ANTI-CANCER POTENTIAL OF *Halimeda tuna* ON HUMAN LEUKEMIC CELL LINES (Molt-4)

A DISSERTATION SUBMITTED TO

ST. MARY'S COLLEGE (AUTONOMOUS), THOOTHUKUDI

AFFILIATED BY Manonmaniam Sundaranar University

In partial fulfilment of the requirements for the award of the degree of

BACHELOR OF SCIENCE IN MICROBIOLOGY

SUBMITTED BY

J.ANNA RATHI (19SUMB01)

P.ARUNA (19SUMB02)

J.P.ASMIKHA(19SUMB03)

A.J.CELESTINA SELVAKANI (19SUMB04)

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R.DURGA DEVI (19SUMB06)

Under the Guidance of

MRS. R. SHYNISHA BEGAM, M.Sc., M.Phil.,

DEPARTMENT OF MICROBIOLOGY

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CERTIFICATE

This is to certify that the project work entitled **"ANTI-CANCER POTENTIAL OF *Halimeda tuna* ON HUMAN LEUKEMIC CELL LINES (Molt-4)"** submitted to St Mary's College (Autonomous),Thoothukudi affiliated to Manonmaniam Sundaranar University, Tirunelveli for the partial fulfillment for the award of Bachelor of Science in Microbiology is a bonafide research carried out by **J.ANNA RATHI ,P.ARUNA J.P.ASMIKHA ,A.J.CELESTINA SELVAKANI, T.DHIVYA DHARSHINI , R.DURGA DEVI** under the guidance and supervision of **Mrs. R. Shynisha Begam, M.Sc., M.Phil.**, Assistant Professor of Microbiology St Mary's College(Autonomous), Thoothukudi, for academic year 2021-2022.

SIGNATURE OF THE GUIDE

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Dr. Joys Selva Mary Albert
Head
Department of Microbiology
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SIGNATURE OF THE PRINCIPAL
Principal
St. Mary's College (Autonomous)
Thoothukudi - 628 001.

SIGNATURE OF THE EXAMINER

PLACE : THOOTHUKUDI

DATE : 25. 5. 2022

DECLARATION

I hereby declare that the project work entitled "**ANTI-CANCER POTENTIAL OF *Halimeda tuna* ON HUMAN LEUKEMIC CELL LINES (Molt-4)**" is a bonafide record of the work completed by me during the academic year 2021-2022 in St. Mary's College (Autonomous), Thoothukudi and submitted as a partial fulfilment of requirements for the award of the Degree of Bachelor of Science in Microbiology prescribed by the Manonmanimam Sundharanar University. I also affirm that this is a original work done by me under the supervision of Mrs. R. Shynisha Begam, M.Sc., M.Phil.,Assistant Professor of Department of Microbiology, St. Mary's College(Autonomous), Thoothukudi.

J. Anna Rathi
P. Aruna.
J.P. Asmikha
A.J. Celestina Selvakani.
T. Dhivya Dharshini
R. Durga Devi

Signature of the Guide

Signature of the Students

Place: Thoothukudi

Date: 25.5.2022

ACKNOWLEDGEMENT

In the name of GOD the most beloved and merciful, first and foremost all praise to be GOD for giving me the opportunity, patience, help and guidance for the completion of this.

I would like to thank Secretary, **Sr. Flora Mary**, St. Mary's college (Autonomous), Thoothukudi.

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To my Parents, and my friends, thank you for bringing me up to be who I am today. My success symbolize and reflects on the undivided support and love from all of you.

I also wish to express my thanks to the laboratory Assistant **Ms. M.Delecta Mary** for helping a lot during my study.

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INTRODUCTION

INTRODUCTION:

The world's ocean covering more than 70% of theearth's surface. Marine algae are one of the natural resources in the marine ecosystem. Sea weeds are recognized as a gorgeous origin of natural and bioactive compounds. They contain polyphenols, polysaccharides, peptides and fatty acids with various functional properties. Various biological properties present in marine environment. Cyanobacteria and marine algae have been discovered by screening programs. Phytoplankton, sea weeds and symbiotic dinoflagellates in corals are marine algae. Sea weeds are classified as Green algae (Chlorophyta), Brown algae (Phaeophyta), Red algae (Rhodophyta), and some filamentous blue green algae (Cyanobacteria). Algae frequently live in extreme environments of light, salinity and temperature. Most algae are easy to cultivate or produce industrial scale. Edible sea weeds contain a significant amount of protein, vitamins, and minerals which are essential nutrition's for human. Many algae produce a different variety of secondary metabolite products which helps to heal human chromosomal disabilities.

Sea weeds are located in every climatic zone from tropical warm waters to freezing polar regions. Environmental characteristics such as light exposure, temperature, depth, tides and intertidal species create particular habitats that determine both the distribution and variety of benthic marine algae. Their habitat also determines the presence of different phytopigments because not all species need the same light intensity to perform photosynthesis thus species differ considerably in many ultrastructural and biochemical features.

Green macroalgae present in coastal waters, and are absorb large amount of light energy, because of their expression of chlorophyll a and b, lutein, violaxanthin, neoxanthin, and enteroxanthin. Brown macroalgae are also found in deep ocean waters and their pigments are chlorophyll a and c and flavaxanthin. Macroalgae are rich in proteins, minerals, vitamins, antioxidants and non-starch polysaccharides such as

carrageenan and alginate. Sea weeds have a high content of essential amino acids therefore their protein qualities similar to that of other vegetables. Macroalgae also synthesize fucose containing sulfated polysaccharides such as fucoidan.

They are also rich in minerals, magnesium, iron, zinc and calcium. Sea weeds 20-76% (dry weight) of polysaccharides as structural and storage compounds cellulose, starch, hemicellulose, fucoidan, alginic acids.

CLASSIFICATION OFMACROALGAE :

Seaweeds or macroalgae refers to thousands of species of macroscopic, multicellular and marine algae.Different species of macroalgae are found in different coastlines of the world which are classified into three taxonomic groups based on pigments.

Brown seaweed (*Phaeophyceae*).The color of brown seaweeds is due to the presence of the xanthophyll pigment ,fucoxanthin .Brown seaweeds are large and measure about 2 to 65 m long and thick and leather-like ,and their small species is about 30-60 cm long . Some Indian brown seaweeds are*Dictyotaceylanica*and *Sargassum wightii* .Japanese brown seaweeds include *Laminaria sp., Saccharina sp., Undariasp .,Nemacystussp . Sargassum sp., (*formerly *Hizikia sp.),Eisenia sp.*, and *Ecklonia*sp .

