Isolation and characterization of antibacterial and antifungal compounds from algae.

Project report

Submitted in partial fulfillment of requirements for the degrees of M.Sc. Honours in Zoology

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CERTIFICATE

This certifies that Priyadarshini Sorokhaibam (11606274) has completed the project entitled ‘‘*Isolation and characterization of antibacterial and antifungal compounds from algae’’* under my guidance and supervision. To the best of my knowledge, the present work is the result of their original investigation and study. No part of the report has ever been submitted for any other degree at any University. The report is fit for submission and the partial fulfillment of the conditions for the award of M.Sc. Hons. in Zoology.

Date: Supervisor Signature

DECLARATION

I hereby declare that the project entitled "**Isolation and characterization of antibacterial and antifungal compounds from algae**'' is an authentic record of my own work carried out at School of Bioengineering and Biosciences, Lovely Professional University, Phagwara, for the partial fulfillment of the award of M.Sc. Hons in Zoology under the guidance of **Mohammad Amin- ul Mannan**, **Ph.D.**

This work is my original work and has not been submitted for any degree/diploma in this or any other University. The information furnished in this report is genuine to the best of my knowledge and belief.

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Date:

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Introduction:

Algae are the photoautotroph organisms, which belong to kingdom Protista. They are adiverse group of organisms with the estimate of 0.03 to 1 million species growing in adifferent variety of habitats. They are classified into two classes based on their size (Hardy *et al*.., 2010). They are:

1) **Microalgae**areunicellular and microscopic organism. E.g. Blue-green algae, Green algae, Brown algae, Red algae.

2) **Macroalgae** are multicellular usually consist of marine algae. E.g. seaweeds.

Freshwater algae are found in lactic water i.e. lakes and running water i.e. rivers. These organisms include both prokaryotic and eukaryotes. The sizes of algae can be categorized in Picoplankton, Nanoplankton, Microplankton, Macroplankton. The general shapes of algae can be in unicellular, colony, motile, non-motile, unbranched filament and branched filament. Green algae are alargegroup of other groups of algae. It has about 17,000 known species and has specific environmental conditions i.e. water condition, light source, temperature, pH, hardness, salinity (Edward *et al*.., 2015).

Algae capture the light energy through the process of photosynthesis to synthesize carbohydrate from carbon dioxide and water. Algae produce important resources like biomass, oils for fuel, food, feed and other co-products like bio-diesels. Algae are rich sources for bio-medicine that they contain biologically active compounds such as polyphenols, phlorotannins, fatty acid, polysaccharides, and peptides. Lipids, proteins, polysaccharides, vitamins, and minerals are also extracted from algae (Jyotirmayee *et al.,* 2014).

β-carotene is produced by some alga and this help in treating cancerous cells. An antiviral vaccine has been produced by some brown algae and it helps to inhibit the virus completely. Besides these, some algae strains like *Chlorella* and *Spirulina* are the good source of protein and are consumed as a food and supplement (S.Pooja, 2014).

Algae produce hydrogen gas, bio-oils, bio-ethanol, antifungal, antiviral, antibiotics, antioxidants are the main source of algae. Algae give some water-soluble antioxidants like polyphenols, phycobilin-proteins and vitamin are found. These products helpto treat some diseases like

chronic inflammation, heart diseases, aging, and cancer. Anti-biotic from algae are depends upon at which factor they grow (Shibu and Danam, 2015).

The cells of algae are having atrue nucleus and they have double membranous organelles i.e. nucleus, the mitochondria and the chloroplast. Some algae are unicellular and some are multi-cellular. The DNA and genetic material are present inside the nucleus of the cells.

Photosynthesis occurs in thechloroplast. It is a biochemical reaction where the light energy converts to carbon dioxide and water. The photosynthetic pigment is varied from one alga to another alga due to their chloroplast pigment present in the cells. The mitochondria also called the powerhouse of the cell which performs the cellular respiration where the breaking down of food molecule into CO2 and H2O (O.P Sharma, 1986, MC Grew hill education).

