OPTIMIZING METHODS FOR ISOLATION OF ANTI-MICROBIAL COMPOUND FROM ALGAE & ITS EFFICACY ON OPPORTUNISTIC ORGANISMS

Project Report

Submitted in partial fulfillment of the requirements for the degree of

M.Sc. Hons. In Zoology

Submitted by:

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DECLARATION

I hereby declare that the project entitled ‘‘*Optimizing methods for isolation of antimicrobial compound from algae and its efficacy on opportunistic organisms*’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.

Place:Punjab (Gagandeep Kaur)

Date: 30 November, 2017 Reg. no. 11616434

CERTIFICATE

This certifies that Gagandeep Kaur (11616434) has partially completed the project entitled ‘‘*Optimizing methods for isolation of antimicrobial compound from algae and its efficacy on opportunistic organisms*’’ under my guidance and supervision. To the best of my knowledge, the present work is the result of her 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 this session 17181 submission towards the partial fulfillment of the conditions for the award of M.Sc. Hons. in Zoology.

Date: Supervisor Signature

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

ALGAE: Algae are the large photosynthetic organisms. They can be unicellular as well as multi-cellular, example *Chlamydomonas* and brown alga respectively and can grow upto 50m in length. Some of them are eukaryotic (nucleus bearing) while others are prokaryotic. (Butterfield et al., 2000). They give a number of pharmaceutical and industrial products for humans. Economically, they are important source of crude oil, and its supplements also. In case of algae, the methods of reproduction are asexual, vegetative and sexual is also there. (Guiry et al., 2012).Some of them can result in different resting stages called cysts, whose habitat is sediments, starting from at-least 10 up to 50 years. (Agarwal et.al, 2001) They are auto-trophic, derive their food and energy from their surrounding in the form of sun light. They plan an important role in the food chains. (Barsanti et al., 2014)

OPPORTUNIST ORGANISMS: The opportunistic organisms are those that cause infection. This infection is caused by pathogens like bacteria, viruses, fungi and protozoa. These microorganisms can lie dominant in body tissues for very long period of time (years). (Boyd 1990)

For example, rhuman herpes virus, which is extremely common but it does not show any symptoms of illness. These microorganisms are activated whenever immune system fails to show required response. They then start to multiply and very soon affect the weak deafness of body. (Pontin 1978).

Organisms have three characteristics:

- These organisms have low pathogenicity.

  Eg. *Pseudomonas aeruginosa, Staphylococcus epidermidis, Candida albicans, Cytomegalovirus, Pneumosysuscarinii.*

- They cause infections usually when host's defense mechanisms are impaired.

  Eg. The patients with AIDS immune-suppressed patients who receive renal or other transplants and patients receiving treatment for acute leukemia.

- They behave as conventional pathogens but under opportunistic conditions, may cause

atypical clinical presentation.

Eg. *Mycobacterium tuberculosis* which can cause a PUO (pyrexia of unknown origin) illness in immune-suppressed patients due to military TB or life threatening infection in immune- suppressed patients caused by *Strongyloides stercoralis*.

CURRENT USES OF MICROALGAE

Algae is widely used as a source of protein or valuable chemicals (pigments, enzymes). Several studies have shown the potential of microalgae as a therapeutic help wound cicatrization. The inhibitory effect on HIV virus are shown by some algae. (Becker,W.,2004)

Algae gives the nutritive products like tablets, capsules and liquids. They can be also incorporated into pastas, snacks, foods, candy bars and gum etc. The algae is used in biotechnological research and industry. In the production of food, chemicals and fuels, they act as a biocatalyst. For the development of solar energy, they play important role as in the solar energy technology and biodegradation. (Liang et al., 2004)

Some species of algae are eaten directly by humans as a food supplements in western world health stores. Some of the microalgae for example- *Spirulina Sps.*, *Chlorella Sps.*, *Dunaliella Sps*. Spirulina is a cyanobacteria that has a protein content and used in aquaculture industry.(Yamaguchi et al.(1997)

REVIEW OF LITERATURE

Types of Algae: Two types of algae on the basis of size-

- Microalgae
- Microalgae

What are Microalgae?

