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Research Article

Isolation of fresh water microalgae C*hlorella sp* and its antimicrobial activity on selected pathogens

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Abstract

The present study was started to isolated and optimize the fresh water algae Chlorella sp. cultivated under controlled conditions. Isolation of *Chlorella* sps was carried out in the laboratory from fresh water using BG 11 medium . Then it was optimized the growth using various medium including Bold basal medium, CFTRI medium, Bangladesh M3 and Zarrouk's medium etc. The Mass cultivation of *Chlorella* was further carried under controlled conditions using BBM. The analysis of Chlorophyll a and b was performed and also the Phytochemical analysis of ethanolic extract of Chlorella sp. was done. Finally Bacterial susceptibility testing using algal extracts by AWD assay was performed.

Keywords: Chlorella sp. BG 11 medium, Bold basal medium, CFTRI medium, Bangladesh M3 and Zarrouk's medium, Phytochemical analysis, Bacterial susceptibility test.

Introduction

Microphytes or microalgae are microscopic algae, typically found in freshwater and marine systems. They are unicellular species which exist individually, or in chains or groups. Depending on the species, their sizes can range from a few micrometers (µm) to a few hundreds of micrometers. Unlike higher plants, microalgae do not have roots, stems and leaves. Microalgae, capable of performing photosynthesis, are important for life on earth; they produce approximately half of the atmospheric oxygen and use simultaneously the greenhouse gas carbon dioxide to grow photoautotrophically. The biodiversity of microalgae is enormous and they represent an almost untapped resource. It has been estimated that about 200,000-800,000 species exist of which about 35,000 species are described. Over 15,000 novel compounds originating from algal biomass have been chemically determined (Cardozo et al. 2007). Most of these microalgae species produce unique products like carotenoids, antioxidants, fatty acids, enzymes, polymers, peptides, toxins and sterols.

Microalgae are a good source of feedstock for the production of renewable energy because of their rapid growth rate. The exploitation of the alga *Chlorella* sp. as feedstock using different animal (goat, cow, pig, grass cutter and poultry) waste as growth medium was investigated. A *Chlorella* sp.

isolated from a fresh water pond was grown in the laboratory under different conditions (artificial: aerated /unaerated; natural: sunlight). The algal growth under natural illumination resulted in a higher biomass and lipid yield than that from the artificial illumination. The poultry waste under sunlit conditions gave the highest biomass yield of about 2.5g/l and 18.32% (w/w) of lipids in wet cells under mixotrophic conditions of growth. Mixotrophic conditions of growth are relatively less expensive to maintain. Biomass from the *Chlorella* sp. using inexpensive growth media formulations such as animal waste is an attractive source of valuable substrates for the neutraceutical, pharmaceutical and biochemical industries including biofuel industries(Agwa *et al*., 2012).

Microalgae have been widely used as novel sources of bioactive substances. Along with this trend, the possibility of replacing synthetic preservatives with natural ones is receiving much attention. In general, microalgae are rich in various phytochemicals like carotenoids, phycocayanine, phenolics, amino acids, polyunsaturated fatty acids, and sulphated polysaccharides. These compounds are providing excellent various biological actions including, antioxidant, antimicrobial, antiviral, antitumoral, anti-inflammatory and anti-allergy effects. Their healthy benefit seemed to be due to different biochemical mechanisms. However, some microalgae species such as *Chlorella*, *Spirulina* and *Dunaliella* species have been used in several areas in nutraceutical, pharmaceutical, cosmetics, nutrition and functional quality of foods. In 2006, World Health Organization has been described *Spirulina* as one of the greatest super-foods on earth serving as an example of the potential of microalgae. This review provides background on current and future uses of microalgae as novel source of health promoting compounds (Abd El Baky H.H and El Baroky, 2013).