Red seaweed *(Rhdophyta).* The color of red seaweeds is due to phycocyanin ,phycoerythrin ,chlorophyll a ,and xanthophyll pigments .They are small in size ,ranging from few centimeters to about a meter long .Some Indian red seaweeds are *Catenellacaespitosa*(formerly *Catenellarepens),Polysiphoniamollis* ,and *Gelidiellaacerosa*and some Japanese red seaweeds are *porphyrasp.,and Gracilaria sp.*

Green seaweed *(Chlorophyta).The color* of green seaweeds is yellow to green due to the presence of beta-carotene,chlorophyll and chlorophyll b ,and xanthophylls. They are small in size similar seaweeds are *Rhizocloniumriparium, Ulva intestinalis* (formely*Enteromorpha intestinalis),*Chaetomorpha*linustica (*formely*Lola* capillaries*),and Ulva lactuca while Monostroma sp.is a Japanase green seaweed.*

Domain	Kingdom	Phylum	Class
Prokaryta	Eubacteria	Cyanobacteria	Cyanophyceae
	Plantae	Rhdophyta	Bangiophyceae
			Florideophyceae
Eukaryota	Chromista	Ochrophyta	Phaeophyceae
	Plantae	Chlorophyta	Bryopsidophyceae
			Siphonocladophyceae
			Ulvophyaceae

Table 1: The General Classification Of Macroalgae

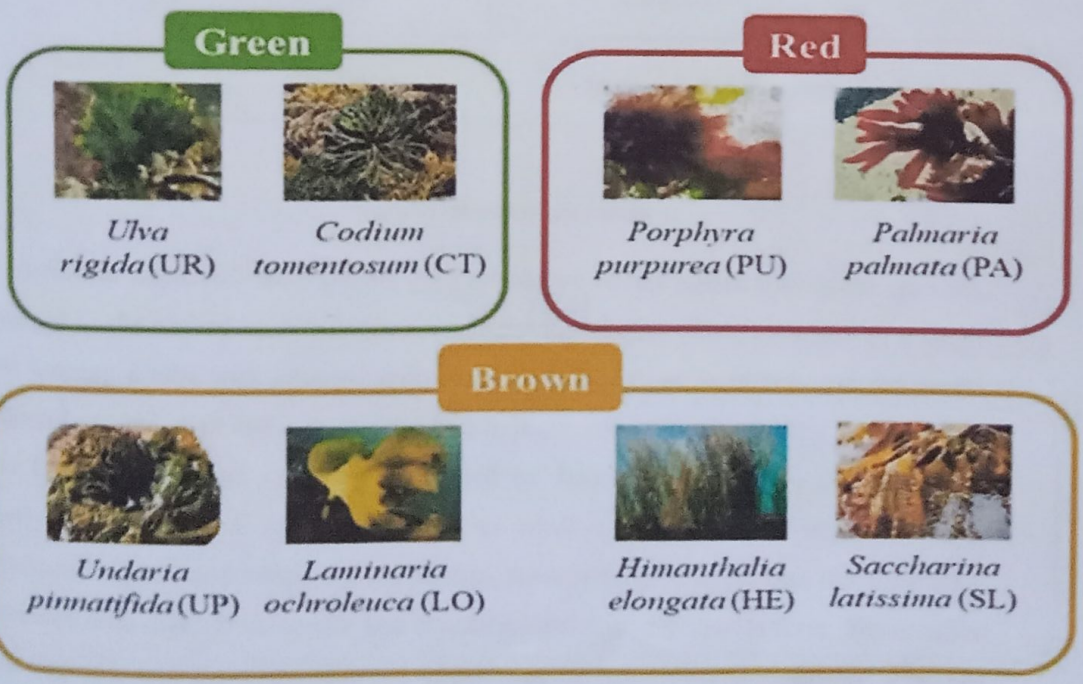


Fig.1Classification Of Seaweeds

SPECIES CLASSIFICATION:

TAXONOMY	
Kingdom	Plantae
Subkingdom	Viridiplantae
Infrakingdom	Chlorophyta
Division	Chlorophyta
Class	Ulvophyceae
Order	Bryopsidales
Family	Halimedaceae
Genus	*Halimeda*
Species	***Halimeda tuna***

Table 2:Taxonomic Hierarchy

*Halimeda tuna(J.Ellis&Solander) J.V.Lamouroux 1816*is acalcareous green seaweed, attached to the seabed by a holdfast.Each individual thallus (frond) consists of a single cell forming a tube with multiple cell nuclei. The cytoplasm is mobile and the nuclei, chloroplasts and other cell contents are free to move around inside the cell wall.The tube has flattened, disc-like segments connected by flexible joints. The surface of these segments have swollen areas called utricles which together make a tabular "cellular pavement". Below and between these utricles, there are gaps and it is here that the fluid is saturated with calcium carbonate and crystalline needles of aragonite form. These stiffen the segments and make the seaweed unpalatable to fish. When the seaweed dies, this

skeletal material breaks down into "sand". Members of this genus are likely to be one of the most important agents of calcification in the marine environment, considerably more productive in tropical seas than stony corals. This species is found in the tropical and subtropical Indo-Pacific region, the Mediterranean Sea and the western Atlantic Ocean. It grows on rocky reefs from the shallow subtidal zone down to depths of about 70 m (230 ft). Dead *Halimeda tuna*formshallow marine sediments. They are sessile organisms. This seaweed has been described as pleasant to eat with oil, vinegar, and salt.

Anti cancer effect of seaweeds:

Cancer is one of the most serious threat to human health in the world. chemotherapy is used as a treatment for cancer. Cancer causes sell to divide uncontrollably.Cancer is a group of diseases involving abnormal cell growth with the potential to invade or spread to other parts of the body. Every healthy individual have cancer cells in them(dormant form) , when favourable situation occur they change themselves into a proliferating cancer cells. Matrix metalloproteinases (MMPs) and vascular endothelial growth factor (VEGF) place major role in angiogenesis and metastasis. They help the cancer cells to spread throughout the body. Ongogeans and tumor suppressor geans plays a crucial role in cancer development. A potent anti cancer drug should have antioxidant, anti-apoptotic,anti-metastasis property for suppressing cancer cell progression and prevention.several ways that are induced to cause normal celsl into cancer cells are radiation including sunlight and X-rays in large or many doses, and exposure to radiation (for example in a nuclear power plant); chemicals and materials used in building and manufacturing (for example, asbestos and benzene.

In cancer cells, the normal cell division goes out of control. Cells change their nature because mutations have occurred in their genes. All the daughter cells of cancer cells are also cancerous.Cancer is generally not contagious in humans, though it can be caused by oncoviruses.

Many different lifestyle factors contribute to increasing cancer risk. Alcohol is an example of a chemical carcinogen.

The World Health Organization has classified alcohol as a Group 1 carcinogen.The most common types of cancer in males are lung cancer, prostate cancer, colorectal cancer, and stomach cancer. In females, the most common types are breast cancer, colorectal cancer, lung cancer, and cervical cancer.

Several risk factors for the development of colorectal cancer include high intake of fat, alcohol, red and processed meats, obesity, and lack of physical exercise. A high-salt diet is linked to gastric cancer.

Other names for cancer are Malignant tumor, malignant neoplasm .The neoplasm or tumor is a group of cells that have undergone unregulated growth and will often form a mass or lump, but may be distributed diffusely.

Chemotherapy and radiotherapy treatments are practices to cure cancers the survival rate is still low and many side effects are reported.Most of the anti cancer drugs currently used in chemotherapy or cytotoxic to normal cells and cause immunotoxicity which affects not only tumor development but also aggravates patients recovery.

Traditional chemotherapeutic agents are cytotoxic by means of interfering with cell division (mitosis) but cancer cells vary widely in their susceptibility to these agents.Anticancer drug, also called antineoplastic drug, any drug that is effective in the treatment of malignant, or cancerous, disease. Due to the continuing failure of the availability of effective chemo preventive agents for cancers, therapeutic agents from biological resources are being experimental to control this malignant disease.