Microalgae are microscopic, eukaryotic cell and contain chloroplasts. The photosynthetic efficacy of microalgae is better than the terrestrial land plants. It synthesizes carbohydrates by utilizing light, CO2 and nutrients. They can also grow in aquatic environments by the use of fertilizer and pesticides which result in less paste and pollution. The algae can be extracted for the proteins and valuable chemicals has a whole algae biomass. (Stanier et al., 1971).

DISEASES PREVALENT TO OPPORTUNISTIC PATHOGEN INFECTION

Gastro intestinal, cryptococcal meningitis, penicilliosis marneffei opportunistic infections are common at various stages of human immunodeficiency virus disease. Opportunistic organisms cause diseases in individuals with healthy immune systems. (Al-Anazi et al., 2009)

Opportunistic organisms examples are *Candida albicans, Pseudomonas aeruginosa, Salmonella, Streptococcus pneumonia*. (Karmakar et al., 2013)

METHODS FOR THE GROWTH OF ALGAE

OPEN POND-These can be divided as natural waters and Artificial ponds or containers. They include lakes, lagoons, ponds and shallow big ponds, tanks, circular ponds, raceway ponds respectively. They are easy to conduct. Algae is cultivated in the raceway ponds because the water and nutrients circulate around the race track. These ponds are placed in shallow because algae need to be exposed to sunlight and can only penetrate the pond water to a limited depth. The carbon dioxide and nutrients are constantly fed to the ponds. The main advantage is that the system results in low production costs and low operating costs.

various B vitamins, Vitamin C, Vitamin K, Choline, Inositol and lipoic acid. This also contains the

PHOTO- BIOREACTOR (PBR)

This is the controlled system that need some type of light source. These are also called as closed system because everything that the algae need to grow like carbon dioxide, water, light are introduced into the system. This is convered by a green house. This system enables the high productivity of algae. By this, there is a better protection from outside contamination. The systems also provide better control of temperature, light intensity, gas transfer and larger surface area to volume ratio. Also, this allows more species to be grown.

Different types of PBRs

- Tanks
- Polyethylene sleeves or bags
- Plastic tubes or glass

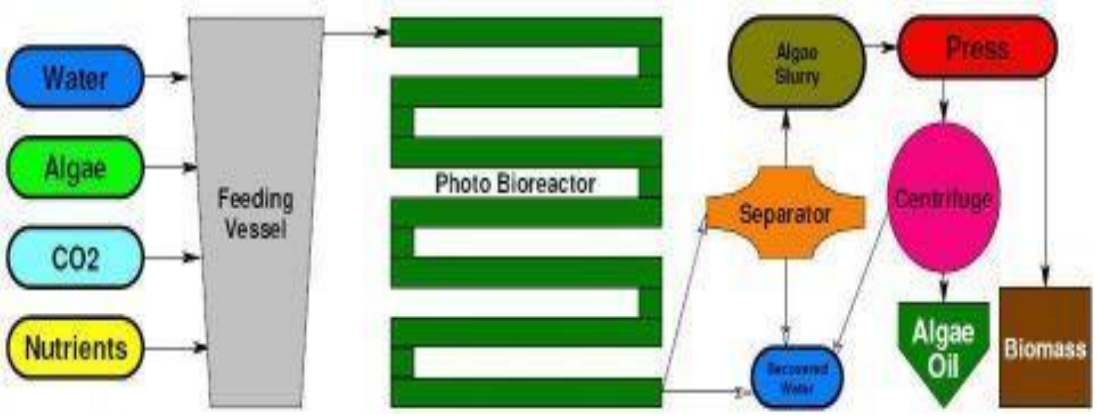

Figure 1. Photobioreactor

Chlorella pyrenoidosa: Genus: Chlorella

Order: Chlorococcales

Family: Chlorellaceae..

