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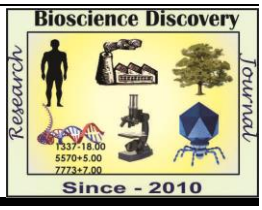
Research Article



In vitro synergism between algae and bacteria isolated from bio-diversity hotspot for better environmental sustainability

Debapriya Roy*, Srijan Bhattacharya, Antara Biswas, Arpan Banerjee, Shinjini Ghosh and Arup Kumar Mitra

Department of Microbiology, St. Xavier's College (Autonomous), Kolkata.
*debapriyaroy026@gmail.com

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Abstract

A water sample containing algae was collected from Manipur. Microscopic examination revealed that it contained filamentous green algae and diatoms. Upon pour plating it gave rise to three types of bacterial colonies which were *Bacillus* spp, *Staphylococcus* spp and *Streptomyces* spp. To artificially prepared BG11 broth respective proportions of algae and bacterial cultures were inoculated followed by constant monitoring of algal biomass after 15 and 30 days accordingly. Each of the bacteria promotes algal growth as indicated from the in-vitro increase in the algal biomass with *Bacillus* spp promoting the maximum growth. The symbiotic interaction between *Bacillus* spp and the algae resulted in the increase of the algal biomass by 10.71%. So, on a detailed study under SEM, it was found out that the algal sample procured nourishment from the nutrients supplied by the *Bacillus* spp as well as the glycocalyx adhesion of *Bacillus*spp with that of cellulosic algal wall. On the other hand, Chlorophyll assay and Carotenoid assay was performed which revealed the ability of the algae to perform photosynthesis with the help of increased Chlorophyll b and production of Carotenoid. The measurement of Chlorophyll a and b at 645nm and 663nm revealed the increase of Chlorophyll b concentration by 3.05%, along with increase in Carotenoid concentration at 470nm was determined to be 0.97µg/ml. The artificial cultivation of diatoms resulted in partial degradation of silica wall which was replenished by bacterial conversion of dead debris of diatoms. Thus it can be concluded that the bacterial association not only facilitate nutrient availability but also helps in partial increase in anabolic process of the algal consortia.

INTRODUCTION

The interaction between algae and bacteria has always been a field of interest. The cultivation of microalgae helps in the removal of pollutants from wastewater, total suspended solids, total dissolved solids etc. Microalgae turned out to be a very promising way out for wastewater treatment where the natural contents of the algae namely the carbohydrate and protein part remain intact in this clearing process. These natural contents are in turn suitable for energy production. The high utility of microalgae involving the wastewater treatment along with the biofuel production settle all the issues related to the expensive and not so environment friendly fossil fuels. Our work thus encompasses an integrated process uplifting the growth of algae in aid of *Bacillus* spp which in turn has multiple future prospects as mentioned above (Raphael Slade and Ausilio Bauen, 2013).Water sample collected from a biological hotspot was examined. Treatment of pernicious anemia by a special diet (Minot & Murphy, 2001**)**.

The aim of this project was to study the interaction between the algal species and the bacterial species isolated from the water sample, when allowed to grow under in-vitro conditions. The increase in algal biomass and further assays revealed that all the bacterial species were promoting algal growth (Harold & Svec, 1966). Microscopic examination of the water sample showed the presence of filamentous green algae such as *Spirogyra* and the average size of the algae measured under 450X magnification was found to be 72µm.

MATERIALS AND METHODS

Pour plate technique

The pour plate technique was performed. Colonies of different bacterial species were obtained after incubating the petri plate overnight. Gram staining was performed to identify the gram characteristics of the bacterial species.

Bacterial Characterisation:

The gram staining protocols were followed (Barthomolew and J.W *et al*., 1962). All the three species exhibited purple coloration indicating that they are gram positive. The characteristics of two isolated colonies were gram positive rods and the third colony was found to be gram positive cocci in cluster.

The inoculums from incubated petri-plate were transferred to a chrome agar medium in the form of quadrant streaking to assume the genus of bacteria from the color they exhibit. (J. Merlino *et al*., 2000). After overnight incubation, the quadrant streaking changed to blue colour with a white halo and green colouration. Comparing the range of colours, they were found to be *Bacillus* spp., *Streptomyces* spp.