In recent years, engrossment in cancer treatment by seaweeds and their phytochemicalshas increased. Though different seaweeds parts have prospects for curative use andchemoprevention, their mechanisms are very difficult to understand. Therefore, extensiveresearch has been recognised for targeted molecular which can have potential to be used as ananticancer agent. There are instance of modifying abilites of many seaweeds and plantproducts on many signalling pathways, along with their anti inflammation and antiapoptotictarget for cancer therapy. There are several phytochemicals like resveratrol, allicin, lycopene,indole-3-carbinol, vitamin c, gingerol,

emodin, natural antioxidant mixture, sulfarophaneellagic acid, myrecitin, vanillin, and eugenol that have anticancer property. They act throughone or more signalling pathways.

Uncontrolled cell growth and proliferation is the main reason for cancer formation.Chemo preventive agents extracted from natural resources have got the attention due to the ability to suppress cancer causing cell formation with lesser or no side effects compared to radiotherapy or chemotherapeutic agents. Basically cell death can be caused by any chemotherapeutic agent through cell arrest by targetting necrotic pathway or apoptotic pathway of respective cells. Bioactive compounds are able to induce apoptosis in cancer cells. This is one of the key mechanism in Cancer therapy because apoptosis is a kind of programmed cell death which can kill only the targetting cancer cells without causing damage to normal surrounding cells. Seaweed having potential bioactive compounds with anticancer effects and able to induce apoptosis in several types of cancer inducing cells .

Cell culture:

Cell culture refers to the removal of cells from an animal or plant and their subsequent growth in a favorable artificial environment.Cell culture is the process by which cells are grown under controlled conditions, generally outside their natural environment.After the cells of interest have been isolated from living tissue, they can subsequently be maintained under carefully controlled conditions.

Cell culture basal media :

There are different kinds of cell culture media which being used routinely in life science including the following:

- MEM (Minimum Essential Medium)
- DMEM (Dulbecco's Modified Eagle's Medium)

The growth factors used to supplement media are often derived from the serum of animal blood, such as fetal bovine serum (FBS), bovine calf serum, equine serum, and porcine serum.

Derivation of MOLT -4 cell line:

MOLT-4 is derived from (T lymphoblast; acute lymphoblastic leukemia).Molt-3 and Molt-4 are T-cell lines originally derived in 1971 from a patient with T-cell acute lymphoblastic leukemia. An unusual T-cell antigen receptor gamma-chain gene (T-gamma) rearrangement detected by Southern blot analysis of Molt-4 prompted an in-depth study of the immunophenotype and karyotype of both cell lines. Molt-3 and Molt-4 had immunophenotypic characteristics of thymocytes with expression of CD1 and CD5. Both cell lines had a hypertetraploid karyotype with two rearranged no. 7 chromosomes.

AIM & OBJECTIVE

AIM & OBJECTIVE:

- To collect *Halimeda tuna* and prepare solvent extraction.
- To maintain and preserve blood cancer (MOLT-4) cell lines in vitro.
- To determine the cytotoxicity of *Halimeda tuna* from MTT assay.
- To evaluate anticancer activity of*Halimeda tuna*.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Kiichiro Teruga*et.al.*, (2009), concluded that enzyme digested fucoidan extract suppressed the growth of anchorage depended and anchorage independent cancer cells. The enzyme digested fucoidan extract stimulated the capase and mitochondrial pathways to induce apoptosis. The enzyme digested fucoidan extract enhanced conA binding of cancer cells and conA induced apoptosis.

KeivanZandi*et.al.*,(2010), reported that red algae *Gracilaria corticate* had anticancer activity and it might be a good candidate for further investigations in order to develope a natural compound as an anticancer agents which can be used for the production of potential anticancer drugs and pharamaceutical leads.

Luis J.Villareal -Gomez *et.al* ., (2010) ., Mentioned that Marine algae and bacteria produced variety of biologically secondry metabolites. This metabolites contribute to the discovery of new drugs.

Gareth H.Williams*et.al.*, (2011), described that cell cycle engine is a trustful diagnostic and therapeutic target in cancer because it of lies downstream at the convergence point complex oncogenic signaling networks and its deregulation is central to the aberrant cell proliferation that characterizes all cancers.

Mohammed A .Deyab*et.al* ., (2012)., Showed that water extract of *Tubinariaornata* as well as oleic acid and palmitic acid upon tumor cells in vitro were shown to be concentration and time dependent.

V. Lavakumar*et.al.*, (2012), demonstrated that ethanol extract of Acanthophoraspiciferapossesanti tumor and anti oxidant activity and the beneficial effect may be due to the presence of bio-active components like flavonoids, terpenoids and tannis.

A.Raja*et.al*.,(2013), highlighted the potential of marine algal compound the increase number of bacterial and fungal metabolites in marine algae had a very good industrial application and as a noval medicine.

K. Indria*et.al* .,(2013), examined antibacterial and antifungal activity in *Halimeda tuna* against ten bacterial strains ethanolic and choloroform extract, methanolic extracts showed exhibited broad spectrum of antimicrobial activity.

MyoungLaecho*et.al*., (2013), suggested that pheephorbide a (pa) isolated from Grateloupiaelliptica is a potential glioblastoma specific anticancer agent without side effects on normal cells.

Catherine Murphy *et .al*., (2014), studied on marine macroalgae that have been investigated for their potential as sources of novel anti-cancer drugs.

Dhivya Balakrishan*et .al* ., (2014), said that bio-active metabolism extracted from seaweed and sponges and their associated bacteria had good antioxidant activity.

Emma M. Brown *et al*., (2014), evidenced the beneficial effect of sea weeds and it's components on marketers of human health and disease status.

FaridehNamvar*et.al* ., (2014), demonstrated that *Gracillaria corticat*methnol extract (GCME) could inhibit the growth of cancer cells and induce apoptosis in human breast cancer.

GhislainMoussavou*et.al*.,(2014),reported that seaweed fight against colorectal and breast cancer.

H.H. Chaminda Lakmal*et.al* .,(2014), Explained that red algae (*Chondrophycusceylanicus* , *Geldiellaacerosa* ,*Gracilaria Corbicata*)And brown algae (*Sargassum cassifolium*) extracts showed antioxidant effect with the radical scavenging activity tested by ESR spectroscopy . *Caulerpa racemosa* extract showed anticancer activity against HL-60 cells

Mohammed M. Safhi (2014), studied on antibacterial activity of seaweed which is an important todevelop newer drugs.

O.Kur*et.al.*, (2014), analysed the neurotoxic , cytotoxic, apoptoic and antiproliferative effects of extracts of*Petalonia fascia, Janialongifurca* and *Halimeda tuna* on breast cancer cell line [MCF-7] and also *Janialongifurca* extract shows more toxic than other two seaweeds.

ThangapandiMarudhupandi*et.al.*, (2014), demonstrated that the fucoidan from Turbinariaconoides could be significantly showed broad spectrum of biological activities.

MeganathanBoominathan*et.al* ., (2015), pointed mechanism of action seaweed carotenoids and strategy to reduce the risk of cancer incidence and mortality rate.

S. Aswin *et.al.*, (2015), demonstrated that chloroform & ethanol extract of *Gracilaria corticata* showed a greater anticancer activity.

Hak Jun Kim *et.al.*, (2016), reported that enzyme digested fucoidan extract suppressed the growth of anchorage depended and anchorage independent cancer cells. The enzyme digested fucoidan extract stimulated the capase and mitochondrial pathways to induce apoptosis. The enzyme digested fucoidan extract enhanced conA binding of cells and conA induced apoptosis.

Denise FernandercoutinhoMoraes*et.al.*, (2017), review the importance of plants as source of drugs and described the anticancer compounds.

