

Marine microalgae and their associated bacteria – A study on growth promotion

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ABSTRACT

Microalgae are the rich source of lipids. The interaction between algae-bacteria reveals the increased algal growth without any harmfulness from their associated bacterial consortia. Many previous investigations discussed that algae when co-cultured with bacteria reach higher cell numbers and exhibit faster growth rates. Likewise our present study aimed to focus the interaction between the green microalgae of *Nannochloropsis sp.*, *Synechocystis sp.* and *Pavlova sp.* and their associated strains *Staphylococcus simiae*, *Citrobacter freundii* and *Bacillus flexus* respectively. With respective to the co - cultivation system , growth rate of algae was monitored at various incubation periods of their corresponding bacterium of 24hr, 48hr,72hr. Hence the proximate analysis of growth rate, total biomass, lipid, protein and carbohydrate were measured before and after co cultivation. Thus enhance the microalgal biomass as well as lipid content. Likewise, lipid content of *Nannochloropsis sp.* (39%) would increase 45.4%. after the co- cultivation experiment likely Thus aids the efficiency of microalgae utilization in biomass/biofuel production in industrial scale at short time period by the influence of extracellular substances from their associated microbes.

1. Introduction

Marine ecosystems are connected through the networks by inter and intra species relationships at a range of spatial scales, includes mutualism, competition and parasitism (Estes *et al.*, 2011). Mostly the inter species interactions are indistinguishable under equilibrium conditions. Either the system must be disturbed or need to longtime observation to perceive the interactions (Armburst, 2009). The Interspecies interactions of bacterial association occur, where the stable platform facilities observed likely interactions of microbe-plant or microbe- mammal in terrestrial environments. In ocean stable platforms exist near – shore / intertidal areas or hydrothermal vents and sediments. However microalgae are the eukaryotic, photoautotrophs as primary producers in aquatic food webs and serves as the primary food source for zooplankton. Thus it is the base for all marine animals. Biotechnologically, algae were exploited for vast applications like biofuel production and high value products (Kouzuma, 2015; Sasso, *et al.*, 2012) to control pollution, food, feed additives, cosmetics, medicine production etc. Diverse studies have been carried out to improve the efficiency of micro algal cultures to ensure the higher valuable products (Adarme-Vega *et al.*,2012; Bougaran *et al.*,2010; EL-Sheekh *et al.*, 2012; Gonzalez, 2000; Sharma *et al.*, 2012; Cheng *et al.*, 2001). In past years, signaling response to chemicals or toxins regarding the association of micro algal and microbial communities were come into focus on production (Asker, *et al.*, 2007). Microalgae concurrently grow extraordinarily with high growth rate on light and chemical energy (Brennan, 2010; Burkholder, *et al.*, 2008; Lee, 2001; Liang, *et al.*, 2009; Yang, 2000). Many microalgae depends on vitamin B12 from heterotrophic bacteria in order to exchange for fixed carbon (EL-Sheekh, *et al.*, 2012).

The bacteria will compete with microalgae for nutrients, either organic or inorganic in mixotrophic culture system. However, the competition between the microalgae- microbial consortia can be abated by adding excessive nutrients or continuously adding nutrients (Subashchandrabose *et al.*, 2011). The growth performances of *Tetraselmis chuii*, *Cylindrotheca fusiformis* and *Nannochloropsis gaditana* were studied during co-culture period of 33 days, to select the microalgal growth promoting bacteria and to determine the feasibility of the bacterium (Lowrey *et al.*,1485; Suman *et al.*,2012;Xu, *et al.*, 2004).

Microalgal - Microbial communities provides the basic understanding of nutritional interdependence between them. Some terrestrial plants and marine diatoms can derive nitrogen as a source from their associated diazotrophic cyanobacteria (Foster, *et al.*, 2011). Haptophytes (prymnesiophytes) can also fixed nitrogen as a source with an associated unicellular diazotrophic cyanobacterium, (Thompson, *et al.*, 2012). In return for fixed nitrogen, the haptophyte partner provides an as-yet unidentified source of fixed carbon. Such symbioses between microalgae and nitrogen-fixing cyanobacteria are likely to be major determinants of marine productivity in oligotrophic waters. To compete other microalgae or cyanobacteria some microalgae produce negative allelochemicals with biofilms (Vanelislander, *et al.*, 2012). The bloom-forming haptophyte *Emiliania huxleyi* collaborates with α -proteobacteria of the Roseobacter clade to provide organic carbon and sulfur in the form of dimethylsulfoniopropionate (DMSP) in return for antibiotics effective against other bacteria (Seyedsayamdost, *et al.*, 2014). This mutualistic phase is terminated by a pathogenic phase involving the bacterial production of algicidal toxins. Such biphasic patterns may also govern the interactions of Roseobacter with dinoflagellates and