What is lacking and what you want to do?

Problem background:

The first algal culture was done by Beijerinck in the year 1890 by using *Chlorella vulgaris* and use of this culture was developed by Warburg in 90s. The various bioactive chemical compound extracted from algae are used to treats or prevents diseases, which are causes by microbial (Mehadi *et al.,* 2015).

Nowadays, numbers of diseases are increasing day by day which is mainly caused by microbes. These can be bacteria, viruses, and fungi. The antimicrobial study was done by disc diffusion method to act against the agents who cause diseases. Algae is using because they can grow fast and they consume carbon dioxide.

External otitis (EO) is the painful infection of the outer ear, it occurs by swelling of the ear canal and hearing loss. It is caused by the fungi or bacterial infection like *Pseudomonas aeruginosa*, *E. coli*are the main agents of this disease. *Dunaliella* algae were shown to produce antibiotics substance by using butanol as a solvent for extraction. From *Chlorella*, chlorellin the anti-bacterium compound is produced which perform the antibacterial activities. The freshwater algae and the marine algae were isolated and cultured at favorable temperature in incubator and they extract methanol by centrifugation and methanol are expressed in the anti-microbial activities (Gianluca *et al*.., 2015)

Algae can acts as medicines (Kolanjinathan *et al.,* 2014):-

1) Antibiotics: solvents are extracted from green brown and red algae that can kill the bacteria or inhibits the growth of bacteria.

2) Antifungal: macro and microalgae have a potential to extract the biologically active substance can act against the antifungal activities.

CURRENT USES OF MICROALGAE

Microalgae are primarily used as a source of nutritional and health supplements, in cosmetics, in the treatment of wastewater and as a major source of oil for biofuels(Aslan and Kapdan, 2006). There are many fields where microalgae are required. The whole algal biomass can be used as a source of protein or valuable chemicals can be extracted. Many studies have shown the activities of microalgae as a therapeutically which help in healing the wounds and some algal compounds have shown active effects on the HIV virus (Smruti *et al.,* 2014)

Macro algae like seaweeds have a soluble fiber which is used for many medicinal purposes. This soluble fiber can bind with water in high rate as they have a strong hydro colloidal property that helps in production short chain of fatty acids that promotes anti-carcinogenic properties. (Kim and Niranjan, 2011)

Table; 1: (Silvia *et a*., 2003) suggested that the anti-microbial compound extracted from algae are:

Compound	**Characteristics**
Alkaloids	This acts on anti-biotics, anti-inflammation, and anti-osteoporotic.
Steroids	Steroid with anti bacterial properties used to treat bacterial infections that have inflammation, itching redness and anti-fungal.
Terpenoid	In*Stoechospermum polypodioides*species which are found in Indian oceans have terpenoid compounds which have the properties of antibiotics.
Polysaccharide	which are found in the fraction of macro algae *Gracilaria corticata* have a rolesto defense from the diseases which are caused by microbes
Fatty acids	In the species *Chaetocero*s fatty acids are found in huge amount and those fatty acids are acts on Anti bacterial activities.
Phenolic compounds	Phenols which are present in algae have a medicinal properties like anti-viral , antibacterial, Anti-fungal anti-inflammatory, anti-carcinogenic.

Table; 2: (Alassali *et al.,* 2016) The solvents used for extracting antimicrobial activities are as follows:

Solvents	**Characteristics**
Hexane	B.P= (68˚C) M.P= (-94˚C) Found in liquid solution.
Methanol	B.P= (42˚- 62˚C) M.P= (-73˚C) Found as a solvent.
Petroleum ether	B.P= (64˚-70˚C) M.P= (-96˚C) Flammable liquid.
Ethanol	B.P= (73˚C) M.P= (-114˚C) Flammable liquid.
Acetone	B.P= (56˚C) M.P= (-95˚C) Flammable liquid.
Chloroform	B.P= (61˚C) M.P= (-63˚C) in dense liquid form.