Catalase is an enzyme which is produced by microbes living in O_2 rich areas, to neutralize toxic forms of oxygen metabolites, H_2O_2 (F C Tenover *et al*., 1994). 4-5 drops of 3% H_2O_2 was placed on a clean glass slide. Small quantity of bacterial colony was transferred into the glass slide containing H_2O_2 solution. Rapid effervescence showed the positive result. The isolated bacteria may be *Staphylococcus* spp.

Blood Agar is an enriched media to grow fastidious organisms and to differentiate bacteria based on their haemolytic properties. The isolated colony was streaked on blood agar and incubated at 37ºC for 24 hours. The colonies showed yellow colouration surrounded by zones of clear beta haemolysis. The isolated bacteria were confirmed to be *Staphylococcus* spp.

Interaction between bacteria

Streak plate technique is used for the isolation of pure culture of the organism (mostly bacteria) from mixed population. T-streak method is a general method showing possible positive and negative interactions between the two genera of Bacteria. The possible interactions between the aforementioned isolated bacteria were observed with the help of T-streaking (Beisher *et al*., 1995). The possible combinations were:

a) Streptomyces spp and *Bacillus* spp.
b) *Streptomyces* spp and *Staphylococcus* spp.
c) Staphylococcus spp and *Bacillus* spp

Estimation of Algal biomass in BG11 Broth

BG11 broth is a universal medium for the cultivation and maintenance of algal growth. This medium supports growth of photoautotrophic algae which requires light as a source of energy. Synthetic nitrogen and carbon sources and also other inorganic salts comprise this medium. The exposure to light intensity optimizes the growth. Isolation of algae after lyophilisation and consequent inoculation in BG11 broth was done. (Ilavarasi *et.al* 2011).Then the algal suspension was kept in tissue culture lab for 30 days under artificial light conditions and increase in algal biomass confirmed the observable growth.

Chlorophyll and Carotenoid assay

Spectrophotometric methods were used for the determination of Chlorophyll a and b content in the filamentous green algae according to (Nayek *et.al* 2014). An extract was prepared by filtering the growing algae through 0.45 µm membrane filter which were solubilized in acetone. The filters were ground in a mortar pestle with 3-4 ml of spectrophotometric grade acetone (90%) for 3 minutes at room temperature. The volume was doubled with 90% acetone after grinding. It was mixed and centrifuged for 10 minutes at 5000rpm. The absorbance of the supernatant was determined at appropriate wavelength

Chlorophyll a and Chlorophyll b were calculated from the following formulas: (Nayek *et al.*, 2014).

$Chl.a = 12.7(A_{663}) – 2.69(A_{645})$

$Chl.b = 22.9(A_{645}) – 4.68(A_{663})$

$Total\ Chlorophyll = 20.2(A_{645}) + 8.02(A_{663})$

The extracts of filamentous green algae for carotene assay were prepared by homogenizing the algae with 10ml of acetone (80%). The homogenized extracts were centrifuged at 10,000rpm for 15 minutes at 4°C (Minerva Lara-Flores, 2013**)**.

The supernatants were separated and 0.5ml of it was mixed with 4.5ml of acetone (80%). Then the solution mixtures were analyzed for carotenoid content in spectrophotometer. The O.D measured at 470nm in spectrophotometer.

Carotenoid was calculated by the following formula: (Nayek *et al.*, 2014).

$C\ x+c = (1000A_{470} – 1.82Chl.a – 85.02Chl.b)/198$

RESULTS AND DISCUSSION

Synergistic interaction between algae and bacteria was observed.

12 mg of algae (*Spirogyra* spp) was re-suspended in BG11 broth and bacterial suspension was made from pure culture. Four different types of set ups were established to analyze the synergistic interaction between algae and bacteria. These are the following working set ups:

Table 1: Synergistic interaction between algae and bacteria

Sl. No	Set-up	Ratio of bacteria and algae	Description
1.	**Control**	-	Only algal species was inoculated in 1 ml of BG11 broth.
2.	***Staphylococcus* spp + Algae**	1:1	1ml of *Staphylococcus* spp suspension inoculated in 1 ml of BG11 broth containing algae.
		2:1	3ml of *Staphylococcus* spp suspension was inoculated in 1.5 ml of BG11 broth containing algae.
3.	***Bacillus* spp + Algae**	1:1	1ml of *Bacillus* spp suspension was inoculated in 1 ml of BG11 broth containing algae.
		2:1	3ml of *Bacillus* spp suspension was inoculated in 1.5 ml of BG11 broth containing algae.
4.	***Streptomyces* spp + Algae**	1:1	1ml of *Streptomyces* spp suspension was inoculated in 1 ml of BG11 broth containing algae.
		2:1	3ml of *Streptomyces* spp suspension was inoculated in 1.5 ml of BG11 broth containing algae.