may cause natural patterns of algal bloom formation and collapse (Wang, *et al.*, 2014). Other antagonistic interactions include the encapsulation of haptophytes (*Phaeocystis spp.*) by *Acantharia*, a group of grazing zooplankton, in exchange for dimethylated sulfur compounds (Decelle, *et al.*, 2012). Physical association may also help to ensure generational persistence of partnerships.

Many microalgae depend on vitamin B₁₂ from heterotrophic bacteria in exchange for fixed organic carbon, which may affect the composition and productivity of microalgae-containing communities (Croft, *et al.*, 2005). Moreover mixed culture comprising B₁₂ auxotrophic greenalgae, *Lobomonas rosata* and a B₁₂-providing bacterium, *Mesorhizobium loti* at equilibrate cell ratio approximately 1:30 although the ratio could be vary when adding external carbon or vitamin B₁₂ (Kazamia, *et al.*, 2012).

A mixed culture comprising a B₁₂ auxotrophic green alga, *Lobomonas rostrata*, and a B₁₂-providing bacterium, *Mesorhizobium loti*, was found to equilibrate at a cell ratio of approximately 1:30, although this ratio could be altered by the addition of external carbon or vitamin B₁₂ (Kazamia, *et al.*, 2012). Some microalgae produce negative allelochemicals to compete with other microalgae or cyanobacteria with biofilms (Vanelander, *et al.*, 2012). Among other factors iron acquisition is the most predominant in algal-bacterial interactions. Iron is an essential element for photosynthesis and respiration but its poor solubility limits primary productivity and bacterial growth which resultant exceedingly low concentration (Coale, *et al.*, 1996; Bruland, *et al.*, 1991; Tortell, *et al.*, 1999).

Many marine heterotrophic bacteria and some cyanobacteria produce siderophores to improve the micronutrients. Siderophores are small organic molecules that tightly bind iron and thereby increase its solubility (Vraspir, *et al.*, 2009). The bacteria then take up the siderophores via outer-membrane transporters that are specific for different groups of siderophores. By contrast, eukaryotic phytoplankton are not known to produce siderophores or to directly take up bacterially derived Fe (III) siderophore complexes. However, many eukaryotic phytoplankton are able to access iron from siderophores or other chelates via ferric reductases and adjacent Fe (II) transporters on their outer cell membranes, for which genomic evidence exists in diatoms and green algae.

2. Materials and methods

Collection and maintenance of samples

The plankton sample collections were carried out using horizontal towing of phytoplankton net (Bolting silk cloth, 10µm) during early morning at Vellar estuary (Lat-11°29'55.0716"N, Long-79°46'28.2108"E). The collected samples were fixed in formalin with 4% buffered solution for qualitative and quantitative analysis of phytoplankton. On the other hand samples were transferred to filtered and sterilized seawater (30 Psu) with Guillard's F/2 medium and brought it to the laboratory immediately. In laboratory the phytoplankton soup was washed with sterile seawater and aseptically maintained using Guillard's F/2 medium under optimum

conditions of 25±2°C and 4000±500 Lux light intensity with 12:12 light and dark cycle.

Morphological identification

The phytoplankton samples were examined using zoom stereo microscope Olympus CX21i according to their morphological characteristics all the microalgal species were identified to their specific key characters with the help of standard taxonomic references as Venkataraman (1939), Subrahmanyam (1946), Prescott (1954) and Steidinger and Williams (1970).

Isolation of microalgae

Microalgae were isolated by continuous serial dilution methods. The mixed species of phytoplankton soup was inoculated into the 30 ml test tube containing 9 ml of F/2 medium up to 10⁻⁹ to isolate the single species. The isolation process was carried out until it reach to get monoalgal strains of targeting species (*Nannochloropsis sp.*, *Pavloval sp.*, *Synechosystis sp.*).