3 cyanobacteria (*Anabaena oryzae*, *Tolypothrix ceytonica* and *Spirulina platensis*) and 2 green microalgae (*Chlorella pyrenoidosa* and *Scenedesmus quadricauda*) were tested in compliance with the agar well diffusion method for their antibacterial and antifungal agent production on various organisms that incite diseases of humans and plants (*Escherichia coli, Bacillussubtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Aspergillus niger, Aspergillus flavus, Penicillium herquei, Fusarium moniliforme, Helminthosporium sp., Alternaria brassicae, Saccharomyces cerevisiae, Candida albicans*). The antimicrobial activity was maintained by using (ethanol, acetone, diethyl ether and methanol). It was found that, *Spirulinaplatensis* and *Anabaena oryzae* had the highest antibacterial and antifungal activity towards the tested bacteria and fungi. *Spirulina platensis* was evaluated for biological activity against *(A. flavus, F. moniliforme, C. albicans, B. subtilis, P. aeruginosa*) by operating the statistical design of Placket-Burman for the degree of significance of the eight different trials by using seven independent variables. The results obtained from Placket-Burman design revealed that highest main effect and t-value were detected with NaCl in case of *A. flavus*. While, they were detected with MgSO and micronutrient (a) in case of *F. moniliforme*. Also, they were detected with FeSO and micronutrient (a) with *C. albicans*. On the other hand, the results revealed that 4 highest main effect and t-value were detected with micronutrient (b) on *Bacillus subtilis*, while they were detected with NaCl and K SO in case of *Pseudomonas aeruginosa (*Ramkumar et al., 1998).

Antifungal and antibacterial activity of some heterocystous cyanobacteria from spaddy-fields in the north of Iran was studied. Soil samples were collected from paddy-fields of Gillan, Mazandaran and Golestan Provinces and cyanobacteria were isolated. Supernatants, methanolic and hexane extracts from biomass of 150 strains of cyanobacteria were isolated and screened against six strains of bacteria and eight strains of fungi. Methanolic extracts and culture supernatants of 21 strains of cyanobacteria exhibited significant antibacterial effect and 13 strains showed antifungal effect. No antimicrobial activity was detected in the hexane extracts and no extract inhibited the growth of E. coli. According to these results, it is concluded that strains of Stigonemataceae including Fischerella and Stigonema species, seem to be more

potential for producing antimicrobial substances than other strains (Younes ghasemi *et al*, 2003).

Aim and objectives

Collection of fresh water sample and Isolation of *Chlorella* sps from fresh water using BG 11 medium.
Optimization of growth using various medium including Bold basal medium, CFTRI medium, Bangladesh M3 and Zarrouk's medium etc.
Mass cultivation of *Chlorella* under controlled conditions BBM.

Separation of algal cells by centrifugation.
Preparation of algal extract.

Phytochemical analysis Bacterial susceptibility testing using algal extracts by AWD assay.

Materials and Methods

Sample collection

Fresh water Algal samples were collected from the sampling site of paddy field of kilambi, kanchipuram. Fresh water samples were collected in sterile container, 250ml Erlenmayer flask which is already pretreated in 0.1N HCl, stream sterilized and cotton plugged. The samples were preserved instantly into the ice bucket which was maintained at 5±1°C and transferred to the laboratory.

Isolation of micro algae (*Chlorella* sp)

The collected sample is transferred to the 100ml of BG11 medium.The flask were kept under sufficient light (1000 lux) and incubated in the flask under room temperature (22-28°C) with a PH of 8.2±1.After 15-18 days, green discoloration was seen in the culture tubes due to the growth of microalgae.

Identification of *Chlorella*

Based on the morphological identification the *Chlorella* culture was identified under the microscope.

Optimization of *Chlorella* growth by using different medium

The following medium such as Bold basal medium, CFTRI medium, Bangladesh M3 and Zarrouk's medium were used for the optimization of growth of *Chlorella sp.*

Measurement of growth in different medium

The growth wwas measured through optical density (OD), cell count (CC) and dry weight (DW). Optical density was recorded by using colorimeter at 670 nm. Cell count examination was performed using haemocytometer.

Estimation of Chlorophyll a and b

Chlorophyll was extracted from samples grown in different media using 100% methanol. The samples were diluted 10-20 times by methanol depending on the cell concentration. The vials containing the samples were wrapped with aluminum foil and stored at 4°C for 30 min. The samples were then centrifuged at 16,000g for 10 min. The absorbance of the green supernatant was measured at two wavelengths, 650 and 665 nm. The chlorophyll a (mg/L), chlorophyll b (mg/L), and total chlorophyll content (mg/L) were then calculated using the equations described by (Hitkins and Baker, 1986). Culture samples were collected from the six types of culture media on the 5th, 10th, 15th and 20th days for the analysis of Chlorophyll a, Chlorophyll b, Total Chlorophyll.

Subculturing of *Chlorella* sp.