These are single celled microscopic plants. It is a greenalgae and grows in freshwater. They multiply rapidly. when the conditions are optimal, single cell divides into four cells in less than 24

hours. The divided cells are called daughter cells. (Araujo et al., 2013)

For the human consumption, Chlorella is cultivated in a fresh water pool under the sun light, according to the article given by Townsend article. A huge amount of present in the Chlorella *pyrenoidosa*. All the essential amino acids are present in this algae and also there is a presence of vitamins like A, B, C, K along with choline, lipoic acid and inositol. Mineral content is also rich in the algae and the important minerals are calcium, iodine, copper, iron, magnesium, zinc and phosphorous. The substance known as CGF which is the growth factor of algae known as Chlorella Growth Factor. (Phang et al., 1999)

In the C. pyrenoidosa, a mutation is observed in the population. And in order to observe such mutation in the alga. He kept population for a food stock of flagellates in the lab and due to certain reasons, flagellates got in the tank of Chlorella and this is the reason that caused mutation from unicellular to multicellular one. (Boraas, 1983)

HEALTH BENEFITS

- Boost the immune system.
- Helps to fight with infection.
- Increases good bacteria in gastrointestinal tract, which helps to treat

- Ulcers

- Colitis- Inflammation of the inner lining of the colon.

- Diverticulosis: When diverticula forms in the wall of colon (large intestine)

- Crohn's disease- The development of inflammation occur at gastrointestinal tract which lead from mouth up to the anus. In the small intestine, it occurs mostly. (Ebrahimzadeh et al., 2008)

- Constipation: The disease usually concern with hardened of faeces, also there is a difficulty in the bowl emptying.

- Fibromyalgia: The disorder which is characterized by which it is widespread in the musculoskeletal pain and there is a complain of memory, mood issues, sleep and fatigue.

- High Blood pressure: The pressure increase in the arteries that carries blood to the rest of the body through heart.

- High Cholesterol: It is a type of fat in the blood. High cholesterol leads to the hardening of the arteries that cause strokes and heart attacks.

- Used to treat Cancer
- Abundance of chlorophyll protect the body against ultra- violet radiation
- It is nutrient- dense super-food. (Young et al., 1993)

The anti tumor activity is suggested in the Chlorella against breast cancer. In Japan, this research is conducted. It remove the radioactive particles during the cancer therapy, After treated with radiation of that cancer body.

Neelam etal.,(2011) In the three algal species (*Spirulina plantensis, Chlorella pyrenoidosa and Nostoc muscorum*) anti-microbial and anti-oxidant properties are investigated against some of the common pathogen in human which are usually bacteria and fungi (*Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus, Bacillus subtilis, Bacillus cercus, Micrococcus luteus and fungus Aspergillus luchuensis, Aspergillus nigar, Fusarium oxysporum*). For the extraction purpose, they used four different solvent- methanol, acetone, n- hexane, water. The antioxidant activity was determined by free radical scavenging activity and they found that methanolic extract for C. *pyrenoidosa* and N. *mucosum* was more effective against P. *aeruginosa*.

O.P. Sharma (1986) described that most basic part of food chain in aquatic ecosystem is algae. They take up water and carbon dioxide with solar energy and produce organic compounds which are secreted as primary and secondary metabolites by the help of chemical compounds like phenol, fatty acids. Microalgae produce several fatty acids and compounds like alpha and beta-ionine,beta- cyclocitrial, neophytodiene and phenol.

Justella et al., (2011) From the Chlorella, the first antibacterial compound is isolated. In which the inhibitory activity of gram positive and gram negative against these bacteria is found due to the Chlorellin that is a mixture of fatty acids.

Phytochemical screening of *Chlorella pyrenoidosa*

In Chlorella *pyrenoidosa* the phytochemicals are present. Phytochemicals are the various biologically active compounds found in the plants. These are the flavonoids, tannin, phenolic compounds, terpenoids, cardiac glycosides, sarponins, carbohydrate.

Table 1: The extraction of antimicrobial activity by C. *pyrenoidosa* with chloroform and ethanol

Organic solvent	Organism	Reference
Ethanol	*Klebsille sp.* *E.coli*	Young, A. J. (1993)
Chloroform extract	*Bacillus sp* *E.coli* *Pseudomonas* *Klebsillesp*	Ashok and Indhumathi, (2015)

The organic solvents are pathogenic to humans. The drastic impacts along with some vector and water borne disease are caused by them. Maximum inhibitory activity against *klebsilla sps*. and minimum inhibitory activity against *E. coli, Bacillus sps., pseudomonas* shown by ethanol. Chloroform shows maximum activity against Bacillus sps. And minimum activity against *E. coli, pseudomonas, klebsilla sps.*

