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A STUDY OF IN VITRO SYNERGISTIC EFFECT OF TWO BACTERIAL SPECIES ON THE PROTEIN AND LIPID CONTENT OF *Anabaena* sps. UNDER OSMOTIC STRESS

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Abstract: Industrial aspects like sewage treatment require the synergy of bacteria and algal species, apart from their natural occurrence in biodiversity hotspots. Recent studies have shown that certain species of micro and macro algae have bacteria closely associated with them, suggesting that they may acquire essential nutrition, especially vitamin B12 by this means. Owing to their colonization of the oceans, algae are responsible for approximately 50% of the world's atmospheric carbon fixation. More than half of these are dependent on bacteria for essential micronutrients. There is also emerging evidence for almost similar interactions between algae and bacteria for the acquisition of other micronutrients. The recognition of these biotrophic interactions will necessitate a change in our understanding of the synergy between algal and bacterial communities, and is likely to have profound implications for the exploitation of this aspect, both as a food source and for biotechnological applications. Thus, in this study, the focus is on how the synergistic combination between algae and bacteria help influence the lipid and protein content of the algal species. Bacterial species of *Staphylococcus aureus* and *Escherichia coli* have been cultured *in vitro* with *Anabaena* sps. under osmotic stress which can predict the future development of such algae-bacteria model systems for industrial use.

Index terms – **synergy, nutrition, micronutrients, interactions, biotrophic.**

I. INTRODUCTION

The synergy between microalgae and aerobic bacteria has long been studied (Borde *et al.*2003). Algae constitute a major natural resource on earth that forms the basis of the food chain for more than two thirds of the world's biomass and are responsible for approximately half of the global photosynthetic activity (Day *et al.*, 1999). Most of the species are microalgae which have aroused an increasing interest in the last two decades. Their industrial applications are numerous, ranging from production of bioactive and pharmaceutical compounds, specialty chemicals, health foods, aquaculture feeds to use in wastewater treatment and agriculture (Metting, 1996).

In nature, most microalgae are found in association with other aerobic microorganisms (Mouget *et al.*, 1995). They produce the molecular oxygen that is used as electron acceptor by the aerobic microflora to degrade organic matter. In return the carbon dioxide released during the mineralization process completes the photosynthetic cycle. This mutualistic association between algae and bacteria is the basis of the BOD removal in aerobic stabilization ponds, described as artificial freshwater environments by Mara and Pearson (1986). Such processes offer other advantages such as the biological aeration is economically interesting compared with the mechanical one (Oswald, 1988). The conversion of solar energy to heat and the Ph-raising due to carbon dioxide fixation during photosynthesis produces a disinfection effect toward many pathogenic bacteria and viruses (Richmond, 1983). Finally, nutrients such as ammonium, nitrate and phosphate known as the main cause of water body eutrophication are concomitantly depleted by algal growth (Laliberte *et al.*, 1994).

Microalgae are mainly used for the treatment of domestic wastewater in tertiary or quaternary treatment units (Laliberte *et al*., 1994). Although they are ubiquitous in the environment and capable of degrading toxic and/or recalcitrant organic materials in heterotrophic modes of nutrition (Semple *et al*., 1999), bacteria and fungi have been more extensively studied for the biodegradation of xenobiotic compounds (Alexander *et al*., 1999). Coming to algal and bacterial coexistence, self-oxygenation in algae–bacteria combined systems is capable of avoiding mechanical aeration which is costly, limited by the poor aqueous solubility of oxygen and may cause the hazardous volatilisation of many organic molecules (Bell et al., 1993) as well as sprays of toxic organisms and compounds.