Cell count

Cell density was determined using manual cell counting (0.1 mm deep neubauer chamber) and measuring Optical Density using spectrophotometer (Spectronic 20, Genesys, Thermos, USA) at 650nm, 650nm, 695nm of *Nannochloropsis sp.*, *Synechosystis sp.*, *Pavloval sp.* respectively. Algal concentration was obtained by measuring the OD value of the culture during the lag, log and stationary phases.

Isolation and identification of associated bacteria

Microalgae in stationary growth phase (OD of 0.8 to 1.0) of three species were inoculated into fresh f/2 medium at a ratio of 1/10 (v/v) and cultured for 20 days. One mL of microalgal culture was diluted into 1000 fold with sterile f/2 medium. From the dilution mixture 1 ml was taken and spread plate into the bacterium solid culture medium of 1.8% agar containing sterile seawater medium and incubated at 37 °C for 24h. In total ± 5 colonies were picked up from each plate and inoculated into 3ml of nutrient broth (Seawater) and cultured at at 37 °C for 200 rpm (Shaker) for one day. After that, the 24 h culture was sub culturing to get 3hrs culture then it was streaked and cultured for 24-48hr. From that single colony was picked up and maintained for glycerol stock was stored at -80 °C in 20 % of glycerol (v/v). Bacterial identification was carried out using protein sequencing by MALDI-TOF (Microbiological Laboratory-Cuddalore).

Co-culture of algae along with bacteria

Initially cell density of mother inoculums were measured at stationary growth phase of each cultures namely *Nannochloropsis sp.*, *Synechosystis sp.*, *Pavloval sp.* was about 2.0 × 10⁷, 2.8 × 10⁵ and 1.9 × 10⁶ cells mL⁻¹, respectively, were inoculated into 100ml of fresh f/2 medium and cultured at 25 ± 1 °C and under 70 µmol photons m⁻² s⁻¹ irradiation under a light cycle of 12 h light and 12 h dark for 15 days. The final cell density was determined with a haemocytometer (Qiujiang, Shanghai, China).

100µl of bacterial isolates from associated microalgae maintained at -80 °C were inoculated into 250 ml of fresh Nutrient broth medium and cultured at 37 °C and 200 rpm for 24 h. The bacterial density was determined with the method described above. Afterwards 1ml of bacterial inoculum was mixed into 100ml of axenic microalgal culture. The microalgae/bacteria density was monitored every 2/3 days respectively, with the method described above.

Time course assay on growth of microalgae – bacteria

Time course assay for algae – bacteria growth was monitored by using six well assay plate of 4ml algal culture and 1ml bacterial culture at different time intervals (12hr , 24hr, 48 hr, 72hr & 96 hr) were inoculated. The experiments were monitored for 2-3 days and their cell densities were measured using Spectrophotometer.

Proximate analysis

Chlorophyll 'a'

Chlorophyll a content was measured using Strickland and parsons (1972). 5ml of 90% acetone was added into 2ml algal culture and vortex for one minute and kept at 4°C under dark for 24 hrs. The refrigerated samples were centrifuged at 5000rpm for 10 min and the supernatant was taken OD at 630nm, 645nm, and 660nm in UV-Vis spectrophotometer (Perkin-Elmer Lambda) 90% acetone used as a blank.

The chlorophyll 'a' content was determined by using the following formula.

$$\text{Chlorophyll 'a'} = 11.93 \times A_{664} - 1.93 \times A_{647}$$

Protein

The protein content was estimated by the following method of Lowry *et al.*, (1951). From this extract, 0.5ml of the sample was taken in a 10ml test tube and 5ml of reagent C was added. The solution was vortex well and made to stand for 10min.in darkness. Later 0.5ml of folin phenol reagent was added with vigorous mixing. The mixture was kept in dark for 30min.the sample was read at 660nm in spectrophotometer. Albumin as a standard.

Calculation

$$\frac{\text{OD of the sample} \times \text{Standard value}}{\text{Weight of the sample taken}} \times 100$$

Carbohydrate

The carbohydrate content was estimated by the method of Dubois *et al.*, (1956)an aliquot (100ul) of the supernatant diluted to 1ml with extraction buffer was mixed with 1ml of 5% phenol (aqueous w/v) and 5ml of concentrated sulphuric acid was added rapidly and mixed through and the tubes were incubated for 10min at 37°C. The color development was read at 490 nm using spectrophotometer. The reagents without the sample served as blank. The amount of sugar was estimated using a standard graph prepared using D-glucose at the range of 10-100µl/ml.