Edward Lopes Casta*et.al.*, (2017), described that the anticancer effects attributed to Fx and PH is colon cancer cells. Further supports the potential of seaweeds as a source of bio-active compounds with bio-medical application.

Marion Zenthoefer*et.al.*,(2017)., demonstrated that examined from crude extracts to the points of purified fractions the anti-cancer potential of the Baltic sea *F.*

vesiculousus proving that this seaweed holds significant cytotoxic activity against human pancreatic cancer cells.

Rai Abdelwahab., (2017), discussed about the important of seaweeds in pharmaceutical & investigator application.

Alejandra Miranda Delgado *et.al.*, (2018), demonstrated that the dichloromethane extract from DictytoKunthii and chondracanthuschamissoi showed high cytotoxic activity against HT-29 and MCF-7.

AnllelyG.Gutierrez -Rodriguez *et. al* .,(2018), determined that seaweeds extracts were applied in food supplements but seaweeds bio- molecules have good source of anticancer drug

DjenisaH.A.Rocha*et .al.*, (2018), discussed about the in vitro activities of 53 secondary metabolities isolated from brown, red, green seaweeds.

Kartika Dwikurniasari*et. al.*, (2018), demonstrated that ethanolic extract of the sea weeds *Gracilaria verrucosa* shows anticancer activity against HCT-116 cancer cells, which is potential to the development as promising new anti- colorectal cancer agent.

Ramalingam vaikundamoorthy*et. al.*, (2018), reported that polysaccharide from Sargassum wightii maybe a candidate for future evaluation as an anticancer agent for human cancer especially the breast cancer.

SalyGhedee*t .al.*, (2018), highlighted the polysaccharides of red marine alga Jania Rubens could be a potential candidate for the natural compounds as antioxidant and anticancer therapy.

Hemasudha*et.al* .,(2019), concluded that *Gracilaria edulis* shows reducing cancer activity against invitro study of breast cancer..

T.H.Ranhew*et.al* .,(2019), mentioned that there are several therapeutic effect for seaweeds due to the presence of unique bio-active compounds.

Tatainaolivares Banuelos *et.al.*, (2019), suggested that egregiamenziesii extract might be good competitor for cancer prevention and the development of novel chemotherapies due to its highest cytotoxicity in transformed cells compare to glia primary cultures.

Thais Rodrigues Teixeira *et.al.*, (2019), described the fungal biotechnology application to obtaining bioactive products from fungi of marine origin mainly anticancer compounds.

Zhiwei Liu *et.al.*,(2019), demonstrated that porphyrans and carrageenans combined with conventional drugs ,these polysaccharides not only showed anticancer activity but also enhance immune competence that had been damaged by drug.

Ali .M. Saeed *et. al* ., (2020) ., Demonstrated that *Ulva lactica* chloroform extract had strong cytotoxic activity against MCF-7 Hela cell liner and *Ulva fasciata* has strong cytotoxic aduchudu against PC3 and Hepg2 cell liner.

Baskaran Babu *et .al.*, (2020), reported that *Acanthophorcespicifera* mediated gold nanoparticles exhibitedanote worthy cytotoxicity outcome against human colorecta adenocarcinoma cells HT-29.

Ferrara *et .al.*, (2020), determined the seaweeds are excellent candidates to be used as ingredients for health by the food industry.

Vangelis smyrniotopoulos*et.al.*, (2020), studied the lipophilic extracts of the Brown alga *Bifurcatabifurcata* showed the enormous capacity of the sea weed to produce a large palatte of acyclic diterpenes which diverse oxygenation and substitution pattens and promising bio activities.

EI Nur E.E. *et.al.*, (2021), said that natural metabolities extracted from the marine algae may be considered as responsible agents for favourable bio-activities. The cytotoxicity of the crude extract displayed extraoridinaryunprecedentented result which were exposed positive selectivity against cancer cell lines.

JelanMofeed*et.al.*, (2021).,deliberated that crude extract of *Ulva lactuca* and *Amphiroa anceps* exhibited the maximum activity against human breast adenocarinoma cell line (MCF-7) colorectal carcinoma cell line (HCT-116).

Samina HyderHaq*et.al.*, (2021), demonstrated that the ethanol extract of chaetomorpha sp. Antioxidant and Anticancer activity compared to aqeous extract. Anticancer activity of chaetomorpha sp. Due to the presence of several active potent antitumor chemicals such as DCA, Oximes and tepinol.

Shimaa*et.al* .,(2021), explained that red and brown seaweeds *Padina pavonica* ,*Taoniaatomaria*, *Janiarubens* and *Corallina elongata* are rich in secondary metabolites and showed antioxidant and anti-inflammatory . Methanol extracts of *J.rubens* and *P.pavonica*& observed the highest cell growth inhibition of the Hela canar cell lines compared to other seaweed extracts.

Yogesh Kumar *et.al.* ,(2021), said that the bio-active and nutritional compounds take place in different classes of seaweeds while targeting on their the therapeutic activities.

MATERIALS AND METHODS

Materials and methods:

Collection of sample:

Fresh plants of *Halimeda tuna* (Bryopsidales, Chlorophyta) (J.Ellis and Solander) J.V.Lamouroux(1816) were collected from the intertidal region (Lat. 8° 40' to 8° 55' N and Long. 78° 0' to 78° 15' E) on the Tuticorin, Tamil Nadu, Southeast coast of India. The plants were cleaned of epiphytes and extraneous matter, and the necrotic parts were also removed. Afterward, the plants were washed with seawater and then in fresh water and then transported to the laboratory in sterile polythene bags . Samples were rinsed with sterile distilled water and were then shade-dried, cut into small pieces and powdered in a mortar pestle.

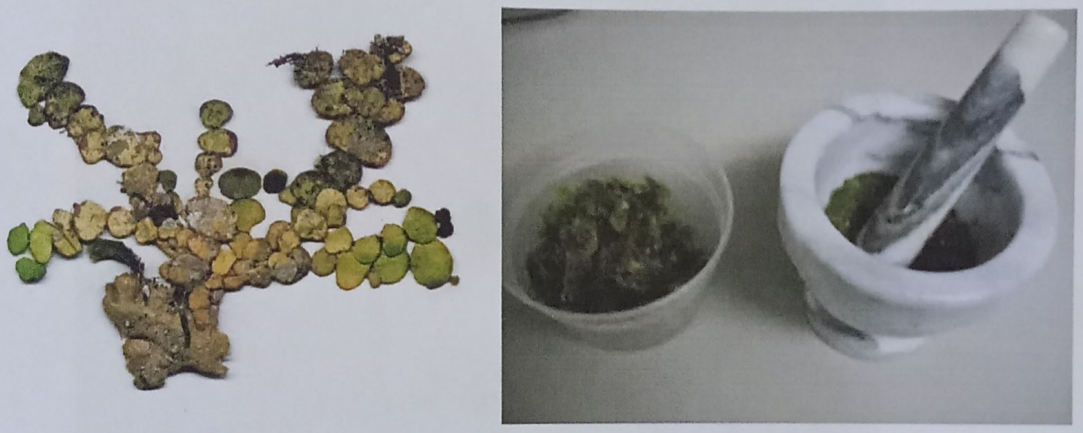


Fig 2: Collection of sample

Extraction of the samples:

The fresh sample of *Halimeda tuna* were washed with running tap water and shade dried. Then the sample was crushed and coarsely powdered. These coarse powders (25g) were then subjected to successive extraction in 250ml of ethanol by using Soxhlet apparatus. The collected extracts were stored and then used for further analysis.