Review of literature:

History of algal culture:

Helena *et al*., (2011) describes that algae are the basics part of the food chain in aquatic ecosystem. They performed photosynthesis and produces complex organic compounds. These organic compounds help to secreted primary and secondary metabolites .by the presences of many chemical compounds like terpenes, phenol, fatty acid, acetogenic and volatile halogenated hydrocarbon. *Chaetoceros muelleri* have a large amount of lipids composition and that helps for the antimicrobial activities. *Dunaliella salina* produces several fatty acids and compound like alpha and beta- ionine, beta-cyclocitrial, neophytodiene and phenol, those responses in the antimicrobial activities in human because of their antimicrobial properties. The anti-bacterial compound from microalgae chlorella was isolated and found that they can acts on both gram positive and negative bacteria because they have a mixture of chlorellin and fatty acids. Thousands of death cause due to Candidiasis in the years (1950-1970) and it got treat by using active compounds and methanol is used as organic solvent to extracts those active compounds from *Chlorella vulgaris.*

Various biological active compounds showing anti microbial properties has been isolated from algae *namely C. vulgaris, D. Salina, Euglena viridis* (B.Digamber Rao, 2015)

Algae are the source of the bioactive compound shown anti-microbial properties. Blue – green alga are rich source of structurally and biologically active metabolic compounds like anti fungal, anti bacterial, anti-viral, anti-allergic. *Chorella, Dunaliella* gives organic solvents by using methanol, diethyl, ether, chloroform. And methanol shows the high antimicrobial activities on *E.coli* and *Staphyloccus acereus* (Hind and Juntawong, 2014).

Micro-algae are very important raw materials for production of amino acid, vitamins and other biological secondary metabolites. So, the cultivation of micro-algae helps in term of many fields like practical application and biotechnology field and they helps in bio medicinal activities (Vivek k.bajpai, 2016)

Algae have Primary and secondary metabolites which can be bioactive compounds in the pharmaceutical industry. Unsaturated lactone, sulphur, phenol, ether, glycosaride are the

compounds which are used to extract the compound from algae that are acts on anti-microbial activities. From green-algae, diatom extracts secondary metabolites which are acts on antifungal activities .Methanol, Ethanol, Hexane are used for the extraction .These extracts acts against on pathogen like *P.mirabilis, E,coli , S,arerus, S.pyrogenes* and the methanolicwhich are extract from *O.echinospermum* acts only one bacterium *S.typhi*. The ethanolic extracts from *S. grantia* shows the antibacterium results on *E. coli* which are rod shaped bacteria and causes many diseases. The rod shaped gram negative bacteria *P.mirabilis* which are responsible for the infection was acts by the solvent ethanolic extracts from *S.grantiana* these shows that the extracts from the algae can responsible of anti-microbial activities. (Justella *et al*., 2011)

Nowadays, there is huge increasing of the diseases that are causes by microbial and it can be fatal. These can be treated by green medicines which are natural product. Micro algae are used because they are found in many diverse in nature, they are unicellular and it can be found individual cells or as a group. They have their biologically active compound i.e. lipid, fatty acid or phenol etc that acts against on microbial activities, which are extracting by using many organic solvents in different temperature (Jyotirmayee *et al*.., 2014)

Chlorella pyrenoidosa

The freshwater alga, *Chlorella pyrenoidosa* which grows in colonies and its natural habitats are ponds and lakes. They are minute uni-cellular organism cannot seen without microscope and they belong in micro alga group. Their shapes are in spherical and sizes are in 2-12 micron(μ) Because of their rich chlorophyll content the color of the alga is in dark green. *C. pyrenoidosa* possess distinct feature from all the chlorella family that is they are highly in nutritive value. So, they are commonly found in market as food or as a food product. (Chlorella guide, 2012, Keats publishing)

The nutrients which are presence in C. pyrenoidosa are vitamin A, B, C and K, chlorine, inosetol and lipoid acid. They also have minerals i.e. calcium, iron, iodine, copper, magnesium and phosphorus. A substance called chlorella growth factor or also called (CGF) are the unique compound which is found inside the nucleus of cells. This substance is highly rich in nucleic acids that help in maintaining various organs. Complex proteins, enzymes and glycoprotein are much known for their cancer preventive quality as well as detoxifying properties (Klamczynska and Mooney, 2017).