Calculation

$$\frac{\text{Standard value} \times \text{OD of the sample}}{\text{Weight of the sample taken}} \times 100$$

Lipid

Typically, cells were harvested by centrifugation at 8500 rpm for 5min and washed once with distilled water. After drying the samples using freeze drier, the samples were pulverized in a mortar and extracted using mixture of chloroform: methanol (2:1, V/V). About 50ml of solvents were used for every gram of dried sample in each extraction step. After stirring the sample using magnetic stirrer bar for 5hrs and ultra-sonication for 30min, the sample were centrifuged at 3000rpm for 10min. the solid phase was separated carefully using filter paper (Advantech filter paper, no.1) in which two pieces of filter papers were applied twice to provide complete separation using rotary evaporator at 40-45°C.

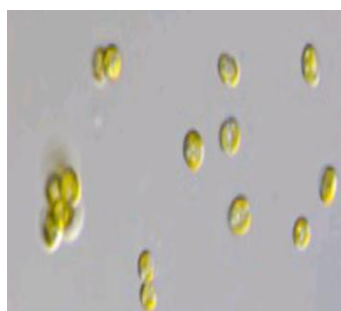
Calculation

$$\frac{\text{Amount of lipid in the sample}}{\text{Weight of the sample taken}} \times 100$$

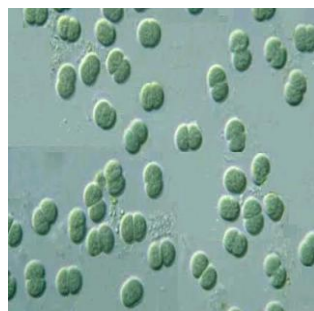
Isolation of microalgae



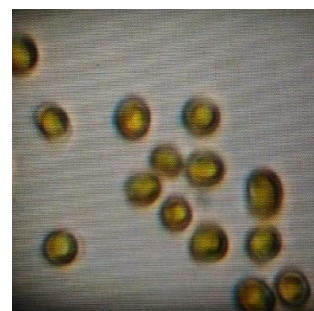
Fig.2 Isolation of monoclonal algal species



Pavlova sp.



Synechocystis sp.



Nannochloropsis sp.

Fig. 3 Algal species observed in 100x magnification

Isolation and identification of associated Bacteria

S.No	Name of the bacterial species	<i>Nannochloropsis sp.</i>	<i>Synechocystis sp.</i>	<i>Pavlova sp.</i>
1	<i>Citrobacter freundii</i>	+++	-	-
2	<i>Clostridium sordellii</i>	-	+	+
3	<i>Staphylococcus sp.</i>	+	+	+
4	<i>Staphylococcus simiae</i>	+	+++	+
5	<i>Salmonella sp.</i>	+	+	+
6	<i>Vibrio mytili</i>	+	+	+
7	<i>Vibrio parahaemolyticus</i>	+	+	+
8	<i>Bacillus flexus</i>	+	+	+++

Table.1.Total number of bacterial species isolated from the microalgae.