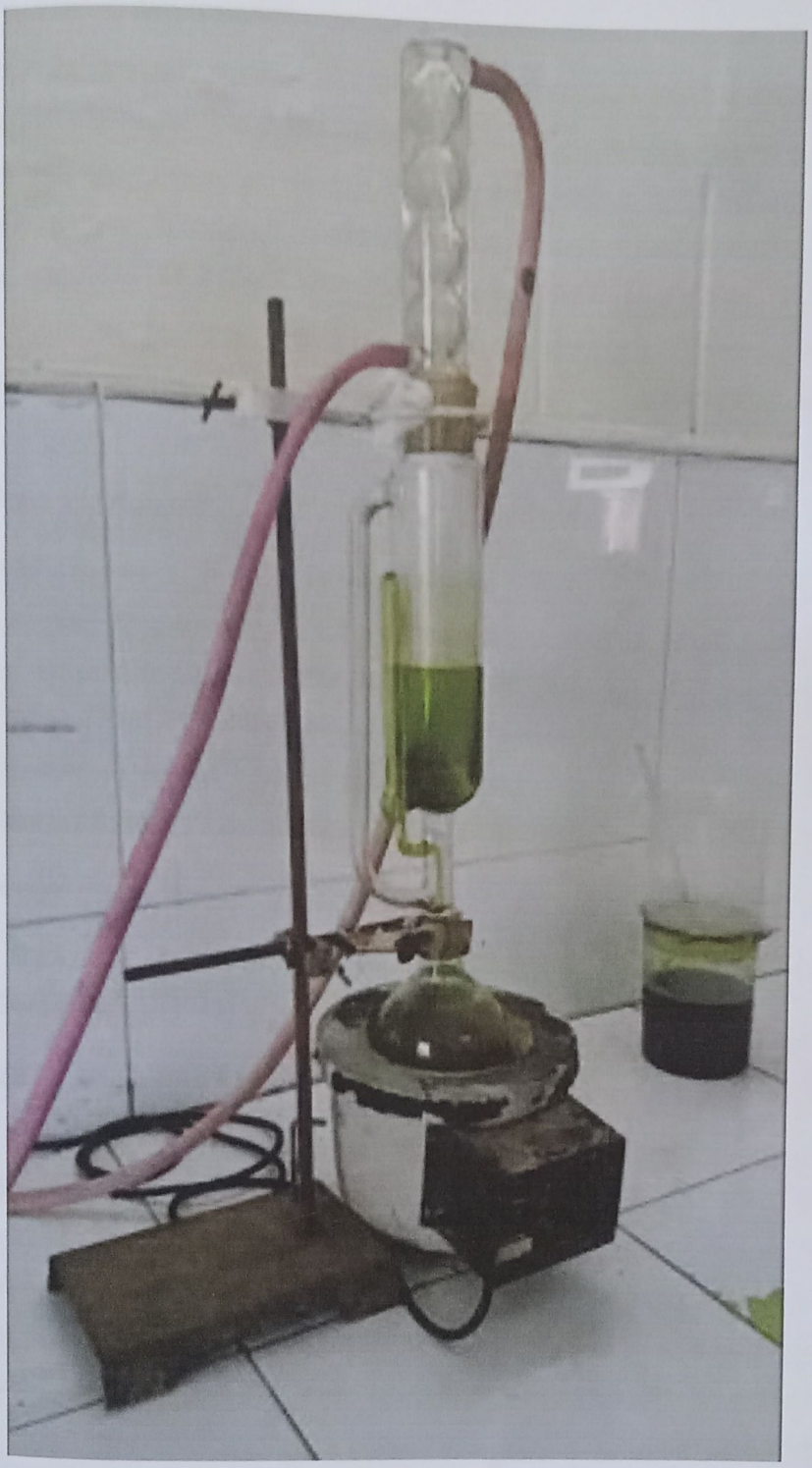


Fig 3: Extraction Of Sample Using Soxhlet Apparatus

Source of chemical and reagents:

Dulbecco's Modified Eagle's Medium (DMEM), streptomycin, penicillin-G, L-glutamine, phosphate buffered saline, 3-(4,5 dimethylthiozol-2-yl)-2,5-diphenyltetrazoliumbromide, 2'7'diacetyl dichloro fluorescein, sodium dodecyl sulfate, trypan blue, trypsin-EDTA, ethylene diamine tetra acetic acid, acridine orange, ethidium bromide, rhodamine-123, triton X-100, ethanol, dimethyl sulfoxide (DMSO), and bovine serum albumin were purchased from Sigma Aldrich Chemicals Pvt Ltd (India). All other chemicals used were of analytical grade, purchased from Hi media Laboratories Pvt. Ltd., India.

Cell culture maintenance:

Blood Cancer (Molt-4) cell line was procured from the cell repository of National Centre for Cell Sciences (NCCS), Pune, India. Dulbecco`s Modified Eagle Media (DMEM) was used for maintaining the cell line, which was supplemented with 10% Fetal Bovine Serum (FBS). Penicillin (100 µg/ml), and streptomycin (100 µg/ml) were added to the medium to prevent bacterial contamination. The medium with cell lines was maintained in a humidified environment with 5% CO_2 at 37°C.

MTT assay:

The cytotoxicity of *Halimeda tuna* on Blood cancer cells was determined by the method of Mosmann, (1983).

Principle:

This is a colorimetric assay that measures the reduction of yellow3-(4,5dimethylthiozol-2-Yl)-2,5diphenyltetrazoliumbromide [MTT] .The determination of cell viability by(3-(4,5dimethylthiozol-2-Yl)-2,5diphenyltetrazoliumbromide [MTT] assay).Tetazolium dye is reduced by mitochondrial dehydrogenase of viable cells yielding a measurable purple formation product. Viable cells contain NAD(P) H-dependent reductase, which reduce the MTT reagent to formazan, with a deep purple colour. Formazon crystals are then dissolved using solubilizing solution and absorbance is measured at 500-600 nm by plate reader.

Reagents:

MTT stock solution:

MTT (50 mg) dye was dissolved in 10 ml of PBS. After vortexing for 1 min, it was filtered through 0.45 micro filters. The bottle was wrapped with aluminium foil to prevent light, as MTT was light sensitive. The preparation was stored at 4°C.

Procedure :

Cell viability assay:

Blood Cancer (Molt4) cells were harvested and counted using haemocytometer diluted in DMEM medium to a density of 1×10^4 cells/ml was seeded in 96 well plates for each well and incubated for 24 h to allow attachment. After that the Blood Cancer (Molt4) cells were treated with different concentrations of the seaweed extract (50 to 300 µg/ml) were applied to each well. Blood Cancer (Molt4) cells were incubated at 37°C in a 95% humidified air and 5% CO_2 for 24 hrs. After incubation, the drug-containing cells were washed with fresh culture medium and the MTT (5 mg/ml in PBS) dye was added to each well, followed by 4 h of incubation at 37°C. The purple precipitated formazan formed was dissolved in 100 µl of concentrated DMSO and the cell viability was absorbance and measured 540 nm using a multi-well plate reader. The results were expressed at the percentage of stable cells with respect to the control. The half maximal inhibitory concentration (IC_{50}) values were calculated, and the optimum doses were analysed at different time period. All experiments were performed at least three times in triplicate.