These respective ratios were kept in tissue culture lab and growth was monitored for 15 and 30 days respectively. After 30 days, 2:1 did not show any possible growth so they were eliminated.

Table 2: Ratio of bacteria and algae on biomass after 15 and 30 days

Ratio	Interaction type	Biomass after 15 days(mg)	Biomass after 30 days(mg)
2:1	1+A	5.5	3.2
2:1	2+A	6.0	3.1
2:1	4+A	6.0	3.1
1:1	1+A	7.9	8.4
1:1	2+A	8.4	9.3
1:1	4+A	8.4	5.4
-	Control	5 .0	3.5

A – Algae
1- *Staphylococcus* spp
2 - *Bacillus* spp
4-*Streptomyces* spp

The percentage increase of the algal biomass in the presence of *Bacillus spp* was found to have risen by 10.71%. Percentage increase of biomass was observed only in the ratios 1:1. The 1:1 ratio of algal biomass in presence of *Bacillus* spp showed maximum growth after 30 days.

Analysis of algal growth by Scanning Electron Microscopy (SEM):

Analysis by SEM revealed the interaction between the *Bacillus* and the algal species. Mainly the controlled culture and the growth of algae in the presence of *Bacillus* spp was examined under SEM.

In the controlled culture (figure2: 3a,3b), where the algae was grown invitro in BG11 broth without any bacterial species revealed the following observations :

Filamentous masses of Spirogyra showed varying diameter range.Filament was relatively less thick and the presence of conjugation bridge was observed. The algae was observed to be in replicating condition. Scalariform conjugation was observed where there is association of two filaments lined side by side partially or throughout their length. SEM study also revealed the presence of diatoms. Nutritional deficiency resulted in the degradation of silica walls in diatoms. Further analysis of interaction between algae and Bacillus grown invitro in BG11 broth revealed the following observations:

Filamentous green algae Spirogyra showed apical growth when grown in interaction with Bacillus spp. in BG11 broth.(figure: 3-5a). Most of the Bacillus spp. was found growing on the algal surface.(figure:3-5b). The filament was found to be relatively thicker in comparison to the filament in controlled culture.

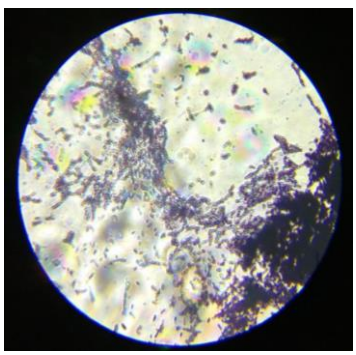
Bacteria remain attached to the algal surface due to adhesion between the glycocalyx and the algal cellulosic wall.(figure: 3- 6a). Algae are showing epiphytic growth in presence of bacteria. Bacteria are providing nutrients promoting the growth of Spirogyra and the diatoms. There is an evidence of the regeneration of silica walls in diatoms in the presence of bacteria which probably came from the dead and degrading diatoms through natural cycling (figure: 3-6b). Thus, SEM study reveals the synergistic interaction between the algae and the bacteria, promoting algal growth.