Cell density and growth of microalgae

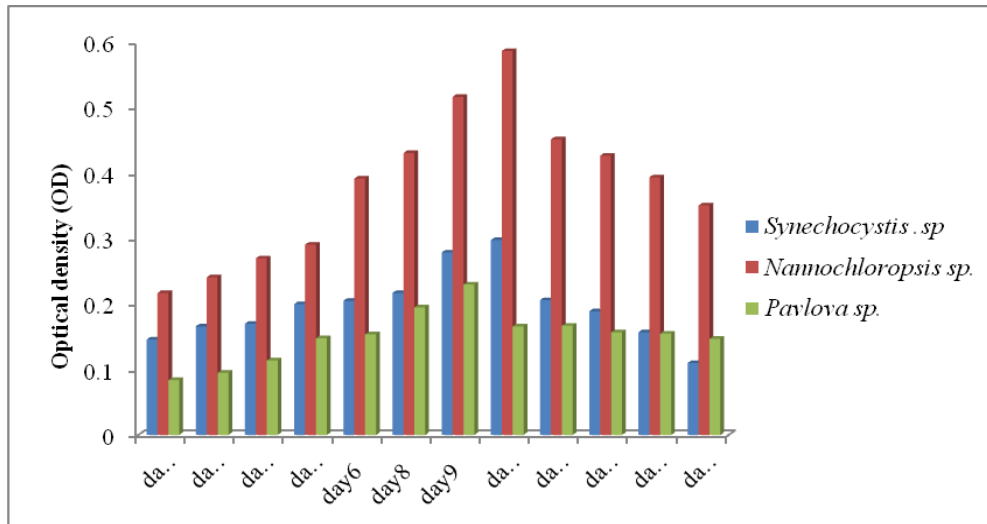


Fig.9 Growth chart of microalgae before co cultivation

S.No	Name of the species	No.of cells/ml
1.	<i>Nannochloropsis sp.</i>	1.37×10^6
2.	<i>Synechocystis sp.</i>	1.43×10^6
3.	<i>Pavlova sp.</i>	5.84×10^6

Table.2 Cell density of microalgal species

The graphical representation shows the microalgal growth before bacterial association. The *Synechocystis* growth are increased at day 10 optical density (0.6) was compared to day 1(0.1). *Pavlova*, *Nannochloropsis* were compared to the day 1

to day 14 growth and optical density. After day 10 the growth was reduced in the stationary phase decreasing the cell density.

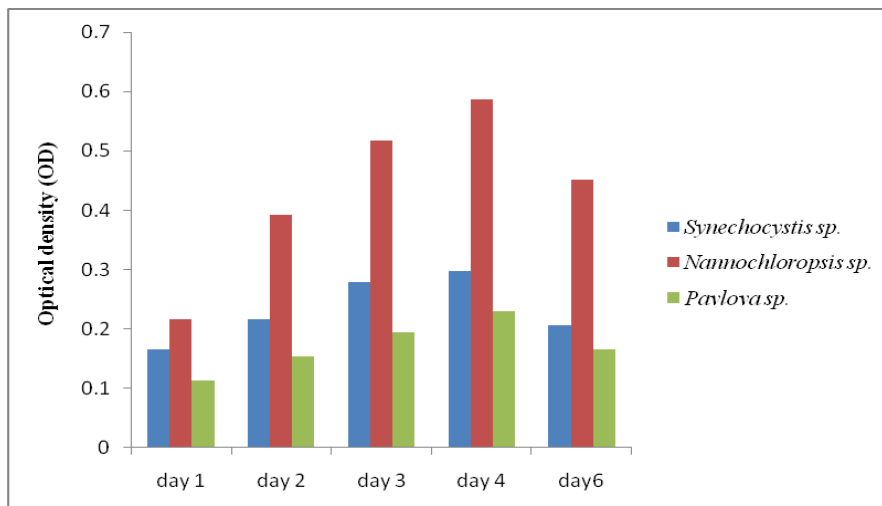


Fig.10 Growth of microalgal species after co cultivation

The graphical representation explains the microalgal growth after bacterial association. The growth was compared to the day 1 and day 4 for increased the cell density

Synechocystis (0.3), *Pavlova* (0.2) and *Nannochloropsis* (0.6) growth optical density was continuously increased in the day 4. After day 6 the cell density was reduced.

Time course assay for Co-cultivation:

