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Cultivation of efficient marine microalgae and their biochemical composition and its antibacterial activity against human pathogens

Abstract

The three marine microalgae (*Chlorella vulgaris, Spirulina platensis and Nannochloropsis bacillaris*) were collected from Vellar estuary, South east coast of India. These three microalgae were cultivated in respective media and estimated their biochemical composition and antibacterial activity. Simultaneously, these cultures were cultivated in flask containing 500 ml of respective media at lab condition for a period of one month and their growth, pH, biomass and CO_2 fixation and carbon content were determined. Based on the growth rate, the pH of three microalgae in media was observed at lab condition. During maximum growth and biomass, the pH was found to be ranged between 9 & 11 for *Spirulina platensis*, 7 & 9 for *Chlorella vulgaris*; 8 & 9 for *Nannochloropsis bacillaris*. *Spirulina platensis* and *Chlorella vulgaris* reached maximum growth rate whereas *Chlorella vulgaris* and *Spirulina platensis* showed maximum biomass produced. *Chlorella vulgaris* and *Spirulina platensis* attained maximum biomass in media at lab condition, also fixed highest level of carbon dioxide in media but they did not produce maximum biomass, though the growth of *Nannochloropsis bacillaris* were found high in media at lab condition. Among the three microalgae, *Chlorella vulgaris* and *Spirulina platensis* produced highest biochemical compounds. Hence *Chlorella vulgaris* and *Spirulina platensis* were selected as efficient microalgae for antibacterial activity against human pathogen. This study revealed that certain green algae and blue green microalgae having high growth, pH, CO_2 fixation, carbon content and biochemical composition paves the way for pharmaceutical activity. Antibacterial activity was evaluated for *Chlorella* and *Spirulina* withtheir potential health benefits.

Keywords: *Chlorella vulgaris, Spirulina platensis, Nannochloropsis bacillaris,* CO_2 fixation, Biochemical composition, Pharmaceutical activity

Volume 5 Issue 4 - 2017

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Received: December 08, 2016 | **Published:** April 07, 2017

Introduction

Ocean covers nearly 70% of earth's surface and possesses nearly three lakh described species of plants and animal from marine sources, representing 34-36 phyla and some of them are exclusively of the marine ecosystem. Microalgae are the most important and basic component in marine and fresh water ecosystem.[1] They are also the primary producers at the base of aquatic food chain and one of the first group be affected by metal pollution and bio indicators of various pollutants.[2]

Algae are known as the most primitive and fast-growing plants in worldwide and they are an important source of useful biomass. Algae do not have morphological features such as roots, stems and non-sterile covering of reproductive cells.[3] Algae have chlorophyll-'a' as the primary photosynthetic pigment for them to convert light energy into chemical energy by the process of photosynthesis.[3] According to Brennan et al.,[4] algae have the capability of trapping energy from sunlight ten times higher than the terrestrial plants. However, some of the species are not able to photosynthesize but live in a parasitic form. Algae are found in a wide range of habitats including marine and freshwaters, deserts and even in snow and ice.

There are fresh water and marine microalgae which have high photosynthetic efficiency and possess high cell division rate compared to terrestrial plants. Therefore, they absorb more carbon dioxide by fixation to produce higher biomass at increased growth rates.[5,6] There are various carbon dioxide sources that may be used for the fixation process which include industrial waste gases, emitted gas from power plants and from the atmosphere.[7] Some microalgae have higher potential to survive and are capable of fixing carbon dioxide under extreme conditions such as low pH and acidic gases compared to the terrestrial plants that exhibit fatal effects at these conditions.[8]

Marine microalgae *Chlorella vulgaris* and *Spirulina platensis* was capable of producing valuable metabolites, such as proteins, carbohydrates, lipids and vitamins for feed additive, pharmaceutical and nutraceutical purpose. They have diversified use ranging from supply of fatty acids and vitamins for fish in aquaculture systems and the nutritional value of microalgae is influenced by their size, shape, digestibility and biochemical composition.[9] The production of planktonic organisms in good nutritional condition to feed fish larvae and fingerlings is a basic requirement in a vast majority of farms to add organic and chemical fertilizers into the hatchery ponds.[10]