Algae and bacteria have coexisted ever since the early stages of evolution. This coevolution has revolutionized life on earth in many aspects. Algae and bacteria together influence ecosystems as varied as deep seas to lichens and represent all conceivable modes of interactions — from mutualism to parasitism (R. Ramanan *et al*). Several studies have shown that algae and bacteria synergistically affect each other's physiology and metabolism, a classic case being algae– *Roseobacter* interaction. These interactions are ubiquitous and define the primary productivity in most ecosystems. In recent years, algae have received much attention for industrial exploitation but their interaction with bacteria is often considered a contamination during commercialization. A few recent studies have shown that bacteria not only enhance algal growth but also help in flocculation, both essential processes in algal biotechnology. These photosynthetic organisms along with cyanobacteria live in the planktonic region of the aquatic habitat and are collectively called phytoplankton (Buchan *et al*., 2014). Phytoplankton and bacterioplankton numerically dominate the ocean and freshwater planktonic community (Sarmento and Gasol, 2012). These plankton communities together influence the global carbon cycle and ultimately the climate.

Therefore, the interactions between these two groups of plankton and the influence of their interaction on each other and on a global scale are areas of recent research interest (Amin *et al*., 2015; Landa *et al*., 2015). Several studies show that heterotrophic bacteria play a ubiquitous role in algal growth and survival (Amin *et al*., 2015; Gonzalez and Bashan, 2000; Kim *et al*., 2014; Seyedsayamdost *et al*., 2011). Thus, it opens the possibility for revisiting the global carbon cycle and other biogeochemical processes (Amin *et al*., 2012, 2015; Landa *et al*., 2015). Heterotrophic bacteria not only decompose plant and animal organic matter, but also promote plant growth by complex communication mechanisms and nutrient exchange (Philippot *et al*., 2013). Also, mass cultivation in algal biotechnology should integrate the essence of evolutionary and ecologically relevant relationship between algae and bacteria. Together they not only influence ecosystems but also could potentially influence the growth of future biotechnology industry (Bose *et al*., 2011; Wang *et al*., 2015). Thus, this review attempts to articulate algal–bacterial interactions in totality, from ecology and evolution, to the use of this knowledge to invigorate their combined biotechnological potential.

Algal–bacterial interactions cover the whole range of symbiotic relationships which are deemed possible. Algae, heterotrophic bacteria and archaea are the primary producers and decomposers, respectively, making them the structural pillars of the ecosystem and its foremost functional entities. The algal and bacterial synergism has many benefits for the environment, but this interaction also helps influence the physiological functions of the individual organisms involved. Algal biomass, carotenoid production, increase in the photosynthesis rate, enhanced growth rate, etc are known to be affected by the bacterial presence along with the algal species. The symbiosis and co-existence of microalgae with bacteria is of great importance in reducing the Biological oxygen demand (BOD) in the marine systems (Lee *et.al*. 2016).Similarly, leguminous plants host Anabaenain the lower forms like ferns, and *Rhizobia* in the angiosperms. *Anabaena sp.* has been an appropriate model system to study the change in protein conformation and gene expression under osmotic stress responses (Apte *et.al*. 1987).

A study on a water sample collected from Manipur by Roy D. *et.al*. 2019 showed that the presence of *Bacillus* sps .increased algal biomass by 10.71%. Glycocalyx adhesion of *Bacillus* sps. to the cell wall of *Spirulina* sps. has brought about changes in the algal biomass. Also, there has been an increase in the chlorophyll and carotenoid content of *Sprilunia* sps. in presence of *Bacillus* sp. and *Streptomyces* sps. An experiment conducted by Borde *et.al*. 2003 showed decreased aromatic pollutants in a system where *Pseudomonas migulae* was artificially introduced into a culture medium of *Chlorella sorokiniana*. This led to photosynthetic biodegradation of toxic pollutants by algae-bacterial microcosms.

Similarly, Lee *et.al* 2006 studied how the co-culture system of microalgae and bacteria enables simultaneous removal of BOD and nutrients in a single reactor if the pair of microorganisms is symbiotic. In this case, nutrients are converted to biomass constituents of microalgae. This study analysed that microalgae and bacteria in the co-culture system could cooperate or compete each other for resources. In the context of wastewater treatment, positive relationships are prerequisite to accomplish the sustainable removal of nutrients. Therefore, the selection of compatible species is very important if the co-culture has to be utilized in wastewater treatment. Croft *et.al* 2005 demonstrated that the source of vitamin B12 for microalgae is through a direct interaction with bacteria. It was even proposed that the nature of this interaction is symbiotic, with the algae supplying fixed carbon in return for vitamin B12. Similarly, the activity of two such bacterial species have been observed in these studies which do not occur with algae in a natural habitat to explore the prospects of a possible system for industrial use.