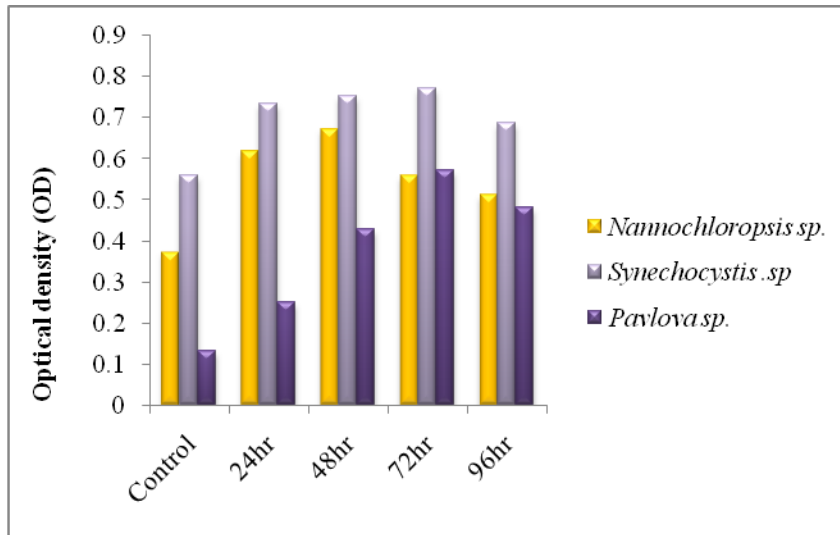


Fig.11 Time course assay for co-cultivation microalgae with associated bacteria

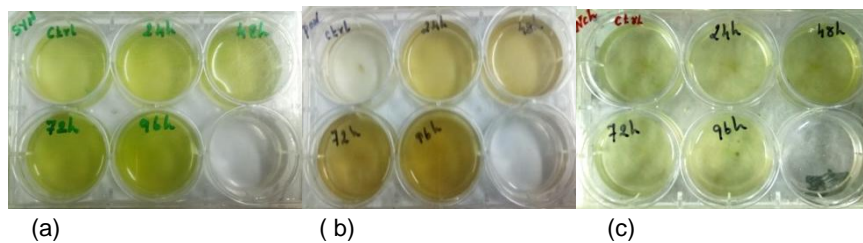


Fig.12 Co- cultivated algae with bacteria in the assay plates

The associated bacteria with cocultivated in the assay plates. The algal growth was observed in the 24 hours to 96 hours. (a) *Synechocystis sp.*with associated bacteria (b)

Pavlovasp. with associated bacteria (c) *Nannochloropsis sp.* with associated bacteria.

Proximate analysis:

Estimation of Total Protein, Carbohydrate and Lipid content:

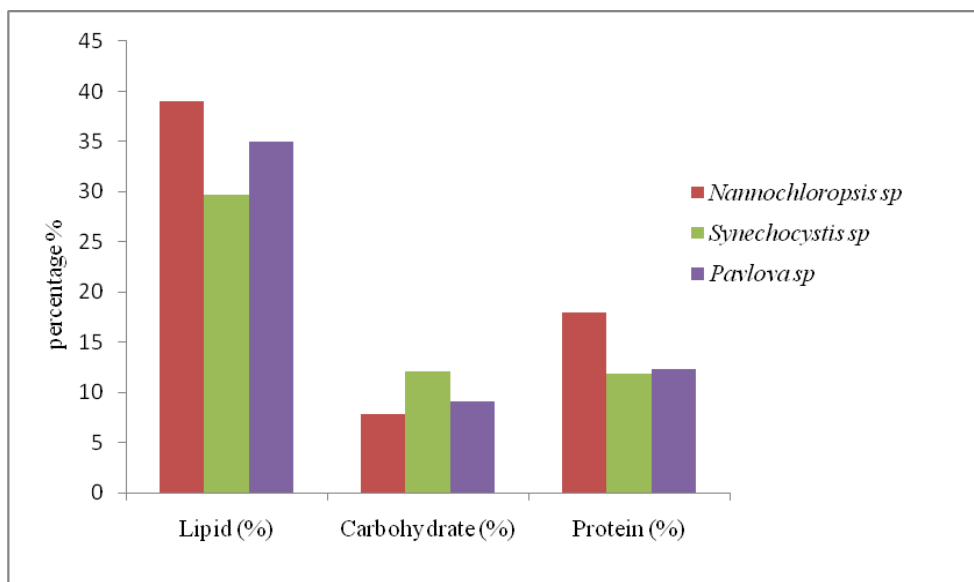


Fig. 13 Biochemical composition of microalgae before co cultivation.

S. No	Name of the species	Lipid (%)	Carbohydrate (%)	Protein (%)
1	<i>Nannochloropsis sp.</i>	39	7.8	18
2	<i>Synechocystis sp.</i>	29.7	12.1	11.8
3	<i>Pavlova sp.</i>	35	9.1	12.3

Table.3 Estimation of biochemical composition of microalgae

The Biochemical composition of Lipids, Carbohydrate and Protein contents of various microalgal species were estimated. The highest lipid and protein content was found in

Nannochloropsis sp. of 39% & 18% respectively. In *Synechocystis sp.* 12.1% of carbohydrate content was observed in maximum when compared to other two species.