The biochemical composition in algae can change with altering environmental conditions. In nature, changes in phytoplankton community can modify the food quality and quantity.[11] The effect of these variations for asset of algae species has been exposed by understanding algal physiology. This pool probably reflects the balance between release and uptake processes, due to the physiological activity of bacteria and phytoplankton biomass and free amino acids in seawater are sometimes not correlated, or may be negatively correlated.[12]

To determine the phytochemicals and screen the antimicrobial potential of *Desmococcus olivaceus*(*D. olivaceus*) and *Chlorella vulgaris* (*C. vulgaris*) against human bacterial pathogens.[38] The present study was aimed to isolate green microalgae *Chlorella vulgaris* from the Pichavaram Mangrove Forest, South East coast of India. After being isolated, they were confirmed through morphological structures of microalgae *C. vulgaris* on cultivation of two different medias. Phytochemicals like phenol, tannins, flavonoids, terpenes, terpenoids, alkaloid and saponins were present in the dried biomass and antibacterial activity was showed better results to control infectious human pathogens[37] .However, there are no reports regarding the antibacterial compounds of microalgae against human pathogens except.[13] The antibacterial study is desirable not only to contribute towards an understanding of ecological interactions but also to assess the potential of algal antimicrobial activity and their possible therapeutic value. The future role of microalgal compounds in drug discovery is especially in the priority areas for development of new medicines, namely to fight viral infections, cancer and combat infections from antibiotic resistant bacteria and fungi.[14]

In view of the above, this study was undertaken with the aim to evaluate three marine micro-algae (*Chlorella vulgaris, Spirulina platensis, Nannochloropsis bacillaris*) cultivation and biomass harvested from medium (at laboratory condition), analysis of growth factor and also to apply for carbon dioxide sequestration, biochemical composition estimation and anti-bacterial activity of against human pathogen from selected two efficient marine microalgae (*Chlorella vulgaris* and *Spirulina platensis*).

Materials and methods

Sample Collection and Isolation

The marine algae were isolated from Vellar estuary, Parangipettai, south east coast of India, Tamilnadu. Green algae and Blue-green algae were isolated by serial dilution method which was developed in the microalgae Culture Laboratory, Faculty of Marine Sciences, Annamalai University Parangipettai, India. The species were identified using the morphological characters of green algae *Chlorella vulgaris, Nannochloropsis bacillaris* and *Spirulina platensis*.[15]

Stock Culture Maintenance

All the three strains were grown in 250 ml conical flasks containing 90 ml medium added with 10 ml (10%) of inoculum. All the cultures were maintained in an incubator shaker set at 100 rpm at 25°C±1. The cultures were illuminated with cool fluorescent lamps with the irradiance of 42 µmolm-2s-1 on 12:12 hour light dark- cycle. The stock cultures were maintained by sub culturing into new medium every two months.

Preparation of Inoculum

The inoculum was prepared from the maintained stock culture for further cultivation. The inoculum was obtained from exponential phase cultures standardized at an optical density of 620 nm.

Optical Density

The optical density of the culture was determined by taking about 2 ml of the sample from the placed in a cuvette. Then, the optical density of each sample was measured for every two days throughout the growth period[16] at 620 nm using Shimadzu UV-Vis Spectrophotometer.

Estimation of pH

pH of the media was determined using digital pH meter.