Propionibacterium acne is the bacteria that caused acne vulgaris which is chronic inflammatory disease and 85% of teenage are facing the problem from this disease but the bacteria get resistance because it has been used for long period of time. Nowadays this inflammatory disease becomes the most challenging in treatment. *P. acne* is gram positive anaerobe which causes inflammation in oil glands mostly in face, neck, chest regions in the body. Lipid extracts from *Chlorella* species were used and tested for inhibition of lipase and inflammatory mediators. ROS products are used with lipid extracts and perform as inhibitory activity and acts in reducing inflammatory cell damage. Lipid can kill the microbes by disrupting their cellular membrane and this proves the fresh water algae i.e. *Chlorella* possesses anti acne properties (G.Sibi, 2015).

Checking of anti bacterial and anti fungal properties from green micro algae and cynobacteria has been done. The test organism that are used for examine are *B.subtilis*, *P.aerugenosa*, *E.coli*, *S.aereus* and *Candidaalbicans*. The extracts of *C.pyrenoidosa* have anti bacterium against B.subtilis and P.aeruginosa and moderate properties against on E.coli and no activity towards S.aereus and the extracts of *Scendesmus* also have anti bacterial effects on *B.subtilis* and *S.aereus* but no effects on *E.coli* and *P.aeruginosa*. the result of these experiments shows that ethanol was the best solvent to extracts the anti microbial agents and also the methanolic extracts of some algae anti bacterial activity by using cup-plate method for the antifungal light experiment has taken and result came positively from microalgae especially *C.pyrenoidosa* and *Scendesmus quadricaula* and some cynobacteria are effective more than contemporary antibiotics and fungicides (Rania and Hala, 2008).

For investigate the properties like anti microbial and anti oxidant, 3 algal species are used i.e. *S.platensis, C.pyrenoidosa* and *N.muscoum*. The solvent methanol, acetone, n-hexane and water are used for extraction of secondary metabolites from the algae species. The report show methanolic extract was most effective against pathogenic microbes. Methonolic extract of S.platensis have higher anti-oxidant properties and acetone extract was most sensitive to B.cereus. The biologically active compound that acts against the microorganism are terpenoid,phenol,methanolic extract, ethanolic extracts and acetone extract. Lipid and fatty acid which are presence in the algae strain have anti microbial properties (Neelam *et al.,* 2012)

Micro algae are found in a large diversity that grows in colonies as well as individual cells. The growth factor are varies from one species to another species. They are well known for its highly nutrient content that helps in pharmaceutical industry and in biotechnology field. The biologically active compounds which are found in algae have different properties that can acts as anti-microbial, anti-oxidant, anti-protozoan. *Chlorella* has fatty acid called chlorellin which was found to be responsible acts on pathogenic gram positive and negative. Butanoic and methyl lactate are the two ingredients which response in antifungal activities. The supernatant extract from *C.pyrenoidosa* and *S.qundricanda* are methanolic and hexane which acts against eight fungi (Yingying *et al.,* 2008).

There are 5 growth phase of micro-algae culture, they are: (P.Perumal *et al..,* 2012)

a) Lag phase

b) Exponential phase

c) Phase of declining relative growth

d) Stationary phase

e) Death phase.

Table no. 3**: Anti microbial activity of *Chlorella pyrenoidosa* extracted with acetone, ethanol and chloroform**. (Ashok and Indhumathi, 2015)

Organic solvent	**Organism**
Ethanol	Klebsille sps, E.coli.
Chloroform extract	Bacillus sps, E.coli, Klebsille sps.
Tetracycline	Pseudomonas , Bacillus sps, E.coli.