Chlorella has the highest antioxidant activities and phenolic content due to this they are considered as antimicrobial agent and biological anti-oxidant. The anticancer and antioxidant product can be developed through the algae. The innovative functional food ingredients is shown from a specific species of the micro algae and also the activity obtained by the compound and for this extraction mechanism is used. Many bioactive substance is shown by this algae which is a unicellular algae and also it has some important properties which are related to medical. The Chlorella which is taken under the experiment observation shows some important results. One of them is its cancer chemopreventive property, anti- tumor effect, antioxidant activity, anti inflammatory activity and anti- microbial activity. (Danielli M.M. Dantas et al., 2015)

ANTIMICROBIAL OR SECONDARY METABOLITIES:

Microalgae are natural source of bio-compounds. Various therapeutically effective bio-compounds are produced from microalgae. These bio-compounds are either obtained from biomass or released extra- cellularly into the medium. Various bioactive compounds like proteins, polysaccharides, enzymes, vitamins, sterols, lipids etc are obtained from these microorganisms.

(Priyadarshani and B. Rath.,(2012)

Table 2**: EFFICACY OF ANTIMICROBIAL SECONDARY METABOLITIES** (S. Bhagyavathy et al.,2011)

AGENT OF ANTIMICROBIAL	**ACTION AND MECHANISM**
Polysaccharide	Inhibition of hualuronidase
Fatty acids and Lipids	Damaging membrane of cell.
Flavonoids	Membrane potential dissipation and inner bacterial membrane permeability increase.
Polyphenols	Cause membrane damage, activity against enzyme inhibitor, cell wall complexibility, shows affinity to adhesions and also deprivation in the substrate.
Carotenoids	Cell wall digestion through enzyme known as lysozyme.

Table 3**: METHODS FOR ISOLATION OF SECONDARY METABOLITIES**

Secondary metabolites are organic component developed from primary metabolites. They are important for adaptation to different conditions. In the mixture of fatty acid the chlorellin in the Chlorella is recovered first anti bacterial compound in gram positive and gram negative bacteria, it shows some inhibitory activity. (Luque et al., 1998)

MICROALGA	ACTIVE COMPOUND	TARGET MICROORGANISM
Chlorella pyrenoidosa	Methanolic extract	*Staphylococcus aureus, E.coli,, Pseudomonas aeruginosa*

They isolated Pseudomonas aeruginosa, Syaphylococcus aureus and E. coli. The Methanolic extract inhibited the growth of Pseudomonas aeruginosa, Syaphylococcus aureus and E. coli. So, this extract has anti-bacterial activity against bacteria that cause some nosocomial infection. That is why this is considered to control the infectious diseases.

OBJECTIVE:

I. Optimization of culturing and harvesting method of microalgae
II. Standardization of isolation of anti-microbial and secondary metabolites from microalgae.
III. To test the efficacy of isolate anti-microbial and secondary metabolites for human opportunistic organisms.

MATERIAL AND METHODS

Culturing and Growth of algal organism:-

Chlorella pyrenoidosa strains was selected for the screening of their antimicrobial activity against some pathogenic bacteria and fungi. *C.pyrenoidosa* was culture by using BG11 (Composition: 0.135gm in 500Ml) broth media in 28˚c with light condition.

Test organisms: -

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

PHYSICAL AND CHEMICAL CONDITIONS

Culture medium/ Nutrients (Anonymous.,1991)

Table 4: Growth conditions

PARAMETERS	RANGE	OPTIMA
Light intensity(Lux)	1000-10000(Depends on volume and density)	2500-5000
pH	7-9	8.2-8.7
Photoperiod(light: dark, hours)		16:8(maximum) 24:0(minimum)
Temperature(°C)	16-27	18-24

Light: Microalgae photosynthesize, it convert that inorganic matter into organic matter. This supplied through two ways, either by natural or through fluorescent tubes.

pH: Give 7-9 range of pH. On the other hand, 8.2-8.7 is a range of optimum.

Aeration/Mixing: The algae sedimentation is prevented has, it is a important one by mixing we came to know that the population of cell gives comparatively same amount of exposure towards

nutrients as well as light are may not.