Determination of Total Biomass

At the final day of growth, the biomass was harvested by flocculation using alum and it was filtered, allowed to dry under room temperature. The weight of the petriplates was calculated initially to avoid numerical errors. The filtered biomass was kept in sterile dried petriplates were weighed initially and calculated the initial weight (fresh weight or wet biomass). After, it was allowed to dry under (sun) light and the dried biomass in the petriplate was weighted (dry weighted).[17]

The total biomass can be calculated by using the formula as follows, Total biomass = Dry weight - Initial weight (wet biomass)

Preparation of Algal extract

After freeze-drying, the culturedmicroalgae were grounded into fine powder and each of the materials was homogenized separately. Then, the homogenized microalgae samples were sonicated at 25°C for 90 min, 3 times each using 80% methanol. Crude methanol extracts were concentrated by evaporating the solvent under reduced pressure using rotary evaporator and further subjected to solvent-solvent partition chromatography. Prior to the *in vitro* assays, the solvent extractions from the microalgae were prepared in 100 mg mL-1 concentration, each using Dimethyl Sulfoxide (DMSO).[18]

Estimation of carbon content and carbon dioxide fixation rate

Dried algal (0.2 mg) samples were placed in 500 ml conical flask and 10 ml of 1N potassium dichromate and 20 ml of conc. H_2SO_4 mixture was diluted with 200 ml of distilled water and 10 ml of hypophosphate (H_3PO_4) and 1 ml of diphenyl amine was added. Finally it was titrated against 4 N ferrous ammonium sulphate (FAS). The end point was brilliant green colour appeared. The carbon content was estimated using the following formula:

$$A=3.951/g\ (1-T/S)$$

where, A is carbon content, g is weight of the sample, T is FAS with blank (ml) and S is FAS with sample (ml). The amount of carbon dioxide fixation rate was estimated using the formula of.[19]

$$R\ CO_2=Cc\times\mu L\ (Mco2/Mc)$$

Where, R CO_2 and µL are the CO_2 fixation rate (g CO_2 $m^{-3}h^{-1}$) and the volumetric growth rate (g dry weight $m^{-3}h^{-1}$) respectively in the linear growth phase. MCO_2 and MC represented the molecular weights of CO_2 and elemental carbon respectively, CC is average carbon content (algal dry weight/ g).

Analytical Methods

The following parameters were determined for all the three microalgae strains: chlorophyll 'a' content, carotenoid content, biomass and biochemical composition such as protein, carbohydrate and lipid contents and the distribution of fatty acids.

Biochemical composition

Biochemical composition analysis viz. for protein, dry matter content was done by following standard methods. Protein,[20] Carbohydrate,[21] Carotenoid and Chlorophyll 'a'[23] and lipid were analyzed following the methods of.[24] Triplicate samples were analyzed and the average values were taken.

Antibacterial Assay

Antibacterial activity was carried out by using the standard disc diffusion method by Matsunaga.[25] The test microbial pathogens were obtained from Rajah Muthaiah Medical College, Annamalai University and were *Klebsiella pneumoniae, Proteus mirabilis, Vibrio cholerae, Salmonella typhi* and *Escherichia coli.* The crude and fractionated extraction of *Chlorella* and *Spirulina* were dissolved in different solvents like Ethanol, Methanol, Chloroform and Diethyl ether. The extracts were applied to 6 mm dry sterile disc in aliquots of 30µL of solvent, allowed to dry at room temperature and placed on agar plates seeded with microorganisms. The bacteria were maintained on nutrient agar plates and incubated at 37oC for 24 hrs. Zones of growth inhibition were measured after incubation from all the extracts and tested twice at a concentration of 30 mg $disc^{-1}$

Results and discussion

The pH of three micro algae grown in medium at lab condition

The present investigation is carried out with the parameters viz. pH, growth rate, Biomass and CO_2 fixation and carbon content rate of three marine microalgae (*Chlorella vulgaris, Nannochloropsis bacillaris* and *Spirulina platensis*).

The pH was found to be at the range between 8 and 10 in medium inoculated with *Chlorella vulgaris,* 8 with *Nannochloropsis bacillaris* and 9-11 with *Spirulina platensis*.