The successful culture was diluted and sub cultured in 200mi and subsequently to 250ml, 500ml and 1 litre Erlenmeyer flask and maintained as stock culture under a luminosity of 1000 lux. The cultured flasks were shaken thrice a day to ensure proper growth. The algae were further mass cultured using BB medium and harvested in the exponential phase for experimental purpose.

Mass cultivation of *Chlorella*

The pilot scale mass cultivation extended from 250-500, 500-1000ml and to mass of 5litres under controlled conditions was carried out in a cylindrical glass tank with an aerator in 12 hr dark/12hr light method using Bold Basal Medium.

Separation of *Chlorella*

The mass cultivated algal cells were recovered from culture by batch centrifugation at 10,000 rpm for 15 minutes. The cells were repeatedly washed in normal saline (0.85% NaCl) for three times by centrifugation at high speed. Extracted biomass were transferred to a pre-weighted dry filter paper using a clean spatula then placed in an oven at 60°C overnight to reach a fixed weight. The dry weight of the microalgal cells was weighted and the results were tabulated.

Preparation of algal extract

Ethanol is used as solvent in this experiment. The separated algal cells (*Chlorella sps*) were collected. A known quantity of algal cells 2.5g was taken in 100 conical flask and added with 50 ml of ethanol. The preparation was kept under room temperature for 48 hours and rapidly stirred using glass rod every 8 hours. After 48 hours the extract were filtered. The filterate was taken in separate beaker and kept in a water bath at 45ºC until the solvent get evaporated. The greasy final material (crude extract) obtained from the algal cell was transferred to sterile screw capped bottles.

Phytochemical Analysis

Qualitative analysis of phytonutrients of algal extracts

Qualitative analysis of phytonutrients was done for ethanolic extract of *Chlorella sps.*

Benedict's test

To 0.5 ml of extract, 5 ml of Benedict's reagent was added. The mixture is then boiled for 5 minutes. Presence of a bluish green precipitate indicated the presence of carbohydrates.

Test for Glycosides

To 2ml of algal extract 1ml of aqueous NaOH solution was added. The appearance of a yellow color indicated the presence of glycosides.

Test for Proteins and Amino acids Ninhydrin test

A small quantity extract solution was boiled with 0.2% solution of ninhydrin. Purple color indicated the presence of free amino acids.

Test for Phytosterols and Triterpenoids

Salkowski test

o 2 ml of the algal extract, 1 ml of concentrated sulfuric acid added. Chloroform was added along the sides of the test tube. A red color produced in the chloroform layer indicated the presence of Phytosterols or if it is yellow in color at the lower layer indicated the presence of triterpenoids.

Antibacterial activity of algal extract on selected pathogens

The selected organisms were tested for their susceptibility against algal extract and standard antibiotics by Kirby – Bauer method on Muller-Hinton agar plates. A swab of the test culture was taken aseptically and inoculated to the surface of the Muller-Hinton agar plates so as to make a lawn.This was allowed atleast 5 minutes for the agar surface to dry before cutting the wells of 3mm using gel punch and applying the standard antibiotic disc. The disc was pressed gently to give a better contact with agar. 20µl of extract were introduced into each well. The agar plates were incubated aerobically at 37ºC for 24 hours. The zone of inhibition was observed around the antibiotics discs. The indication whether test organisms is resistant (No zone or inhibition) or sensitive (clear zone of inhibition) to the antibiotics was observed.

Results and Discussion

pH Isolation and Identification of micro algae *Chlorella* sps

The *Chlorella* were isolated from the collected fresh water by using BG11 medium. The sample were identified based on the morphological identification .

Optimization of *Chlorella* growth by using different medium

The results for the optimization of isolated Chlorella cultured using different medium was shown in under controlled condition

Measurement of growth in different medium

The measurement of growth of *Chlorella* in four different media was given in Table – 1 and graph – 1. The estimation of chlorophyll a and b test results were shown in Table2 and 3.

Preparation of algal extract

The Chlorella sps harvested from the mass cultivation tank and separated the algal cells by centrifugation .After the separated algal cells extracted by using three solvents. These result were shown in figure -8.

Qualitative analysis of phytonutrients of algal extracts

Benedict's test

Presence of a bluish green precipitate indicated the presence of carbohydrates

Test for Glycosides

The appearance of a yellow color indicated the presence of glycosides .

Test for Proteins and Amino acids

Ninhydrin test

Development of purple color indicated the presence of free amino acids .

Test for Phytosterols and Triterpenoids

Salkowski test

A red color produced in the chloroform layer indicated the presence of Phytosterols or if it is yellow in color at the lower layer indicated the presence of triterpenoids .