Table 3: Determination of Chlorophyll a, Chlorophyll b and Carotenoid content in the filamentous green algae:

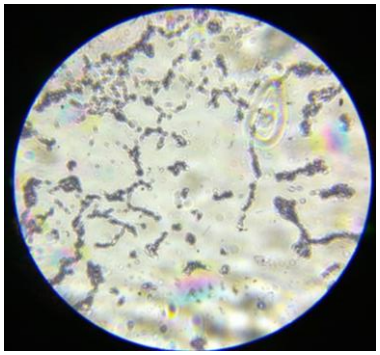
Sample	Chl.a (µg/ml)	Chl.b (µg/ml)	Total Cholorophyll (µg/ml)	Carotenoid (µg/ml)
Control	4.61	1.31	3.31	0.47
***Bacillus* spp + Algae (1:1)**	0.67	1.35	2.02	1.43

Thus, the results show that there is a decrease in chlorophyll a concentration, while increase in chlorophyll b concentration in the algal species grown in presence of *Bacillus* spp, but on the other hand there is a high increase in carotenoid concentration. Increase in chlorophyll b concentration was found to be 3.05% while carotenoid concentration was found to increase by 0.97µg/ml (Banerjee and Ragsdale, 2003**)**.

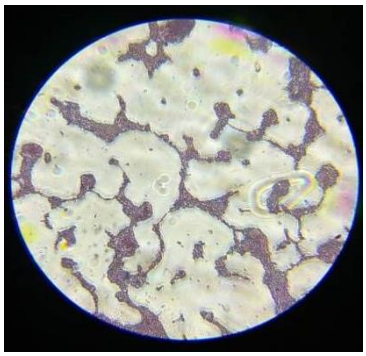
The synergistic interaction was observed between the algal and bacterial species, which is playing a major role in promoting algal growth. The bacteria is providing nutrients which is utilized by the algal species to carry out various anabolic processes (Carlucci & Bowes, 1970). Increase in concentration of the photosynthetic pigment chlorophyll b and carotenoid indicates that the algae, when grown under invitro conditions in presence of bacteria is utilizing chlorophyll b and carotenoid as the photosynthetic pigments to perform photosynthesis for their survival. Furthermore, the artificial cultivation of diatoms resulted in partial degradation of silica wall of diatoms which was regenerated by the bacterial conversion of debris of diatoms (Bartholomew *et al.*, 1962; Kay Bidle, Farooq Azam, 2001). Thus, there is an evidence of symbiotic association between algae and *Bacillus* spp which is enabling them to survive and replicate in invitro conditions.



1A



1B



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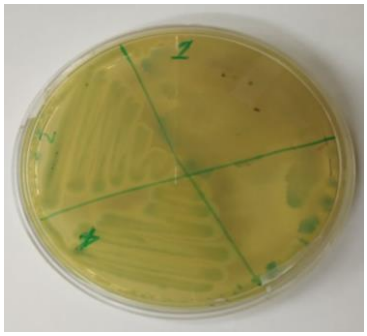
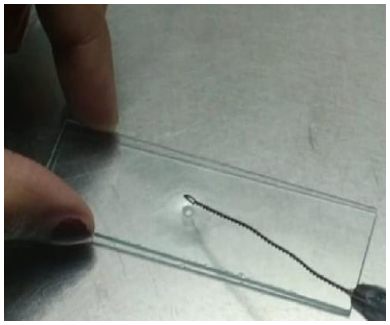
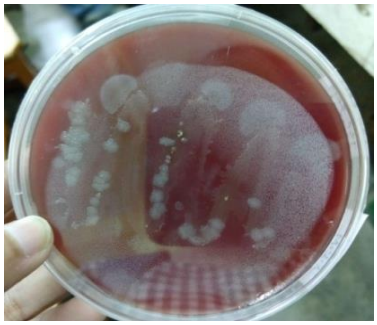