Optimum pH for most cultured algal species ranges between 7 and 9, with the optimum pH being 8.2-8.7. The complete culture collapses due to the disruption of many cellular processes result from failure and maintain an acceptable pH. In the case of high intensity algal culture, the addition of carbon dioxide allows correction for increased pH which may reaches the limiting values of pH during *Chlorella vulgaris* and *Nannochloropsis bacillaris* growth.[26]

The growth rate of three micro algae in medium at lab condition

The growth rate found to be at range between OD at 1.28 and 2.93 in *Chlorella vulgaris,* maximum range between OD at 0.606 and 2.19 in *Nannochloropsis bacillaris* and thehighest growth rate was observed at the OD value of 3.29 during 28th day in *Spirulina platensis*.

Biomass of three microalgae in medium at lab condition

The highest biomass was obtained in *Spirulina platensis* (0.585g/500ml) followed by *Chlorella vulgaris* (0.448 5g/500ml) and *Nannochloropsis bacillaris*produced low biomass (0.429g/500ml). The harvesting, thickening and dewatering of microalgae cultures have been extensively reviewed by Kaur I.[27] Key properties of microalgae which influence their separation are (a) shape [rods, spheres or chains or filaments], (b) size [generally between 2 and 30 µm], (c) specific weight [1.05–1.1], (d) surface charge [usually negative]. Microalgal cultures to be harvested usually between 0.2 to 2 g L^{-1} solids and for lipid extraction a concentration of at least 20 g L^{-1} solids is required. Filamentous algae such as *Spirulina* can be harvested by filtration,[29] but almost all of the algae under consideration as a source of biofuels (e.g., *Nannochloropsis* or *Chlorella*) are unicellular and too small for effective filtration. Centrifugation is too energy intensive[26] and not practical for the extremely high volumes required to be processed for algal biofuels production. Sedimentation is also a possibility, but is generally too slow to be effective.[27, 28]

The most commonly considered processes are flocculation followed by floatation or by settling as the first step. Flocculation is the first stage in the bulk harvesting process and is used to aggregate the cells, so increasing their effective particle size and thus easing subsequent centrifugation, filtration or sedimentation steps.[29] Flocculation can be achieved by the use of inorganic flocculants such as alum[27-29] or organic flocculants such as chitosan[16] although the cost of these flocculants is substantial. The flocculant used must be compatible with the need to recycle the water back to the growth system without complex pre-treatment of this recycled water.

Carbon content and CO_2 fixation rate by microalgae biomass in medium

The biomass of *Chlorella vulgaris* contain maximum carbon content (22.874 mg/g) followed by *Spirulina platensis* (19.367 mg/g). Among the three microalgae, *Chlorella vulgaris* (0.083 $gml^{-1}d^{-1}$) and *Spirulina platensis* (0.070 g $ml^{-1}d^{-1}$) fixed highest level of CO_2 whereas *Nannochloropsis bacillaris* fixed low level of CO_2 (0.056 $gml^{-1}d^{-1}$).

Highly CO_2 tolerant microalgae and cyanobacteria suitable for biological fixation of CO_2 are *Anacystis, Botryococcus, Chlorella, Rhodobacter, Scenedesmus, Spirulina* and *Synechococcus*.[31]

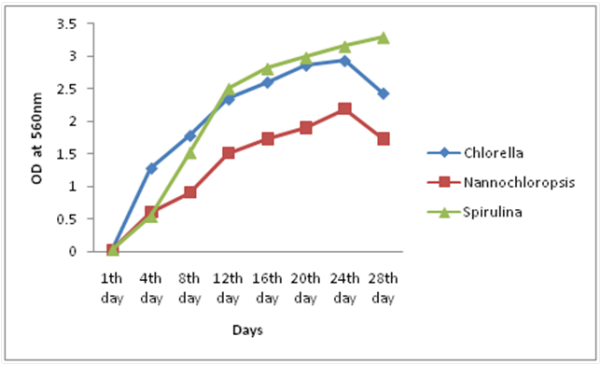

Figure 1 Growth rate of microalgae in medium at lab condition.