Antibacterial activity of algal extract on selected pathogens

The bactericidal activity against selected human bacterial pathogens were screened with standard antibiotics where as extract of Chlorella species. The standard bactericidal activity were shown in Table – 4.

The present study was planned to investigated the optimal media for the biomass production of *Chlorella* sp. is an important microalgae which involved in the aquatic food chain, and rich in protein content (Richmond 2004). They are also rich in other sources like vitamins, minerals, lipids, and chlorophyll and carotenoids (Burlew 1953). Since *Chlorella* sp is an autotrophic, and heterotrophic, it can be easily grown in chemically defined media and in sewage. (Iwamoto 2004).

Comparing to other microalgae C. vulgaris was reach in fatty acid, protein, and pigment concentration (Richmond 1986; Thompson et al., 1993; Jafar Seyfabadi et al., 2011). The aquatic algae culture based on the importance of trace methods such as Fe, Mn, Mo, Cu, and Zn for algal growth is indicated by this requirement as constituents of laboratory growth media (Huntsman and Sunda, 1980). Other element such as boron(b) and vanadium (v) may also be necessary, but the requirement is low level of this elements presence as impartment sufficient to support growth of algae. Hence the micro algae *Chlorella* sp has taken for the analyses for this nutritive sources. This type of

Table.1 Measurement of growth in different medium

Culture Days	BBM	Bangladesh M3	CFTRI	Zarrouk's M
15 days	0.270	0.192	0.158	0.198
20 days	0.372	0.205	0.214	0.234
25 days	0.513	0.245	0.312	0.298

Graph.1 Measurement of growth in different medium

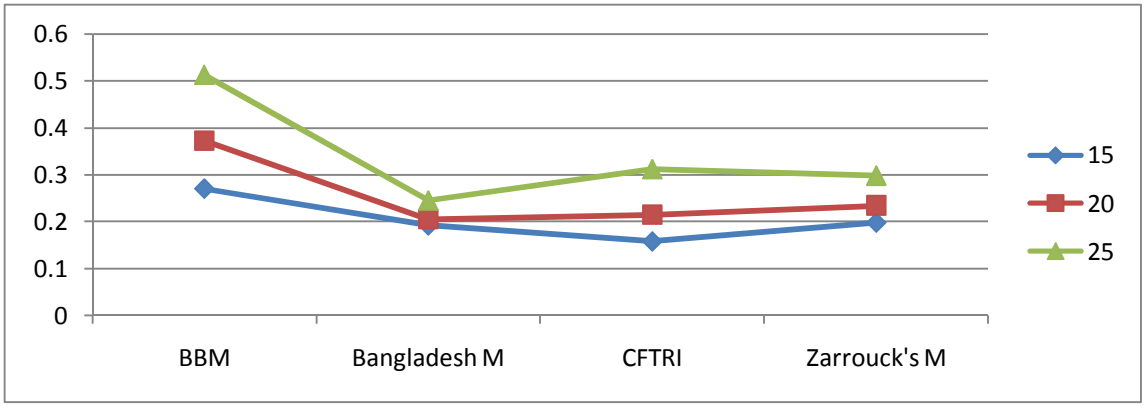

Table.2 Estimation of Chlorophyll a

Culture Days	BBM	Bangladesh M3	CFTRI	Zarrouk's M
15 days	1.97	1.52	1.10	1.00
20 days	2.00	1.83	1.23	1.28
25 days	2.30	2.03	1.52	1.64

Table.3 Estimation of Chlorophyll b

Culture Days	BBM	Bangladesh M3	CFTRI	Zarrouk's M
15 days	0.35	0.28	0.10	0.08
20 days	0.47	0.32	0.18	0.14
25 days	0.52	0.43	0.26	0.20

Table.4 Antibacterial activity of algal extract on selected pathogens

S.No	Bacteria	Zone of inhibition(mm)	
		Algal extract	Standard Antibiotics (Gentamycin)
1	*S.aureus*	15	16
2	*E.coli*	18	14
3	*Klebsiella* sp.	20	17
4	*Vibrio* sp.		24

studies has been reported in Spirulina platensis by (Pandey et al., 2010). In culture of *Chlorella* sp in conical flask has its own limitation in providing the complete information related to growth, development and production of nutrients. Research has been conducted extensively on *Chlorella* sp to enhance their mass culture.

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