II. RESEARCH METHODOLOGY

2.1 Culturing of microorganisms - The whole process was conducted in an aseptic laboratory condition. Algal sample of *Anabaena* sps. and bacterial samples of *Escherichia coli* and *Staphylococcus aureus* were used.

For culturing of algal samples, a loopful of *Anabaena sp.* pure culture was taken and streaked on sterile petriplates Thereafter the petriplates were left for incubation in the aseptic Plan Tissue Culture laboratory for 7-10 days at 28˚C and 3000 lux illumination. The cultures were then transferred to standard BG 11 broth used for microalgal cultures in conical flasks.

For culturing of bacterial samples, a loopful of bacterial inoculum were taken and streaked on sterile petriplates. The petriplates along with standard nutrient agar media were first autoclaved at 121˚C and 15 lbs pressure for about 45 minutes. The nutrient agar

media was then poured onto each petriplate at about 20mm in thickness. With a sterile loop, the microorganism cultures were inoculated in the nutrient agar. The petriplates were sealed and then kept in the incubator for 24 hours at 37˚Celsius.

For the growth of *Anabaena sp.*, the following four conditions were maintained:

- Algal growth in the presence of NaCl
- Algal growth in presence of *E. coli* and *S. aureus*, respectively
- Algal growth in the presence of NaCl and *E. coli*
- Algal growth in the presence of NaCl and *S. aureus*
- Control was taken as only 5mL of the BG-11 broth medium, without any inoculation.

The bacterial and algal cultures were taken in 1:1 ratio. 2ml of nutrient broth containing a 24 hour culture of two species of bacteria was inoculated into 2ml of BG-11 broth containg a 72 hour culture of *Anabaena* sps. After the inoculation of the bacterial colonies in the algal cultures, they were kept for incubation. In between, the seven days of incubation, the wet biomass of the algae was measured. On completion of the incubation period the qualitative and quantitative estimation of proteins and lipids were conducted on the growing algal species.

2.2 Sample preparation

The samples from the culture tubes were transferred into centrifuge tubes. The samples were homogenized using a centrifuge at 3000rpm for 20 minutes. After centrifugation, the pellets containing the debris of the cells were discarded and the supernatant was used for further analysis. A 1% solution was made for each sample using distilled water. Aliqouts of the homogenized 1% samples were used for the following qualitative and quantitative tests for proteins and lipids.

2.3 Qualitative tests

- For protein: Ninhydrin Test
  A 2% solution of ninhydrin was prepared by dissolving 0.2 grams of ninhydrin in 10ml of either ethanol. A 1% solution of each sample was prepared after homogenisation. A few drops of the 2% ninhydrin solution were added to this solution. The test tube was kept in a warm water bath for approximately 5 minutes. The development of a deep blue/violet colour indicated the presence of amino acids in the respective samples.

- For lipids: Solubility Test
  Three test tubes were taken for each sample which contained 5ml of homogenized sample solution to be tested. 5ml of water was added to the first test tube and the solution was observed. To the second test tube was added 5ml of alcohol. To the third test tube was added 5ml of chloroform. The change in solubility of the respective samples was observed for the presence or absence of lipids.

2.4 Quantitative tests

- Protein estimation: This was done by Lowry's Method which is described as follows.
  Reagents used
  A. 2% Na2CO3 in 0.1 N NaOH
  B. 1% Sodium potassium tartrate in H2O
  C. 0.5% CuSO4.5 H2O in H2O
  D. Reagent I: 48 ml of A, 1 ml of B, 1 ml C
  E. Reagent II- 1 part Folin-Phenol reagent [2 N]: 1 part water
  F. BSA (Bovin Serum Albumin) standard solution- 1mg/ml

  Procedure
  0.2 ml of BSA working standard was taken in 5 test tubes and the volume was made up to 1ml using distilled water. 1ml aliquot of each sample was taken in 8 test tubes. A test tube with 1 ml distilled water was taken as the blank. 4.5 ml of Reagent I was added to each test tube and incubated for 10 minutes. After incubation 0.5 ml of reagent II was added to each test tube and incubated for 30 minutes. The absorbance was measured at 660 nm using a Colorimeter and the standard graph was plotted. The amount of protein present in the respective samples was estimated from the standard graph.