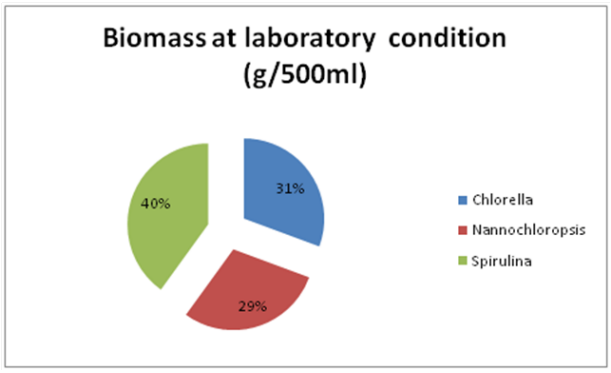

Figure 2 Biomass of three microalgae in medium at lab condition.

Bioactive molecules of pharmaceutical activities of efficient selected microalgae

The collected three microalgae were found to be grown in media, the maximum growth rate and biomass were obtained only in *Chlorella vulgaris* and *Spirulina platensis* not in *Nannochloropsis*

bacillaris. Hence, *Chlorella vulgaris* and *Spirulina platensis* were selected and its growth and biomass production on the basis of efficiency for bioactive molecules of pharmaceutical activity when compared to *Nannochloropsis bacillaris*.

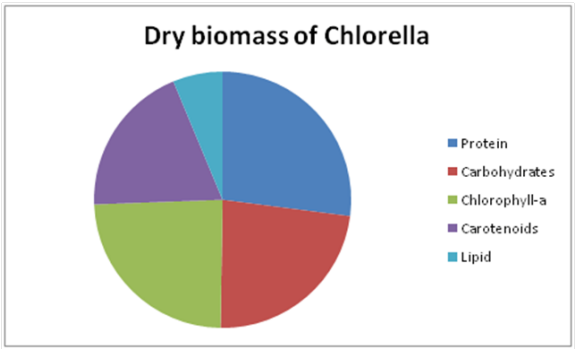

Figure 3 Biochemical composition of *Chlorella vulgaris* dry biomass.

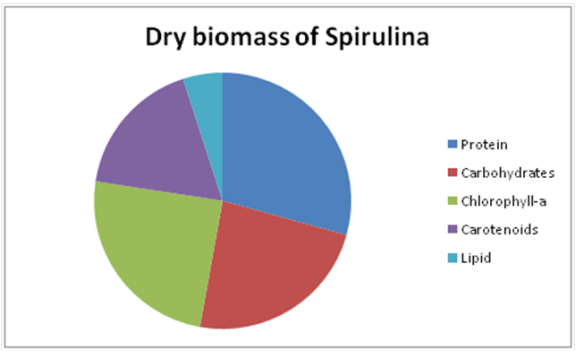

Figure 4 Biochemical composition of *Spirulina platensis* dry biomass.

Biochemical composition of *Chlorella vulgaris* dry biomass

The protein content showed 44.31±1.33 mg/g and carbohydrate holds 38.09±0.92 mg/g. The total chlorophyll 'a' was also analyzed and it was found to be 39.64±0.81 mg/g. The carotenoids were analyzed and showed 31.63±0.79 mg/g dry biomass. The lipid content of *Chlorella vulgaris* obtained from the dry weight as 10.3±0.12 mg/g.

Biochemical composition of *Spirulina platensis* dry biomass

The protein content showed 48.63±1.81 mg/g dry biomass and carbohydrate content showed 39.09±1.92 mg/g. The total chlorophyll 'a' was also analyzed and it was found to be 40±1.73 mg/g. The carotenoids were analyzed and it showed 29.18±0.93 mg/g. The lipid content of *Spirulina platensis* obtained from the dry weight as 8.3±0.16 mg/g.

Anti-Bacterial activity assay

The results obtained from the present study were recorded and analyzed using different solvent against gram negative human pathogens. It is clear from the study that the diameter of the inhibition zone varies with the type of the solvent used and hence varies in anti-bacterial activity (Table 1&2).