Temperature: For the culture of algae, the temperature range lies between the 20-24°C. Algae can tolerate the temperature up to 16 (minimum) and 27°C (maximum). However, if the temperature gets lower, then the minimum range directly affect the growth of algae that is slows down and in comparison to maximum range, it increase the growth of algae.

GRAVITY SEDIMENTATION:

The method gravity sedimentation is mostly used to separate the micro algae from water as well as in waste water treatment. The gravity sedimentation is increased by the flocculation. Depend on the density of the micro algal particles enhance the removal of solids through gravity settling. (Brennan et al.,2009)

HARVESTING AND CULTURING OF ALGAE: (John et al., 2013)

It can be harvest by various well known processes.

Table 5: The common methods are mentioned below:

Harvesting techniques	Advantages	Disadvantages
Centrifugation	For rapid cell harvesting of most algal types can be handle.	Operational cost and capital is in the high rate.
Chemical flocculation	Mostly low cost prices or it may vary, availability of flocculants in the wide range.	Removal of flocculants, chemical mixture of unknown source may present.
Filteration	Availability of various filter and membrane.	More depend in algae, more suited to large algal cells, also following our clogging on tissues.
Flotation	Much faster than sedimentation, gases transfer also possible.	Specific algae species also related to high in capital as

		well as operational cost.
Sedimentation	Mostly low in cost, reduced energy input and also subsequent cost stages	Specificity in algae, mostly for dense as well as non- motile cells, rate of separation as well as concentration is low.
Ultra- filteration	Only delicate cell are handled	Operational cost and capital is in the high rate.

Sample should be monitored every day 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 andDewatering.

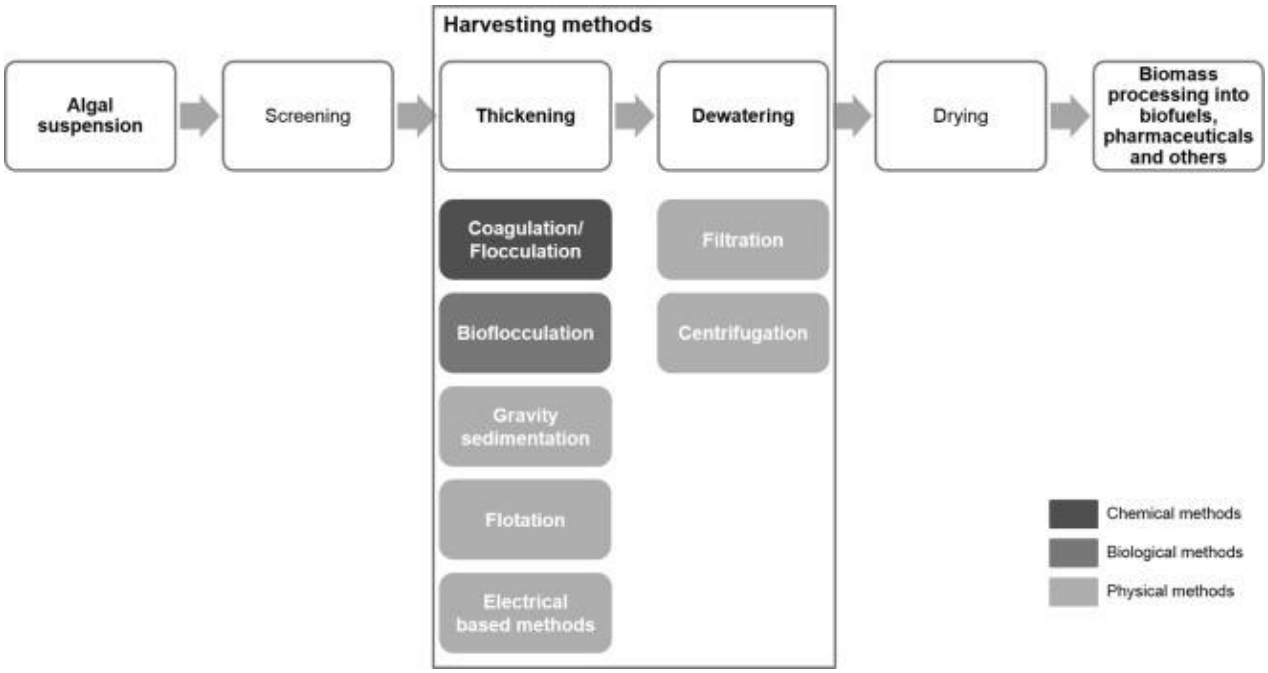

Figure2. Micro-algal harvesting and drying techniques (Barros et al., 2015)

CENTRIFUGATION:

In the process of centrifugation, the sedimentation rate is heterogenous with that of centrifuge and during this process centrifugal force play important role. The liquid broth is used to recover the micro algae in the centrifugation process.