- Lipid estimation: Gravimetric method was used to quantitatively estimate the lipid content under different conditions.
  The gravimetric method consists of the lipid extraction using solvents and lipid quantification achieved by recording the weight of extracted lipids after evaporating the extracting solvents. 5 ml aliquots from each 1% homogenized sample solution were taken in eight 50ml beakers using a measuring cylinder. Before transferring each sample, the weight of the empty beaker was measured using a weighing balance. 5 ml of absolute ethanol was pipetted out into each beaker containing the samples. The beakers containing the samples were kept inside the hot air oven at 60˚C till all the solvent got evaporated. The final weights of the beakers were recorded. The weight of the empty beaker was subtracted from the final weight of each beaker which gave the amount of lipid present in each sample.

III. RESULTS

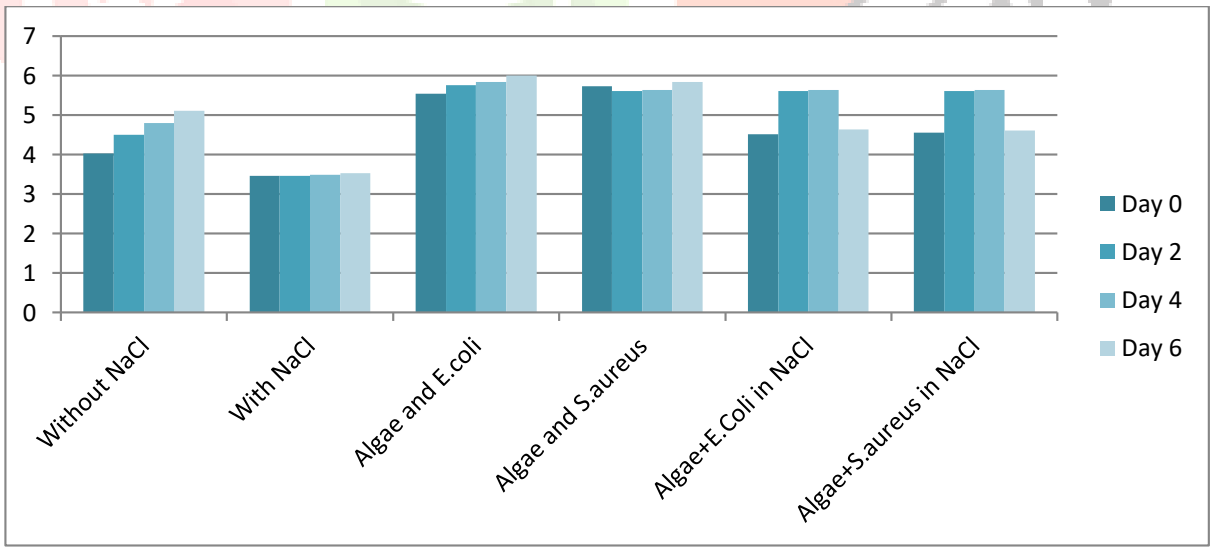
3.1 Wet Biomass estimation

Weight of empty test tube = 35.67g
Weight of only *E.coli* culture broth = 1.52g
Weight of only *S.aureus* culture broth = 1.71g

Table 1: Estimation of wet biomass of the culture tube every alternate day, for a span of 7 days.