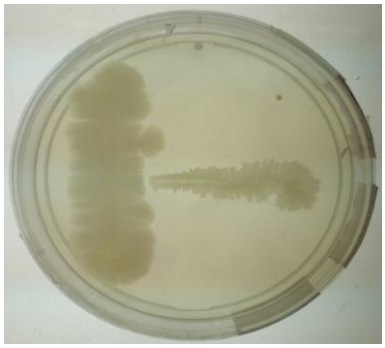
2A



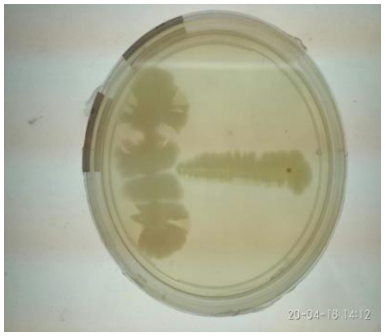
2B



2C



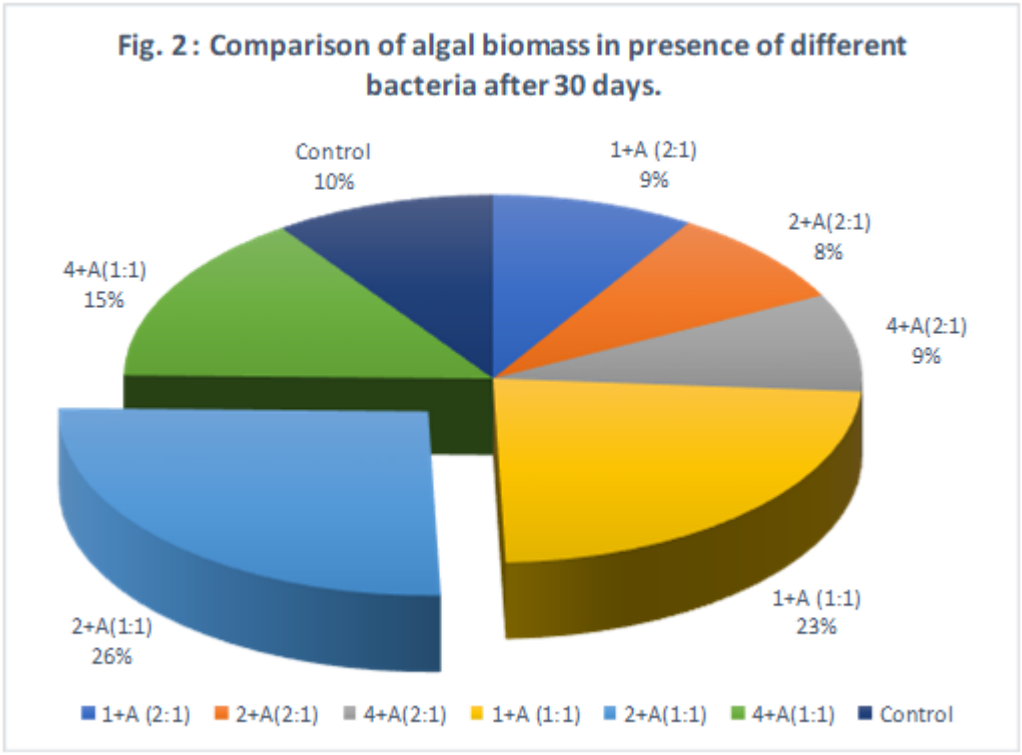
3A



3B

3C

Figure:1 (1A, 1B, 1C) Gram staining of the bacteria observed under 450X magnification. (2A) Chrome agar plates after streaking. Colonies showing blue coloration with white halo. (2B) Catalase test. Rapid effervescence showing the positive result for *Staphylocccus* spp. (2C) Colonies exhibiting yellow coloration surrounded by zones of clear beta haemolysis. (3A, 3B, 3C) Streaking between two genera of bacteria. (3A) *Streptomyces* spp and *Bacillus* spp. *Streptomyces* spp inhibited the growth of *Bacillus* spp due to the possible secretion of an antibiotic. (3B) *Streptomyces* spp. and *Bacillus* spp. *Streptomyces* spp inhibited the growth of *Staphylococcus* spp due to the possible secretion of an antibiotic. (3C) *Staphylococcus* spp and *Bacillus* spp. *Bacillus* spp inhibited the growth of *Staphylococcus* spp.