CULTURE VESSEL

- It should be non- toxic.
- Reasonable transparent to light.
- It should be easily cleaned and sterilized.
- Microalgae should be grown in conical flasks.
- It should be performed in Bio- reactor.

STERLIZATION

- Autoclave (steam sterilization)at 121 °C for 15-20 minutes.
- Oven (dry heat sterilization) at 180°C for one hour.

Preparation of media:-

For agar media,

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

For broth media,

0.1627 gm 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*.

SOXHLET METHOD

The compound which contain the source material can be extracted is placed in the thimble which

lie inside. In the soxhlet extractor of the main chamber, thimble is loaded. Also in distillation flask, the extraction solvent which is used is placed in it.

Table 6. Soxhlet method

Extraction method	Soxhlet extraction
Biomass	*Chlorellapyrenoidosa*
Chemicals used	Acetone
Temperature/Pressure	120-180°C
Time(min.)	480
Chemicals extracted	Lipids
Analytical method	Lipids were separated applying L-L separation and then drying
Reference	Araujo et al.,2013

DISC DIFFUSION METHOD

Due to the high degree of reliability, the disc diffusion method is mostly preferable towards the standardization in the concentration of anti- biotic, which shows relatively performance ease. The primary function in DD technique of the culture medium is to supply nutrition which is optimum to environment which ultimately supports the test organism for growth.

Table 7. Disc diffusion method

Extraction method	Disc diffusion method
Biomass	*Chlorella pyrenoidosa*
Chemical used	Muler- Hinton agar
Temperature	35-37˚C
Time	18-24 hours
Reference	Olga cades et al., (2001)

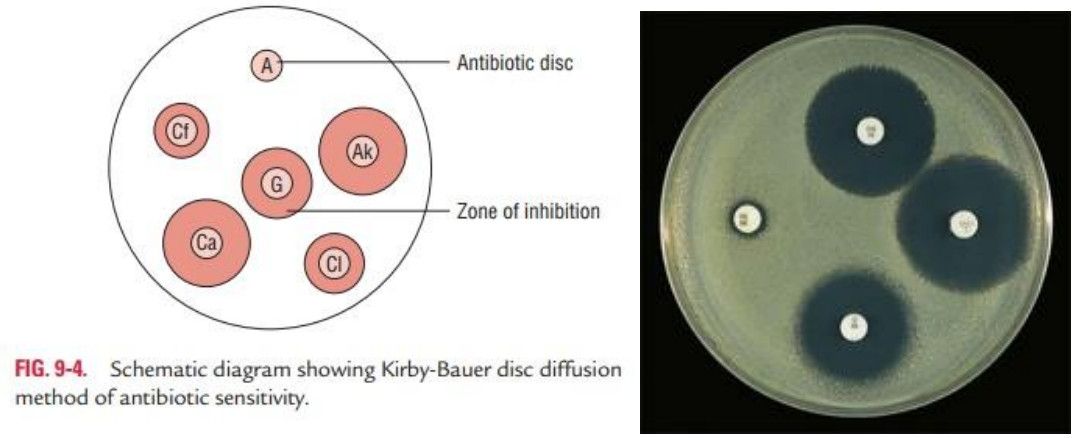

FIG. 9-4. Schematic diagram showing Kirby-Bauer disc diffusion method of antibiotic sensitivity.

Figure 3: Disc Diffusion Method

ANTIBACTERIAL BY PHENOL:

We can determine the total phenolic compounds by the help of Folin- Ciocalteau methods (Gouveia et al., 1996).