Sample:	Day 0 (in g)	Day 2 (in g)	Day 4 (in g)	Day 6 (in g)
Algae culture without NaCl	4.02	4.5	4.8	5.1
Algae culture with 1M NaCl	3.46	3.46	3.49	3.52
Anabaena+E.coli	5.54	5.60	5.63	5.68
Anabaena+ S.aureus	5.73	5.78	5.83	5.84
Anabaena+ E.coli + NaCl	4.51	4.53	4.58	4.63
Anabaena+ S.aureus + NaCl	4.55	4.59	4.59	4.60

Figure 1: Graph showing the gradual increase of algal wet biomass over a period of 7 days.



3.2 Quantitative estimation of protein and lipids

Table 2: Represents the presence/absence of proteins and lipids in the samples.

Sample	Protein	Lipid
Without NaCl	**Present**	**Present**
With 1M NaCl	**Present**	**Present**
Algae+*E.coli*	**Present**	**Present**
Algae+*S.aureus*	**Present**	**Present**
Algae+*E.coli*+NaCl	**Present**	**Present**
Algae+*S.aureus*+NaCl	**Present**	**Present**
Control (Only media)	**Absent**	**Absent**

3.3 Quantitative estimation of proteins

Table 3: Represents the quantitative estimation of proteins by Lowry's method.

Sample.	Volume of sample (ml)	Volume of distilled water (ml)	Volume of Reagent I (ml)	Volume of Reagent II (ml)	OD Value at 660nm
Algae culture without 1M NaCl	1	0	4.5	0.5	0.41
Algae culture with 1M NaCl	1	0	4.5	0.5	0.23
Anabaena+E.coli	1	0	4.5	0.5	0.46
Anabaena+ S.aureus	1	0	4.5	0.5	0.21
Anabaena+ E.coli + NaCl	1	0	4.5	0.5	0.48
Anabaena+ S.aureus + NaCl	1	0	4.5	0.5	0.18
Blank	0	1	4.5	0.5	0.00

Figure 2: Represents the standard curve for protein estimation.