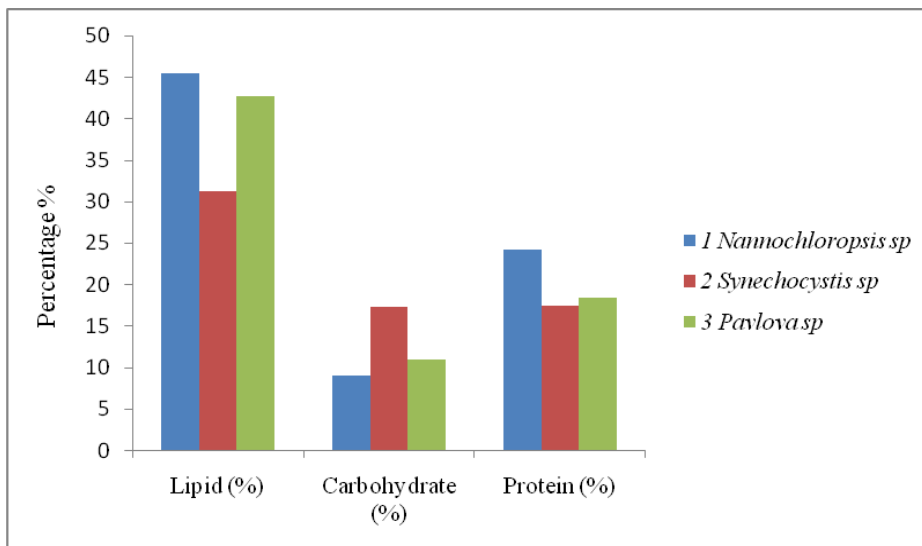


Fig. 14 Estimation of total lipid, carbohydrate and protein content of with bacterial association

S.No	Name of the species	Lipid (%)	Carbohydrate (%)	Protein (%)
1	<i>Nannochloropsis sp.</i>	45.4	9.12	24.2
2	<i>Synechocystis sp.</i>	31.3	17.4	17.5
3	<i>Pavlova sp.</i>	42.7	11	18.45

Table.4 Estimation of biochemical composition of microalge with bacteria

The biochemical composition of microalgal species with bacterial association was measured. Total lipid and protein content was found maximum in *Nannochloropsis sp.* of 45.4%

& 24.2% respectively. In *Synechocystis sp.* 17.4% of Carbohydrate content was observed maximum.

Estimation of chlorophyll a :

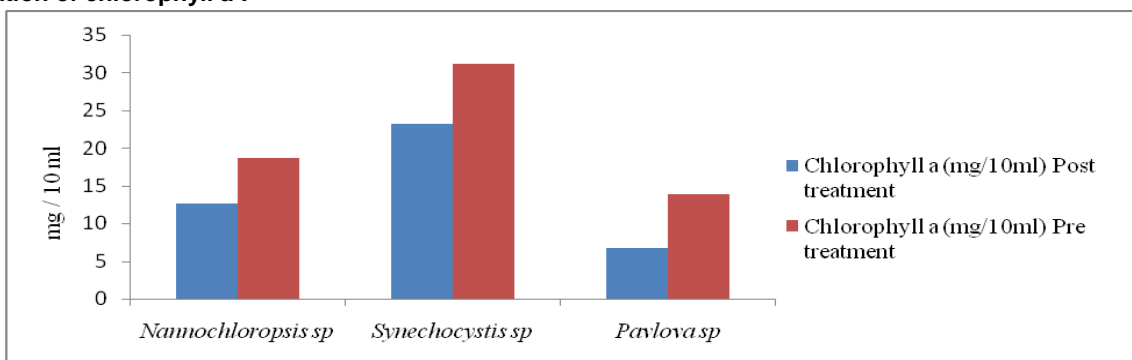


Fig. 15 The estimation of chlorophyll a content in the post treatment and pre treatment

S.No	Name of the species	Chlorophyll 'a' (mg/10ml)	
		Before co cultivation	After co cultivation
1	<i>Nannochloropsis sp.</i>	12.66618	18.6471
2	<i>Synechocystis sp.</i>	23.22827	31.21917
3	<i>Pavlova sp.</i>	6.71005	13.84115

Table.5 Chlorophyll content of microalgae before and after co-cultivation.

The chlorophyll a content of various microalgal species were measured. The highest Chl 'a' content was found in *Synechocystis sp* (Post treated) of 23.22827 (mg/10ml) and the pretreated of 31.21917 (mg/10ml).