Table no. 4: **Principal bioactive compounds extracted from micro-algae are as follows:**

Micro-algae	**Bioactivecompounds**	**References**
Chlorella pyrenoidosa	Peptides.	(Wang *et al*.., 2013)
Chlorella vulgaris	Cantaxanthin,astaxanthin,peptide , Olic acids	(Priyadarshini and B.Rath.., 2012)
Dunelia salina	Trans β-carotene,cis β-carotene,olic acids, linolenic acids, palmitic acids.	(Mule *et al..,* 1991)

Secondary metabolites:

Algae are rich source in medicinal value. The compound like alkaloids, aromatic compound, peptide, terpenes are found as a secondary metabolites. The secondary metabolites are rich source of medicine that can treat microbial infection in human. The secondary metabolites are the compound which are not involved in the growth or reproductive system in the organism (Gabriele and Anthony, 2009)

Micro-algae are the intrinsic derivation of biological active compound. And those compounds are used by many pharmaceutical companies and many researchers, due to their conceivable applications in life science. (Michele *et al..,* 2014)

The methanol has the higher efficiency of extraction of organic solvent from *D.salina*. Methanol and chloroform are mixed together and then extract the solvent, that solvent acts for both gram positive and gram negative bacteria. In opposite chloroforms and ethyl acetate shows the efficiency to acts against anti fungal. (Daniel, 1993)

Algae based medicine have been used from the ancestors. Algae have its active compound that is used in pharmaceutical. Polysaccharide is one of the chemically active compounds that have anti-inflammation properties and anti-cancer. Photodynamic therapy is the therapy which is used to treat cancer by using phycobitin pigment from algae. (Bhumanath and Dhananka, 2017)

Terpanoids is the organic compounds and it's found in alga *Sargasssum microcanthum* species.methanolic extracts are used to extracts terpanoids that have high inhibitory activity on lipid peroxidation and acts as anti-viral activity against mouse cytomegato virus and maize chlorotic motile virus. Quinone metabolites green algae have anti-bacterial properties as well as used to treat from bee venom. (Borowitzka, 1995)

Chlorella vulgaris, Chlorella pyrenoidosa were used as the test organism and the extraction of secondary metabolites from those organisms have an antibacterial substance. They can grow in any nutrient solution if Co2 is present. The extraction can be done directly from the culture or from the cell free medium. Agar cup tests have higher efficiency of anti bacterial properties

against some bacteria like *B.subtilis*, *Bact.coli*. Chlorellin is the serum presence in the micro-algae that can acts against bacteria. (Pratt *et al..,* 1944)

Nystatin ia a compound which is used to treat many fungal or bacterial infection even this is used to treat candidiasis which is cause by yeast. This compound is mainly effectives on fungi because they are sensitive organism which can absorb the polyene in liquid medium. *C.pyrenoidosa* was used to extract nystatin compound as a secondary metabolites and used to acts on fungi and bacteria. *Scendesmus obliques* also have various antimicrobial properties.(Lampen and Peter, 1961)

Objectives:

1) To optimize the growth conditions for the growing algae.

2) Isolation of various antibacterial compounds from algae.

3) Isolation of various antifungal compounds from algae.

4) To check the efficiency of above mentioned antibacterial and antifungal for common human opportunistic organisms.

5) To characterized the secondary metabolites using biochemical and HPLC.

Materials and methods:

Culturing and Growth of algal organism:-

Chlorella pyrenoidosa strains was obtained from NCL,Pune (Accession number-2738).

This strain was selected for the screening of their anti- microbial activity against test organism. *C.pyrenoidosa* was culture by using BG11 broth media in 28˚C temperature with proper light condition.

Test organisms:-

Thetest organisms are the bacteria *Pseudomonas aeruginosa* (MTCC 424), *Bacillus subtilis* (MTCC 736)*, Escherichia coli and* the yeast strains are *Candida albicans*. *Saccharomyces cereviseae* (MTCC 179).