Anti-bacterial activity of *Chlorella vulgaris* against human pathogen

In the present study, *Chlorella vulgaris*was tested for the anti-bacterial activity in crude ethanol, methanol, chloroform and diethyl ether. It showed moderate anti-bacterial activity against five pathogens viz. *Klebsiella pneumoniae, Proteus mirabilis, Vibrio cholerae, Salmonella typhi* and *Escherichia coli* were assayed (Table 3). Of the test bacteria, *Salmonella typhi* and *Vibrio cholerae* was the most sensitive against ethanol and methanol extracts followed by 9.0±0.7 mm and 8.5±0.5 mm respectively. In test for *C. vulgaris*, gram negative bacteria *Salmonella typhi* treated with ethanol extract showed the maximum inhibition zone (13.0 mm), whereas in gram positive bacteria, *C. botulinum* treated with chloroform extract of *C. vulgaris* showed the maximum inhibition zone of 15 mm.[37]

The antibacterial activity of *Chlorella vulgaris* extracts were assayed against bacterial strains viz. *Bacillus subtilis, Staphylococcus aures, Micrococcus cuteus* and *Klebsiella pnemoniae* by evaluating the inhibition zones and zone diameter values *S. typhi* is a gram negative rod shaped bacteria, a causative agent for enteric fever, sepsis and infectious diarrhea in human beings. The ethanolic extracts of *Chlorella* showed the antibacterial activity against the human pathogen *S. typhi* and hence it recommends that in adding up to the available drugs, the extracts of *Chlorella* can be used against the enteric fever, septic and infectious diarrhea or gastro enteritis. It is also suggested that the extracts of *C. vulgaris* may be used to treat urinary tract infections, diarrhea pyogenic infections and septicemia. The ethanol extract of *Chlorella* showed anti-bacterial activity against *B. subtilis*. Hence, the extract is recommended to treat infections like *Bacillus subtilis* and *S. typhi*. Generally *Chlorella* were found to be effective against only on two pathogen and the anti bacterial activities was found to be dose dependent the phenomenon was in agreement with the findings of.[32] After being isolated, they were confirmed through morphological structures of microalgae *C. vulgaris* on cultivation of two different Medias. Phytochemicals like phenol, tannins, flavonoids, terpenes, terpenoids, alkaloid and saponins were present in the dried biomass and antibacterial activity was showed better results to control infectious human pathogens.[38]

Anti bacterial activity of human pathogen from various extracts in *Spirulina platensis*

In the present study, *Spirulina platensis* was tested for the anti-bacterial activity in crude ethanol, methanol, chloroform and diethyl ether. It showed moderate anti-bacterial activity against five pathogens viz. *Klebsiella pneumoniae, Proteus mirabilis, Vibrio cholerae, Salmonella typhi*and *Escherichia coli* were assayed (Table 4). Of the test bacteria, *Klebsiella pneumoniae* and *Proteus mirabilis* were the most sensitive against ethanol and diethyl ether extracts with 10.0±0.8 mm and 7.0±0.6 mm respectively followed by *Proteus mirabilis* and *Vibrio cholerae.* In chloroform extract, *Klebsiella pneumoniae* was the most responsive one. The result showed the highest zone of inhibition was observed in *Spirulina platensis* and the lowest zone of inhibition was observed in *Chlorella vulgaris.*

Extracts of *Spirulina platensis* obtained by different solvents exhibited different degrees of antimicrobial activity on gram negative micro organisms.[33,34] While diethyl ether, ethanol and methanol were the best organic solvents for extracting the antibacterial agents from *Spirulina plantensis* in the present evaluation. Statistically, the effects of the three diethyl ether, ethanol and methanol were insignificant. Comparatively diethyl ether and ethanol and methanol showed a marked activity against *Klebsiella pneumoniae* followed by *Proteus mirabilis* and *Salmonella typhi* exhibiting 10 mm, 8 mm and 7 mm of inhibition zone respectively. The same results were also reported by other workers of.[35,36]

The present study indicated that the antibacterial property of the two algal species against the selected strains of human pathogenic bacteria varies depending upon the solvent medium used for extraction. The most sensitive bacteria are *Salmonella typhi, P. mirabilis* and *Klebsiella pneumoniae* which were inhibited by ethanol and diethyl ether extracts of *C. vulgaris*. *Proteus mirabilis* and *Salmonella typhi* were inhibited by ethanol, methanol and chloroform extracts of *S. platensis*.

