 1 M potassium iodide and its absorbance was read at 390 nm [27]. The H₂O₂ concentration (μmol H₂O₂/g FW) was determined with the help of a calibration curve. O₂ content of microalgal cells was measured by harvesting it by centrifugation and the homogenized with 5 mL of potassium phosphate buffer (65 mM, pH 7.8). This homogenate was centrifuged at 14,000 × g for 10 min and. 1 mL supernatant of obtained aliquot was mixed with 0.9 mL of potassium phosphate buffer (65 mM, pH 7.8) and 0.1 mL of hydroxyl ammonium chloride (10 mM). The solution was incubated at 25 °C for 20 min, afterwards, 1 mL of sulphanilic acid (17 mM), and 1 mL of α-naphthylamine (7 mM) were added to it. It was again incubated for 20 min, and the absorbance of was read at 530 nm [28]. OH content, microalgal cells was measured by harvesting it by centrifugation and homogenized with 2 mL of potassium phosphate buffer (50 mM, pH 7.0). This homogenate was again centrifuged at 14,000 × g for 10 min. Consequently, 0.5 mL of supernatant was mixed with 0.5 mL of potassium phosphate buffer (50 mM, pH 7.0). At 35 °C in dark, the reaction was established for 1 h. 1 mL of 1% TBA and 1 mL of acetic acid was added to 0.05 M sodium hydroxide, and the mixture was boiled for 30 min that was again cooled on ice immediately. The absorbance of this solution was read at 532 nm [29].

Statistical analysis

All the experiments were carried out in triplicates. Statistical data presented here are generated from three independent experiments. All statistical significance comparisons between indicated groups were performed using a one-way ANOVA with Fisher's post-test.

Data presented are mean values and their standard deviation.

Declaration of competing interest

Ethics approval and consent to participate

No conflicts, informed consent, or human or animal rights are applicable to this study”

Consent for publication

Not applicable.

Availability of data and materials

All data generated and analyzed in this study are included in this published article.

Abbreviations

CAS- Chrome Azurol Sulphonate

DCW - Dry cell weight

DW - Dry weight

Fe²⁺ - Ferrous oxide

[Fe-S]- Iron-Sulphur cluster

F_v/F_m - Maximum quantum yield of photosystem II

FW - Fresh weight

GL- Glyco Lipid

H₂O₂ - Hydrogen Peroxide

MDA- Malondialdehyde

NL- Neutral Lipid

O₂ - Superoxide radicals

OH[·] - Hydroxyl radicals

PL- Phospholipid

PS II - Photosystem II

ROS- Reactive Oxygen Species

SO₂ - Sulphur dioxide

SOD - superoxide dismutase

TBA - Thiobarbituric acid

ZM- Zarrouk's Media

Declarations

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Consent for publication

Not applicable.

Availability of data and materials

All data generated and analyzed in this study are included in this published article.

Competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author's contributions

Dr Sandhya Mishra Conceptualized the idea for co-cultivation strategy to enhance the growth, lipids and carbohydrates. Further designing for the experiments, which were actually performed, by Soundarya Rajapitamahuni and Pooja Bachani, Vamsi Bharadwaj S.V. has helped in the molecular identification of the strains used in the experiments. Dr Sandhya Mishra edited manuscript.

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Figures

Figure 1

a Effect of different concentration of standard siderophore on hydroxyl radicals (OH) contents in *C. variabilis*. Values are presented as the mean $\pm$ standard deviation ($n = 3$). Values with the different letters represent a significant difference ($P < 0.05$) between treatments. b Effect of different concentration of standard siderophore on superoxide radicals (O_2^-), contents in *C. variabilis*. Values are presented as the mean $\pm$ standard deviation ($n = 3$). Values with the different letters represent a significant difference ($P <$

0.05) between treatments. c Effect of different concentration of standard siderophore on hydrogen peroxide (H₂O₂), contents in *C.variabilis*. Values are presented as the mean $\pm$ standard deviation (n = 3). Values with the different letters represent a significant difference (P < 0.05) between treatments.

A

B

C

Figure 2

a Effect of different concentration of purified siderophore on hydroxyl radicals (OH) contents in *C.variabilis*. Values are presented as the mean $\pm$ standard deviation (n = 3). Values with the different letters

represent a significant difference ($P < 0.05$) between treatments. b Effect of different concentration of purified siderophore on superoxide radicals (O_2^-) contents in *C.varibilis*. Values are presented as the mean $\pm$ standard deviation ($n = 3$). Values with the different letters represent a significant difference ($P < 0.05$) between treatments. c Effect of different concentration of purified siderophore on hydrogen peroxide (H_2O_2) contents in *C.varibilis*. Values are presented as the mean $\pm$ standard deviation ($n = 3$). Values with the different letters represent a significant difference ($P < 0.05$) between treatments.

A

B

C

Figure 3

a Effects of different concentration of axenic and co-cultured microalgae on hydroxyl radicals (OH) contents in iron deficient conditions. Values are presented as the mean $\pm$ standard deviation (n = 3). Values with the different letters represent a significant difference (P < 0.05) between treatments. a

Effects of different concentration of axenic and co-cultured microalgae on hydroxyl radicals (OH) contents in iron deficient conditions. Values are presented as the mean $\pm$ standard deviation (n = 3). Values with the different letters represent a significant difference (P < 0.05) between treatments. c

Effects of different concentration of axenic and co-cultured microalgae on hydrogen peroxide (H2O2) contents in iron deficient conditions. Values are presented as the mean $\pm$ standard deviation (n = 3). Values with the different letters represent a significant difference (P < 0.05) between treatments.

Figure 4

Effect on co-cultured on biomass productivity of microalgae. YA, YAB are axenic microalgae and co-cultured microalgae with bacteria in the presence of iron. NA and NAB are axenic microalgae and co-cultured microalgae with bacteria in the absence of iron (data represent mean $\pm$ standard deviation of three replicates)

Figure 5

Effects of axenic and co-cultured *C. variabilis* on carbohydrate, lipid, and protein contents under iron sufficient and deficient conditions. YA and YAB are axenic microalgae and co-cultured microalgae with bacteria in the presence of iron. NA and NAB are axenic microalgae and co-cultured microalgae with bacteria in the absence of iron. Values are presented as the mean $\pm$ standard deviation ($n = 3$). Values with the different letters represent a significant difference ($P < 0.05$) between treatments

Figure 6

Variations in the lipid class composition of axenic and co-cultured microalgae under iron sufficient and deficient conditions under nitrogen deficiency. Phospholipids (PL), neutral lipids (NL), and glycolipids (GL). Values are presented as the mean $\pm$ standard deviation ($n = 3$). Values with the different letters represent a significant difference ($P < 0.05$) between treatments.

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