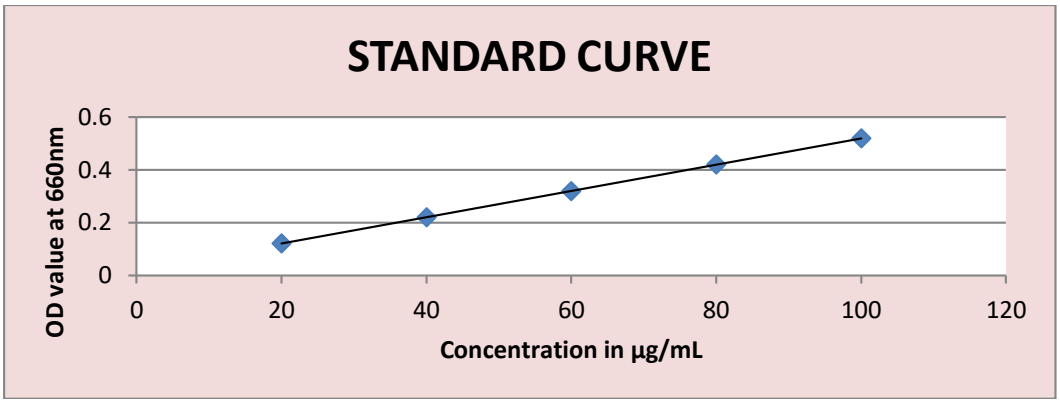
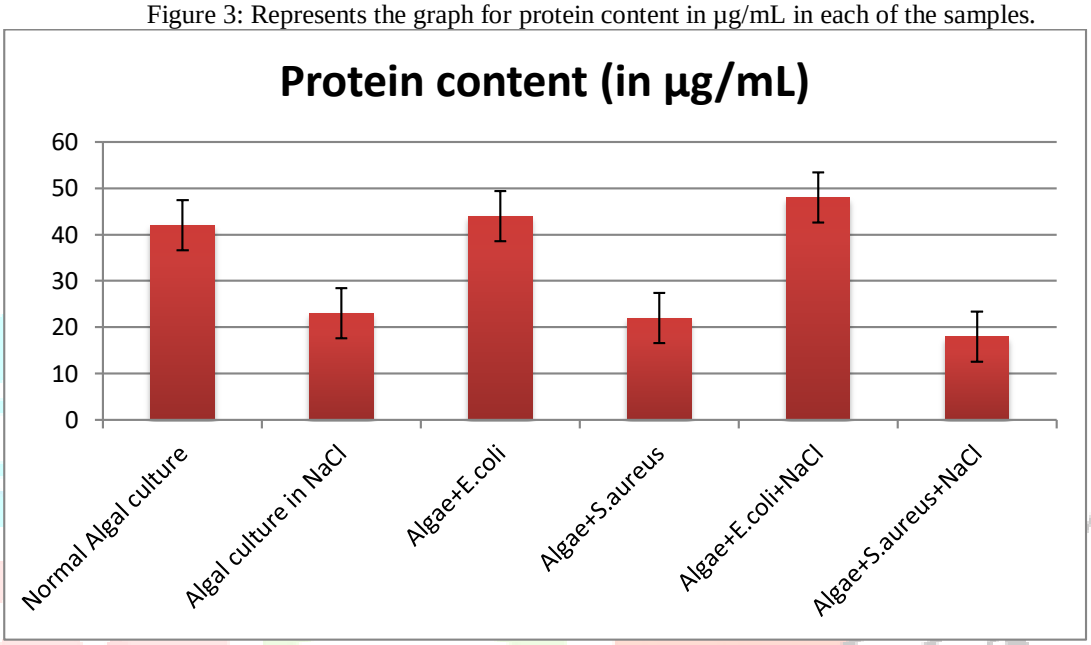
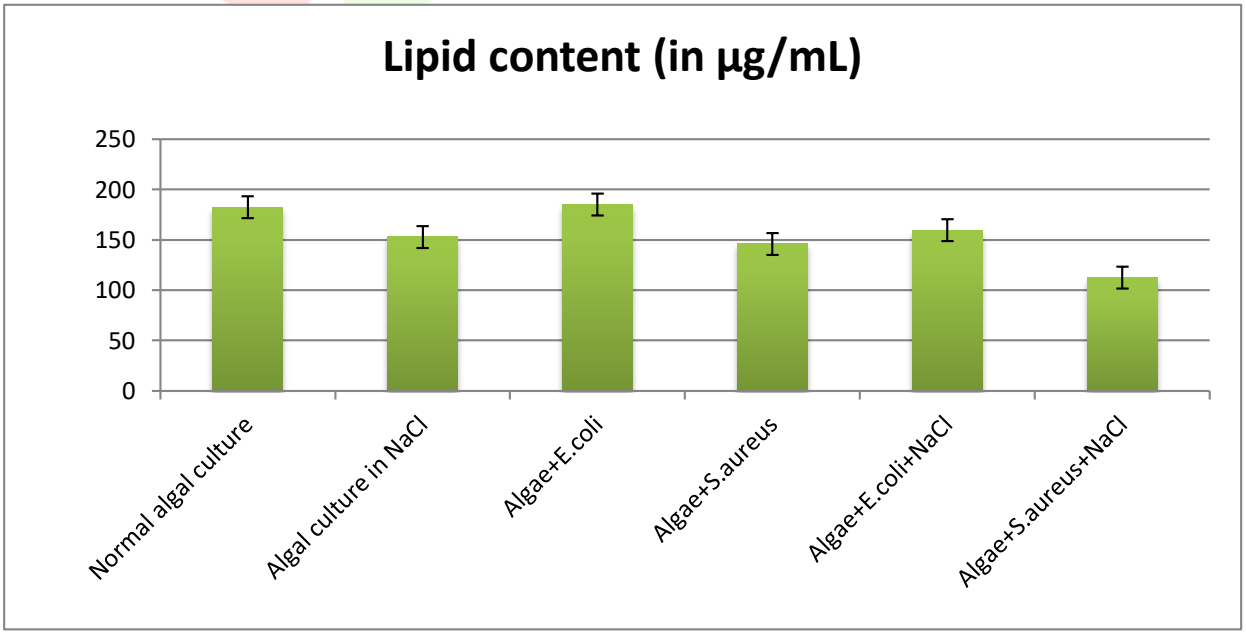


Figure 3: Represents the graph for protein content in µg/mL in each of the samples.