IC50 values and graphics were done using GraphPad Prism 8 software using the inhibitor vs normalize response method.

1. Prism can easily fit a dose response curve to determine the IC50
2. From the Welcome dialog, choose the XY tab, drop the list of sample data sets and choose "RIA or ELISA".
3. Note that the X values are concentration. Also note that this sample data set includes unknown values. These are Y values without corresponding X values. Prism can interpolate these X values.
4. Click Analyze and then Non-linear regression.
5. On the Nonlinear regression dialog, open the "Dose-Response -- Inhibition" family of equations, and choose "log(inhibitor) vs. response -- Variable slope (four parameters)". At the bottom of the dialog, check the option to "Interpolate unknowns from standard curve".
6. Click OK and view the results.

RESULTS AND DISCUSSION

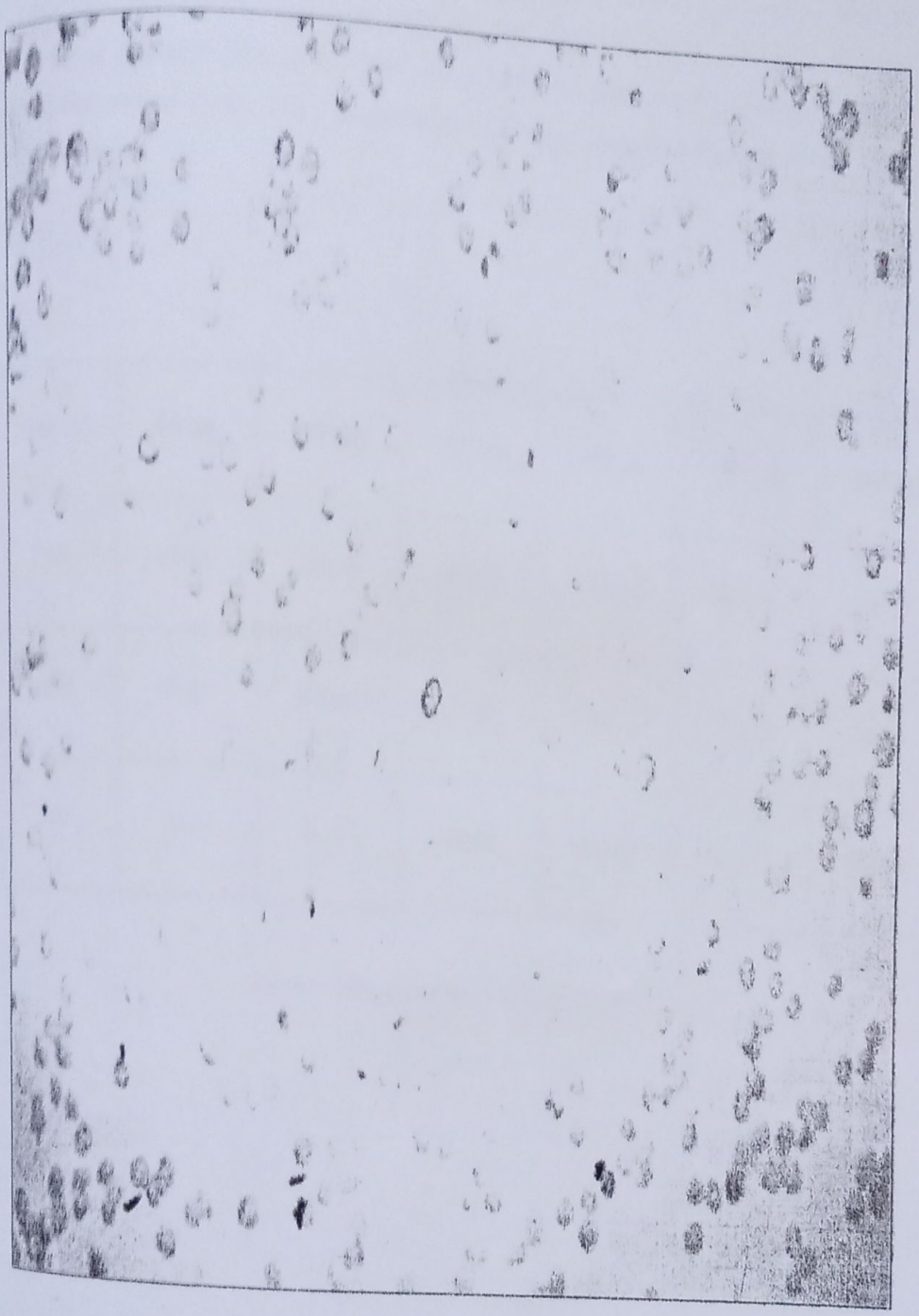
RESULTS AND DISCUSSION:

The seaweed extracts were screened for their potential efficiency against cancer cell lines.The MTT assay used here described in methodology section. Results obtained suggest that crude extracts of the *Halimeda tuna* exhibit significant activity against cancer cell lines. Highest concentration tested 200 µg/ml following 24 hrstreatment .Cell were treated for 24 hrs as an increasing concentration of from 50,100,150,200,250 and 300 µg /ml using the seaweeds extracts *Fig 5.* Shows graph lines representation of cell viability using MTT assay.It showsvery good activity highest concentration tested of 50µg /ml 24hrs of treatment. *Table 3* gives details of data points of cell viability using MTT analysis.the extracts on the cancer cell lines is was measured using in the IC 50 value principle , which is a principle based on the concentration of the plant extract that causes cell death the lower the IC 50 value of an extract on a cell line more potent it is considered to be. The aqueous extract of the *H.tuna* exhibited considerable levels of inhibition percentage of activity against the cancer cell the presented in fig 5 describes the IC50 value of the extract . the extract found exhibit cytotoxicity to Molt 4 cells cancer at IC 50 at 50 µg /ml. these exhibited high level of cytotoxicity. In the study, the water crude extract of *Halimeda tuna* was studied for its probable anti cancer activity against molt-4 cell lines which are kinds of human leukemic cell lines. Based on previous experience, filtration method is the best way for seaweed extract sterilization. We are not using autoclave for sterilizing extract because of the heat sensitivity of some biological constituents of seaweed extract. The most effective concentration against molt-4 cells where 250 and 300 respectively.MTT assay was used to measure significance variations in the inhibitory activity of *Halimeda tuna* extracts on molt-4 leukaemia cancer cell lines. The best thing about MTT is that it does not have limitations and one can test as many times as possible. It was used to measure IC 50 value, the concentration of *Halimeda tuna* extract that induces 50% cell death on the molt-4 cancer cells.

The IC50 is the concentration of drug required for 50% inhibition. IC 50 values used to determine drug effectiveness and it's potency low IC - value means that the drug is potent at low concentration, and thus will show low systemic toxicity when administered to molt to cell line. The total number of cells that remained after 24 hours of the stimulated time is recorded.

Control

200 µg



250 µg

Fig 4: Morphological changes in control and sample seaweed treated blood cancer (Molt-4) cells for 24 h.