A – Algae
1- *Staphylococcus spp*
2 - *Bacillus spp*
4-*Streptomyces spp*

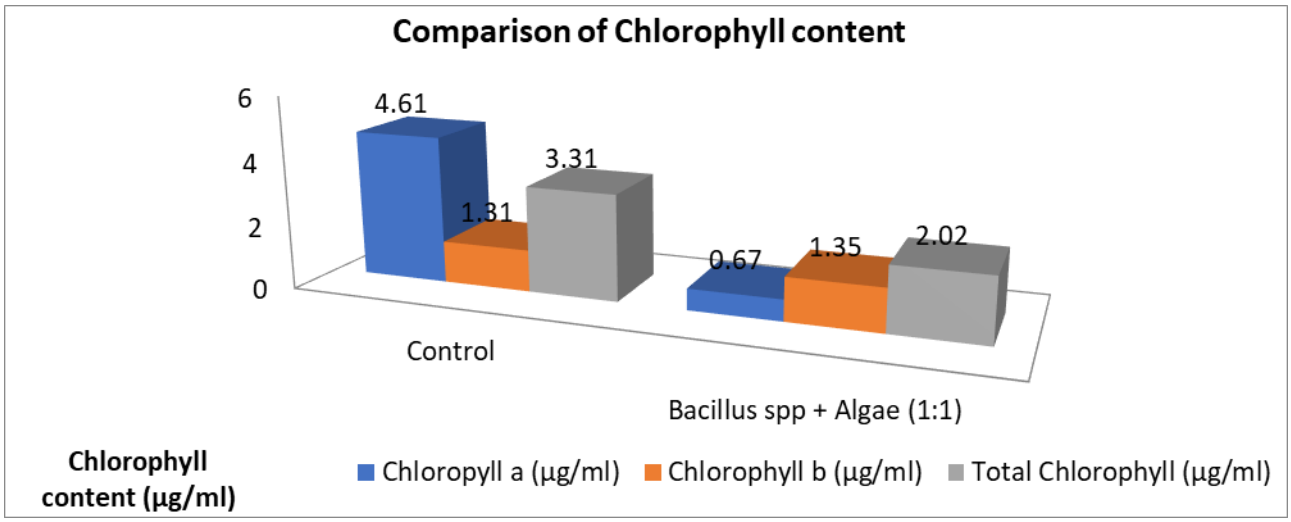
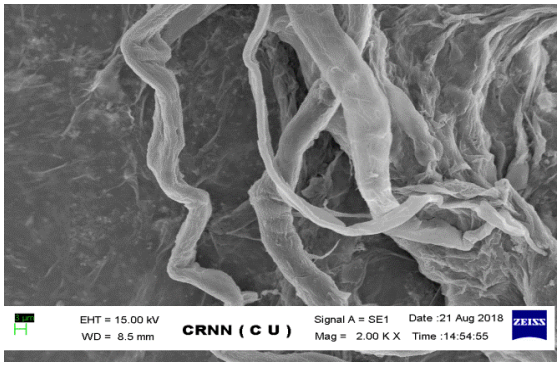
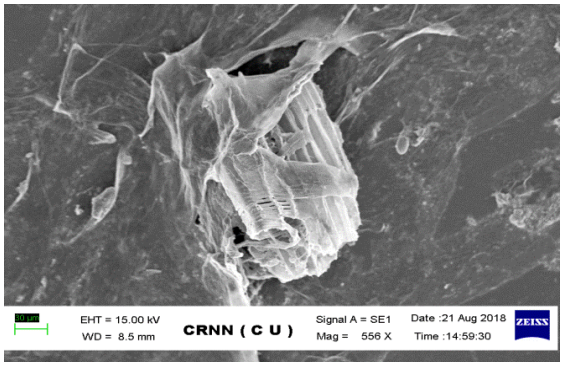


Figure: (3) Comparison of chlorophyll content.