3.4 Quantitative estimation of lipids

Figure 4: Represents the graph for lipid content in µg/mL in each of the samples.



IV. DISCUSSION

The presence of *E.coli* as well as *S.aureus* increases the wet biomass of *Anabaena sp*. Each of the bacteria promotes algal growth as indicated from the in-vitro increase in the algal biomass. The protein content decreases in the presence of 1M NaCl alone due to osmotic stress, but increases when *E.coli* is acting synergistically with that of *Anabaena*. The presence of *S.aureus* does not show any significant change in the protein content. The lipid content increases in presence of *E.coli*, but gets affected by saline conditions. Whereas, the presence of *S.aureus* shows a non-significant decrease in the lipid content. When *E. coli* was grown presence of 1M Sodium chloride, it showed synergistic effect on algae by increasing the protein content. Presence of *E.coli* alone increased the lipid content of algae. The algal sample procures nourishment from the nutrients provided by the disintegration of the *E. coli* cell. But an *S. aureus* cell proves to be incompatible with that of *Ananbaena* sps. Hence, the lipid and protein contents show a minimal decreasing trend with that of *S.aureus* cultures.

The results of biomass estimation are precisely close to the findings of Roy.D et.al (2019) regarding the synergism of *Spirulina* sps., *Spirogyra* sps. and diatoms. But the results obtained with *S.aureus* differ from what has been reported till date. This particular strain does not occur with microalgae in natural habitats, which might be a possible reason for its incompatibility and antagonistic nature towards Anabaena sps. A similar study on nutrient profile showed that scetate and glucose as the most efficient carbon sources for promoting heterotrophic growth of *Chlorella vulgaris* and the carbon source, N and P were assimilated by both *Chlorella vulgaris* and *Bacillus licheniforms* in the *Chlorella vulgaris-Bacillus licheniformis* symbiotic system (Perez-Garcia et al., 2011). During this process, *Bacillus licheniforms* released CO2, while *Chlorella vulgaris* carried out photosynthesis, thereby achieving transition cycles of mass and energy between *Chlorella vulgaris* and *Bacillus licheniformi*, which is also the probable reason for the increase in the protein content in presence of *E.coli*. A few recent studies have shown that bacteria not only enhance algal growth but also help in flocculation, both essential processes in algal biotechnology. Hence, there is a need to understand these interactions from an evolutionary and ecological stand point, and integrate this understanding for industrial use. It is important to reflect on the diversity of such relationships and their associated mechanisms, as well as the habitats that they mutually influence, which can potentially be the future prospects of this study.

V. CONCLUSION AND FUTURE PROSPECTS

The available literature has clearly emphasized and proved beyond doubt that microalgae are efficient in nutrient removal from different types of wastewaters. But the difficulties encountered with the use of monocultures of microalgae, such as growth in diverse environments and harvesting problems highlight the need of a consortial approach. In such a situation, a symbiotic system of algae-bacteria may be a more effective alternative for wastewater treatment. Such consortia, especially showing synergistic interactions would have wider potential in treating different types of wastewater, than microalgal monocultures. Algae and bacteria have coexisted ever since the early stages of evolution these interactions in key evolutionary events such as endosymbiosis, besides their ecological role in biogeochemical cycles. 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. Hence, we conclude from this study that an artificial system between *E.coli* and *Anabaena* sps. can be successfully employed for various biotechnological process, including nutrition enhancement of the algal species, stress tolerance and better nitrogen fixation capacity. Future studies can be carried on from here on creating an artificial system by induction of microalgae and aerobic bacteria symbiotic systems for better biodiesel production from algae. Model systems of leguminous plants with Algae+Bacteria microzone for better yield and stress tolerance can hypothesized using similar interactions between other bacterial species and nitrogen fixing algal cultures, which do not occur in symbiosis in the natural environment. Molecular approaches can be also be undertaken to reduce environmental pollution using microalgae+bacteria synergy models, through relevant bioinformatic tools.

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