Photomicrograph represents morphological changes in Blood cancer cells such as shrinkage, detachment, membrane blebbing, and distorted shape induced by sample seaweed treatment (200 µg and 250 µg for 24 h) as compared with control. Control showed normal intact cell morphology and their images were capture by light microscope

Control	50 µg	100 µg	150 µg	200 µg	250 µg	300 µg
100	94.91	89.35	81.02	56.48	40.74	29.17
99.9	97.69	87.96	73.61	53.7	33.33	27.31
100	96.3	86.11	76.85	55.09	45.37	19.44

Table 3: Concentration vs Cell viability %

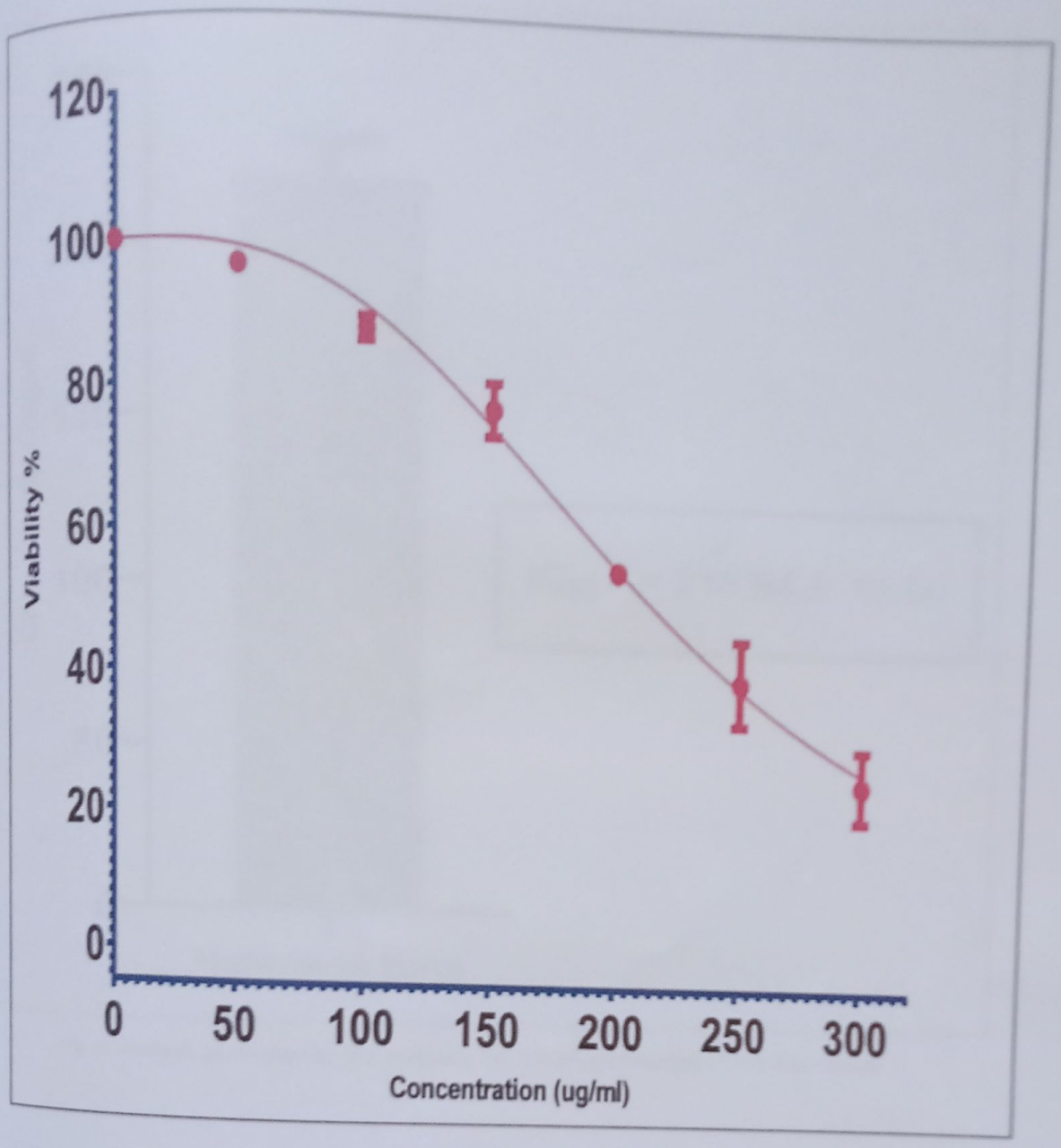


Fig 5: Determination of IC50 values from extract of *Halimeda tuna*for 24 h of incubation in Molt4 cells using GraphPad Prism 8. Results of the viability assays of the *H. tuna*.

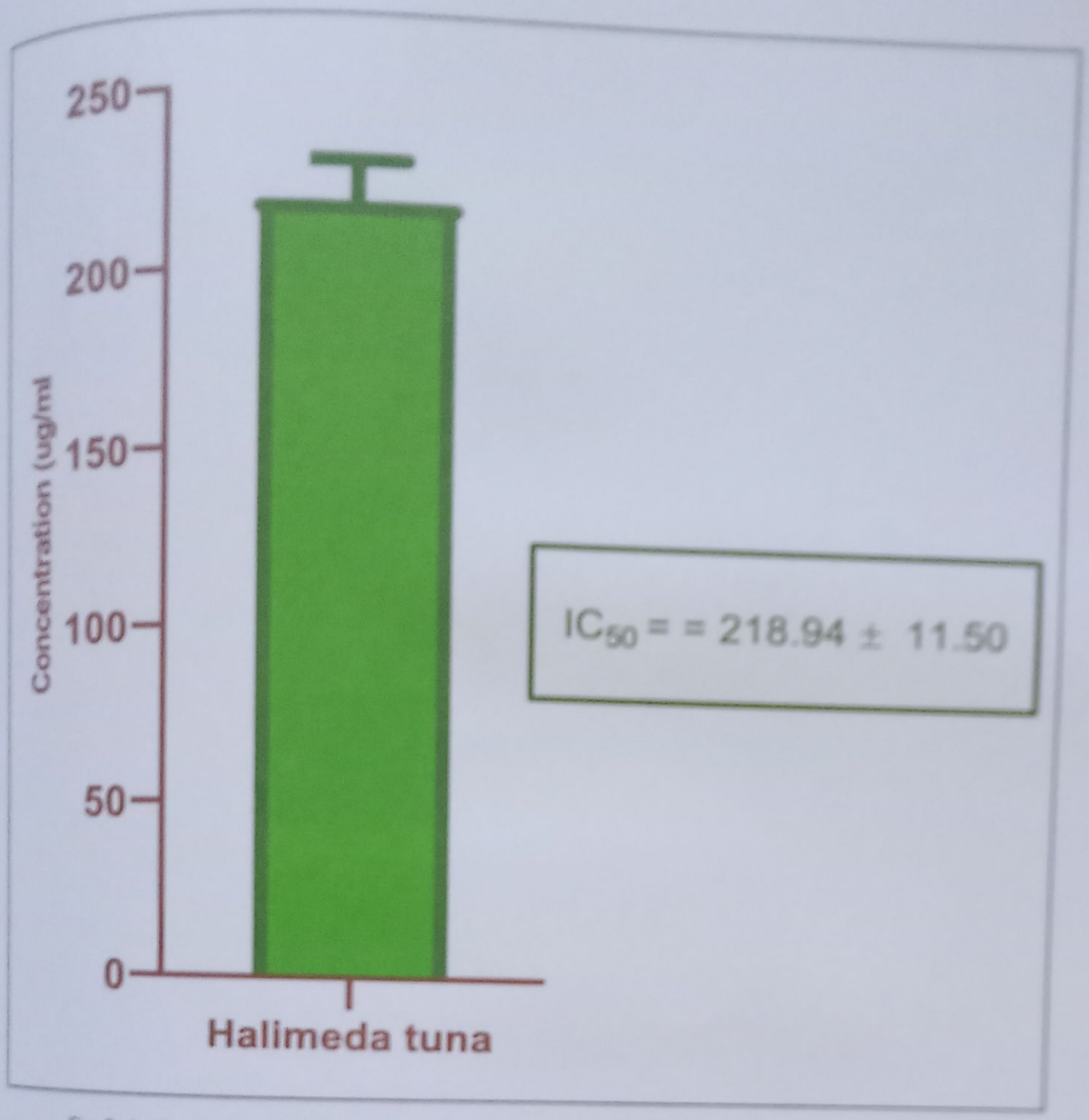


Fig 6: Analysis generated by the software for the determination of the IC50 values

CONCLUSION

CONCLUSION:

The ideal cancer treatment eradicates tumor cells without damage to healthy tissues. Due to the side effects of current treatment methods ,more attention is given to the selective toxicity of seaweed extraction that are nontoxic to normal cells ,but are toxic to tumor cells. Several in vitro and in vivo studies have demonstrated certain seaweeds have strong and effective anticancer properties.