3. Discussion

In this study, the positive effects of co-culture system increase the biomass of microalgae feasible and highly efficient. On the other hand, the results of our present study explore the specific bacteria (*Citrobacter freundii*, *Bacillus flexus*, *Staphylococcus simiae*) can drastically increase the growth of specific microalgae *Nannochloropsis sp.* *Synechocystis sp.* and *Pavlova sp.* The co-cultivation system with specific bacterium induces different effects on different microalgae has been reported earlier (Baggesen, 2014; Suminto Hirayama, 1997). Moreover the co-cultivation method of suitable environment can benefit the microbial growth e.g., increasing carbon dioxides, secreting vitamins and reducing photosynthetic oxygens (Croft, 2005; Fukami, et al., 1997).

In limnology chlorophyll 'a' concentration was most used variables to determine the phytoplankton biomass. In general, bacterial metabolism provides favourable microenvironments to enhance the growth of microalgae. e.g., increasing carbon dioxide, secreting vitamins and reducing photosynthetic oxygen tension (Croft et al., 2005; Fukami et al., 1997). The isolated microalgal species of *Nannochloropsis sp.* *Synechocystis sp.* and *Pavlova sp* were co-cultivated with symbiotic bacteria (*Citrobacter freundii*, *Bacillus flexus*, *Staphylococcus simiae*) enhance the algal biomass as chlorophyll 'a' respectively 18.6471(mg/10ml) 31.21917 (mg/10ml) 13.84115 (mg/10ml). Previous studies have proven that bacteria give high growth of algal biomass, so it is a benefit of the industrial production, mass cultivation and their further applications (Wang et al., 2015).

In this study microalgae – bacteria co-cultivation increase the biomass of economically important microalgae at short time period (3-4 days) instead of 8-10 days. Time course assay for this co-cultivation experiment was carried out at different bacterial incubation periods of 24 hr, 48hr, 72 hr, 96 hr. Thus explores the algal growth effectively in 48 hr *Staphylococcus simiae* co-cultivation with *Nannochloropsis sp.* at the same time 72hr incubation period of *Citrobacter freundii* and *Bacillus flexus* influence the algal growth of *Synechocystis sp.* and *Pavlova sp.* respectively. Whereas the reports on co cultivation in *Tetraselmis chunii*, *Nannochloropsis gaditana* and *Cylindrothecus fusiformis* with their associated bacteria shows influence on growth followed by inhibition except the *Nannochloropsis gaditana*. The

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extracellular compounds secreted by *Tetraselmis chunii* may also play a role on the continuous decline of bacterial density as it has been observed in other microalgae (Amaro, et al., 2011; Kokou, et al. 202; Naviner, et al. 1999).

Microalgae have been well-reviewed as a potential factory for lipid production for biofuels (Gong, et al. 2011). To reduce the cost of raw materials for biodiesel production, the co-culture system presented a proficient and safer alternative. Recently, *Chlorella sp.* U4341 and *Monoraphidium sp.* FXY-10, potential feedstock for biodiesel production. In the current studies on microalgae associated bacteria to improving the cell biomass and its using the valuable biodiesel production and the feedstock of animals and rotifers. In recent studies, B12-producing rhizobia improved the growth rate of *Chlamydomonas reinhardtii* during high temperature stress. Likewise the extra cellular compounds from associated bacteria improves the growth of microalgal species with respect to the incubation time of bacteria as it was in Table:5.

Among the inter-algal associations cited in literature, the assembly between microalgae bacteria is very advantageous at laboratory scale and it was investigated for many purposes, including wastewater treatment, metal, and nutrient pollutants removal (Subashchandrabose, et al., 2011). The phytoplanktons are naturally found in fresh water and aquatic environments, so they are remove the water pollution and waste water treatments. Besides, co-culture model was reported to lead higher release of free fatty acids (FFA) into the extracellular medium (DellaGreca, et al., 2010). The co-culturing was producing high amount of lipid into the F/2 medium. As discussed previously, during lipid production, the role of bacterium in co-culture model has aimed. Among the reported species, Chlorophyll 'a' of *Synechocystis sp* with bacterial association 31.21917 (mg/10ml) has higher than the chlorophyll 'a' of *Synechocystis sp* alone 23.2282(mg/10ml).

4. Conclusion

The results of our present study accomplishes the microalgal growth was enhanced by their associated microbes with the co-cultivation system. thus results the enhanced biomass as well as lipid content which is most predominant for algal metabolism. Algae- bacteria co-cultivation mainly focused to improve their growth short period would aid the largescale production in industry level. Moreover the positive effects of their associated microbes play a key role in future aspects of bio functional products in it

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