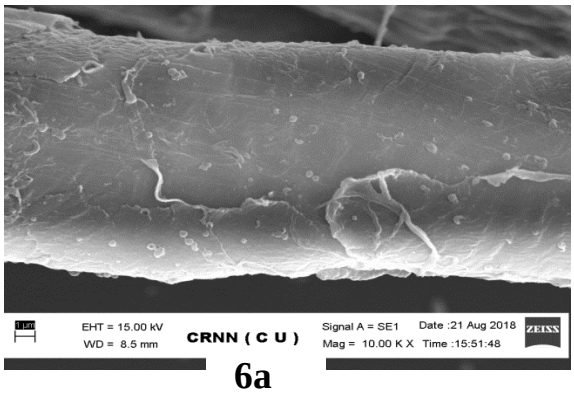


5a

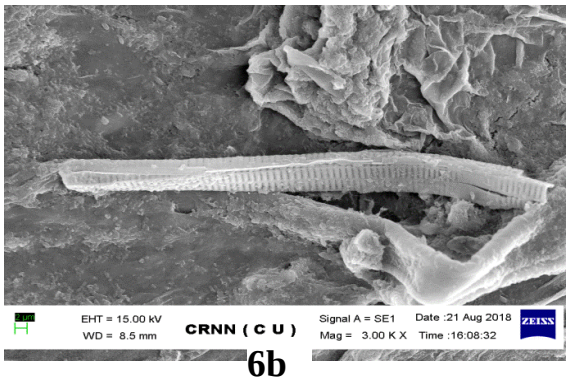


5b

Fig. 4 Graphical representation of increase in algal biomass in the presence of *Bacillus spp* with time. (5a, 5b) Control culture, where the algae was grown in-vitro in BG11 broth without any bacterial species.



6a



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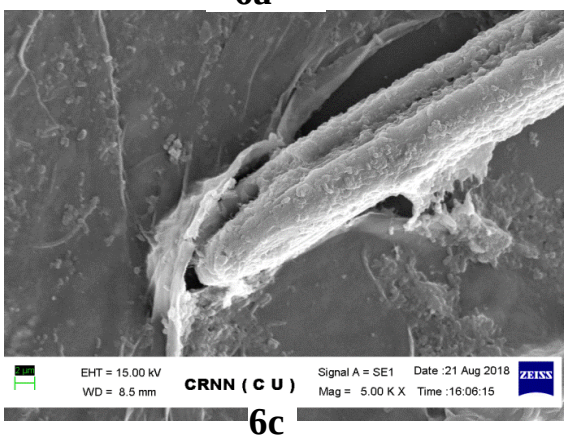
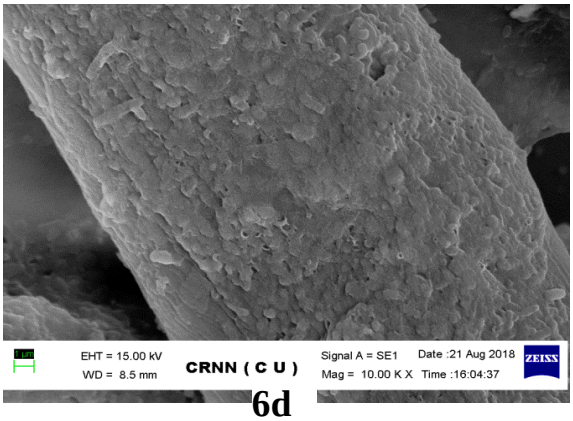


6c



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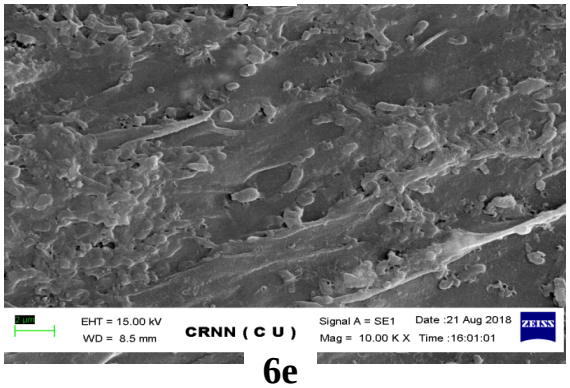

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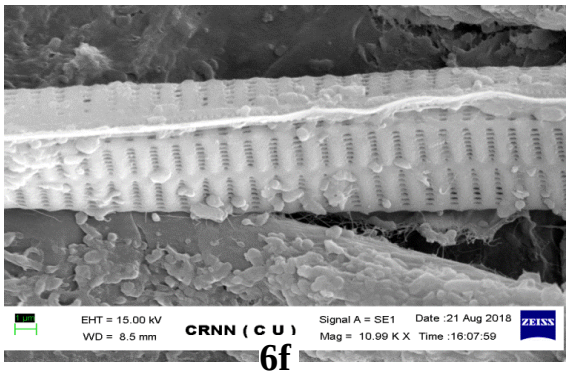

6f

Figure: 6 (a,b)Interaction between algal species and Bacillus spp. studied under SEM.

(c) Filamentous apical growth of Spirogyra in presence of bacteria. (d) Magnified view of bacteria growing on Spirogyra filament. (e) Epiphytic growth is observed. Glycocalyx interaction with algal cellulose wall. (f) Bacterial control of silica regeneration.

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