Micro-algal culture

A culture has three distinct components;

- Culture medium contained in a suitable vessel,
- The algal cells for growing in the medium,
- Air, to allow exchange of carbon dioxide between medium and atmosphere.

Factorinfluencing for algal growth are

- Temperature: Temperature should be maintained at which the organisms were collected. Temperature controlled the incubators usually use constant temperature for the culturing.
- Light: Natural light is sufficient to maintain cultures in the laboratory. Culture should not be exposed to directly to sunlight. Light intensity (LUX) 1000-10,000.
- Amount of carbon dioxide: used gas and liquid transfer rate in microbial culture.
- pH: pH should be maintained at the range 7-9 for determining free carbon dioxide concentration in the media (Y.Azov,1982)

Culture vessel:

- It should be non toxic.
- Reasonable transparent to light

- Should be easily cleaned and sterilized.
- Microalgae should be grow in conical flasks.
- And it should be performed in bio-reactor.

Sterilization:

- Autoclave (steam sterilization) – 121˚C for 15 mins.
- Oven (dry heat sterilization) - 180˚C

Preparation of media:

For agar media,

0.3254 grams in 200 ml of double distilled water + 5 grams of agar.

For broth media,

0.1627 grams in 100 ml of double distilled water.

Growth condition:-

Cultures were grown in sterile test tube with cotton and Petri-plate. Sunlight or light source was maintaining because they are photosynthetic organism and Temperature was set on 28˚C for growing *C.pyrenoidosa*.

Harvesting and cultivation

- Sample should be monitored everyday for the cellular growth rates.
- Culture got aerated using air pumps with air stones.
- Constant mixing of the algal culture in the tank was provided by the aeration.
- Culture medium should be maintained in a temperature 21°C-32°C
- Then for harvesting there are chemical, mechanical, biological and physical methods.
- There are two steps for harvesting micro-algae, they are
- Thickening and
- Dewatering.

Algal suspension

▼

Screening

▼

Thickening

▼

Dewatering

▼

Drying

▼

Biomass procession, Bio-fuels and Pharmaceuticals.etc.

Fig: diagram of Micro-algal harvesting and drying techniques (Adapted from Ana *et al*.., 2015)

Solvent for extraction:

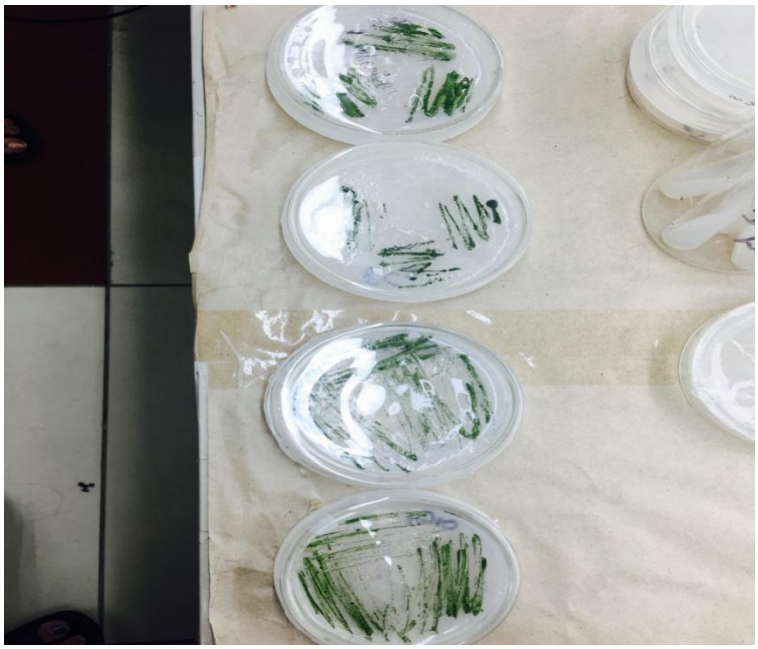
The organic solvents like hexane, petroleum-ether, terpenes, lipid, chloroform, methanol, benzene are used to extract secondary metabolites from algae. (Maschek and Barkar, 2008)
The process of getting secondary metabolites from micro-algae is used in vivo extraction.