Table 1 pH of three micro algae grown in medium at lab condition

Algae	1th day	4thday	8thday	12thday	16thday	20thday	24thday	28thday
Chlorella vulgaris	7.8	8.4	8.9	8.9	9.1	9.9	10.1	10.3
Nannochloropsis bacillaris	7.4	7.8	8.0	8.3	8.3	8.5	8.6	8.9
Spirulina platensis	7.9	8.8	9.1	9.8	10.1	10.6	10.9	11.3

Table 2 Carbon content and CO_2 fixation by three microalgae biomass

Parameter	*Chlorella vulgaris*	*Nannochloropsis bacillaris*	*Spirulina platensis*
Carbon content (mg g^{-1})	22.874	15.521	19.367
CO2 fixation (g ml^{-1} d^{-1})	0.083	0.056	0.070

Table 3 Anti bacterial activity of various extracts in *Chlorella* against human pathogen

S.No	Name of the Human Pathogen	Concentration/Zone of Inhibition (mm)			
		Ethanol Extract	Methanol Extract	Chloroform Extract	Diethyl Ether Extract
1.	*Klebsiella pneumoniae*	7.0±0.5	6.0±0.3	7.0±0.4	5.0±0.5
2.	*Proteus mirabilis*	5.0±0.6	6.0±0.5	--	8.0±0.5
3.	*Vibrio cholerae*		2.0±0.7	8.0±0.5	--
4.	*Salmonella typhi*	9.0±0.7	--	--	--
5.	*Escherichia coli*	8.0±0.6	8.5±0.5	--	5.0±0.6

Table 4 Antibacterial activity of various extracts in *Spirulina platensis* against human pathogen

S.No	Name of the Human Pathogen	Counteraction/Zone of Inhibition (mm)			
		Ethanol Extract	Methanol Extract	Chloroform Extract	Diethyl Ether Extract
1	*Klebsiella pneumoniae*	10.0±0.8	5.0±0.5	6.0±0.3	6.0±0.5
2	*Proteus mirabilis*	4.0±0.9	3.0±0.2	--	7.0±0.6
3	*Vibrio cholerae*	2.0±0.3	--	--	--
4	*Salmonella typhi*	8.0±0.6	--	5.0±0.3	--
5	*Escherichia coli*	5.0±0.4	6.0±0.3	6.0±0.3	--

Conclusion

Microalgae are known to be the most biochemical composition and bioactive compound source that has high potential for sequestering carbon dioxide, a greenhouse that leads to global warming. They are extensively exploited for their high lipid production, maximum biomass production and other useful compounds that have great significance in industry and pharmaceuticals. Bio-mitigation of carbon dioxide by the microalgae is one of the promising ways of bioremediation as it can reduce the elevated level of carbon dioxide in the environment.

Microalgae have the potential to change the pharmaceutical industry, providing a solution to transform the existing systems for antibacterial, anti oxidant and anticancer activity and enabling new applications of existing technologies, provided that one can improve its production cost to a point competitive with pharmaceuticals activities.

Acknowledgements

Authors are thankful to Dean and Director, CAS in Marine Biology, Faculty of Marine Sciences, Parangipettai for providing necessary lab facilities. We are also grateful to the UGC-RGNF; JRF Govt. of India for the financial assistance (Name of the awardee R. Dineshkumar Award letter number and date of UGC Circular number and date F1-17.1/2015-16/RGNF-2015-17-SC-TAM-24190 & January 2016).

Conflicts of interest

None.

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