Growth Curve (Nakai et al., 1999)

Five Phases-

- Lag or induction phase: little increase in cell density occurs
- Exponential phase
- Phase of declining relative growth: Cell division slows down when nutrients, light, pH, carbon dioxide or other physical and chemical factors begin to limit growth.
- Stationary phase: limiting factor and the growth rate are balanced, which results in a relatively constant cell density.
- Death Phase: Cell density decreases rapidly

EXPECTED OUTCOMES:

- Isolation of algae by different methods.
- Optimization of the methods of harvesting.
- Optimization of various methods of isolation of antimicrobial compound.
- To study the efficacy of antimicrobial compound on opportunistic organisms.

Results:-

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

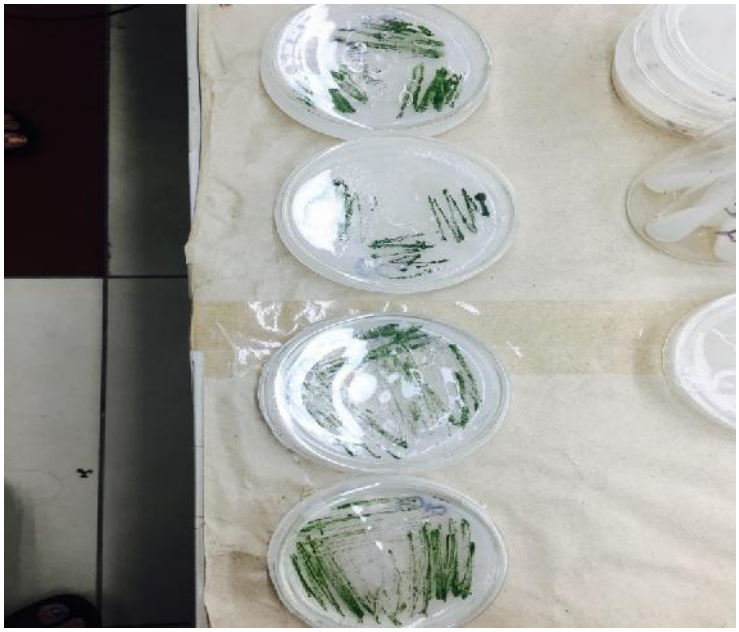

Figure 4. Algae culture in Petri-plates

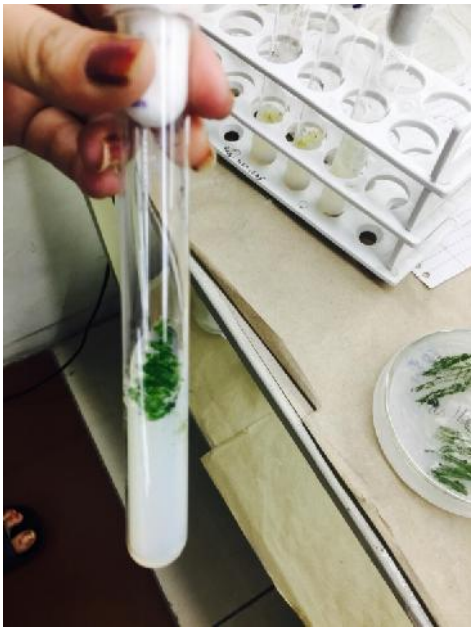

Figure5. Algae culture in test tube.

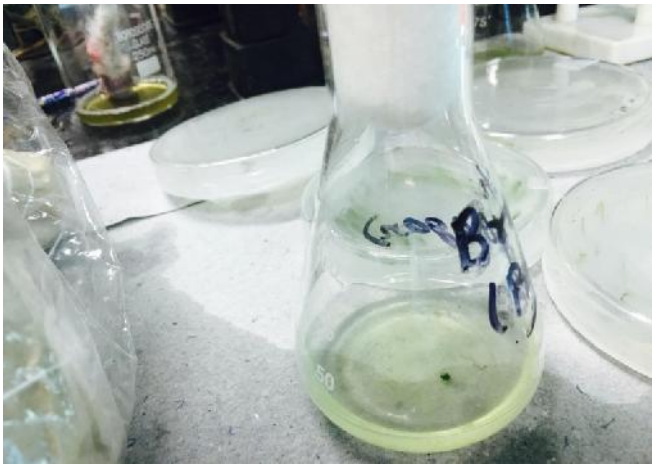

Figure6. Algae culture in broth medium.

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