Methods of extraction

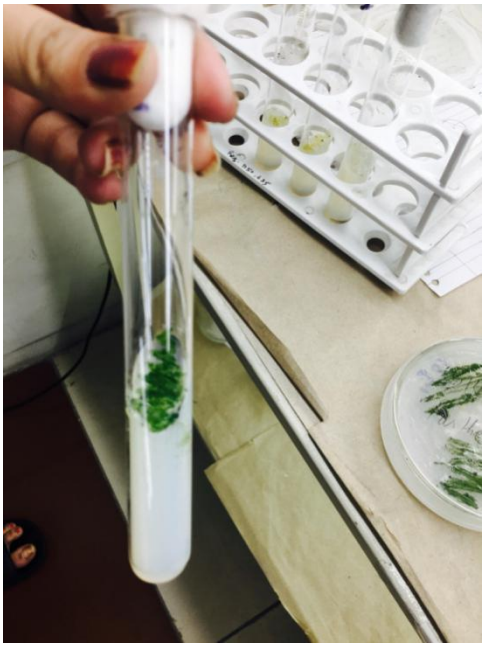
Methods	Secondary metabolites	Instruments	References
Soxhlet	Lipids	L-L separations then drying	(Cardozo *et al..,* 2007)
Liquid-liquid extraction	Carotinoids	HPLC	(Rosenberg *et al.,* 2008)
Sonication	Tocophenols	HPLC	(Efsthathic *et al*..,2007)
Pressurization liquid extraction	Anti oxidant activity	Gas chromatography	(scott , 2010)
Esterification	Fatty acids	Gas chromatography	(Rodhiguenz *et al*.., 2008)
Superficial fluid extraction	Carbohydrate, carotenoids, lipids	Spectrophotometer, Gravimetric, LC uv rays	(Ebada *et al..,*2011)
Autoclaving	Sulphate, sugars, neutral sugars	Barium chloride, gelatin method, phenol-sulfuric acid method,HPLC.	(Lane *et al*.., 2011)

Results:-

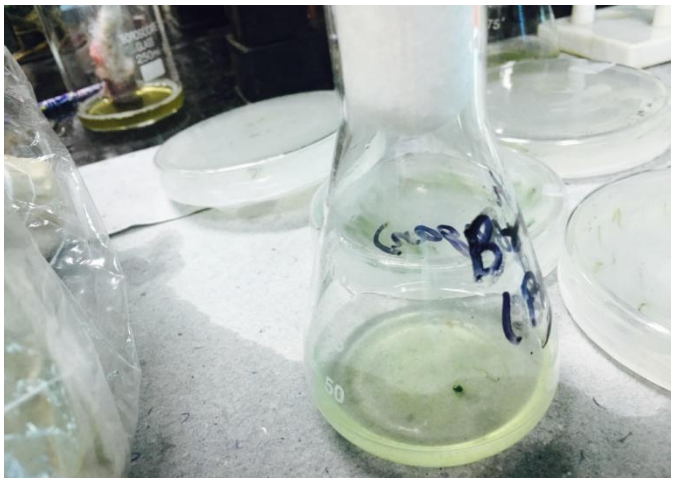
Chlorella pyrenoidosa strains were cultured and make it grown in sterile test tube and Petri-plates.



A) Fig: Algae culture in Petri-plates.



B) Fig: Algae culture in test tube.



C) Fig: Algae culture in broth medium.

Expected outcome:-

- I will learn about different types of algae and their growth condition,
- I will learn about different types of algae culture method,
- I will learn about opportunities pathogens and how to treat by using algae secondary metabolites.

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