The therapeutic effect of seaweeds wasexplored due the presence of novel bioactive compounds. In this context the anticancer effectof seaweed plays a major role by inducing apoptosis. Seaweed are comprised with manysecondary metabolites and some of them have been isolated and further studies are requiredfor isolation of other therapeutic compounds from seaweeds since the most of them showcytotoxic activity against cancer cells and not in normal cells. Thus further studies will openthe gate to reveal new chemotherapeutic agents which are able to fight against cancers whilehaving no or less impact on normal cells.

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ELIMINATION OF HEAVY METALS FROM INDUSTRIAL WASTE WATER USING MICROBIAL CONSORTIUM AND ABSORBENT

A PROJECT SUBMITTED TO

ST. MARY'S COLLEGE (AUTONOMOUS), THOOTHUKUDI

Affiliated by Manonmaniam Sundaranar University,

In partial fulfilment of the requirement for the award of the degree of

BACHELOR OF SCIENCE IN MICROBIOLOGY

SUBMITTED BY

FATHIMA. K (REG NO- 19SUMB07)

HAMZA VARTHANI. R (REG NO-19SUMB08)

HEMA. A (REG NO- 19SUMB09)

INDHUMATHI. A (REG NO-19SUMB10)

ISWARYA. T (REG NO-19SUMB11)

JEFA SHERLIN. B (REG NO-19SUMB12)

Under the Guidance of

Ms. P. RAJA RAJESWARI

DEPARTMENT OF MICROBIOLOGY

St.Mary's College (Autonomous) , Thoothukudi-628 001

May-2022

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St.Mary's College (Autonomous) , Thoothukudi-628 001

May-2022

CERTIFICATE

This is to certify that the project work entitles **"Elimination of Heavy metals from Industrial waste water using microbial consortium and absorbent"** submitted to **St. Mary's College (Autonomous) , Thoothukudi** affiliated to **Manonmaniam Sundaranar University, Tirunelveli** for the partial fulfillment for the award of Bachelor of Science in Microbiology is a bonafide research carried out by **Fathima. K , Hamza Varthani. R , Hema. A, Indhumathi.A, Iswarya.T and Jefa Sherlin.B** under the guidance and supervision of **Ms. P. Raja Rajeswari** Assistant Professor of Microbiology St. Mary's College (Autonomous), Thoothukudi, for academic year 2019-2022.

SIGNATURE OF THE GUIDE

SIGNATURE OF THE HOD

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Director
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St. Mary's College (Autonomous)
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St. Mary's College (Autonomous)
Thoothukudi - 628 001.

SIGNATURE OF THE EXAMINER

PLACE: THOOTHUKUDI

DATE: 25-5-2022

DECLARATION

I hereby declare that the project work entitled **"Elimination of Heavy metals from Industrial waste water using microbial consortium and absorbent"** is a bonafide record of the work completed by us during the academic year 2019-2022 in St. Mary's College (Autonomous), Thoothukudi and submitted as a partial fulfilment of prescribed by the Manonmaniam Sundaranar University. We also confirm that this is a original work done by us under the supervision of **Ms. P. Raja Rajeswari M.Sc., (Ph.D.)** Assistant Professor, Department of Microbiology, St. Mary's College (Autonomous), Thoothukudi.

K. Fathima

R. Hamza Varthani

A. Hema

A. Indhumathi

T. Iswarya

B. Jefa Shelin.

SIGNATURE OF THE GUIDE

SIGNATURE OF THE STUDENTS

PLACE: THOOTHUKUDI

DATE: 25 - 5 - 2022

ACKNOWLEDGEMENT

In the name of the GOD the most beloved and merciful, first and foremost all praise to be GOD for giving us the opportunity, patience, help and guidance for the completion of this.

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ABSTRACT

Rapid industrialisation and urbanization have resulted in significant pollution of the environment. Because inorganic and organic wastes are released into the river, water pollution is one of the most important issues. Heavy metal-containing industrial Waste water could be a substantial cause of contamination. Due to a lack of effective environmental planning, harmful heavy metals may be released into the environment, causing major environmental hazards. Lead, mercury, nickel, cadmium, and chromium (VI) rank first among metal ions in terms of toxicity. Metal ions accumulate and their levels grow along the food chain due to their non-biodegradability. Heavy metal decontamination from wastewater has long been a difficult task. Because of the non-renewable resources utilized, the high expense, and the formation of toxic sludge, traditional technologies are costly, and their harmful effects are more pronounced in animals at higher trophic levels. Bioremediation with heavy metal resistance microorganisms is a less expensive solution. Biosorption is a biotechnological breakthrough as well as a cost-efficient and effective method for eliminating heavy metals from aqueous solutions. Using bacterial species and chitosan, an effective bio sorbent may easily remove hazardous metals from industrial Waste water. The goal of this study is to look at how heavy metals can be removed from power plant Waste water utilizing bacterial species and chitosan for nickel and chromium entrapment.

INTRODUCTION

In past years rapid population growth and urbanization leads to development of industrial areas. The industrial areas liberate toxic Waste water. These toxic Waste waters are wide spread in water bodies. The toxic substance contaminates the environment is increasing that produce a vital concern to the local areas. The water is contaminated by foreign matter is called water pollution. It worsens the quality of water. Water pollution has a liquid forms like ocean pollution and river pollution. Aquatic environment is continuously polluted by many contaminants particularly due to increased industrialization, technological development, human population. Among these, heavy metals are a major class of contaminants that cause harmful effects on both human health, terrestrial, aquatic communities and ecosystems. Heavy metal refers to any natural elements which have relatively high atomic mass and high density. Heavy metals are non-biodegradable and tend to bioaccumulate. These have toxic effects even at low concentration and that are long lasting. Various adverse health hazards are known due to long term and continuous exposure to heavy metals and many of them are known to be toxic or carcinogenic. Nowadays, heavy metals are becoming one of the most significant environmental problems. Amid the pollutants, heavy metals accumulation in the marine ecosystem is of global significance. Heavy metals can enter the water supply by industrial and consumer or even from acidic rain. It breaks down soil and it produce heavy metals into streams, lakes, rivers and ground water. They cause serious problems to the human population and the fauna and flora of the receiving water bodies. The earliest commonly known heavy metals such as iron, copper and tin. Precious heavy metals such as silver, gold and platinum. The heavy metals need to be treated with caution for physical and chemical characterisations. As the metals involved are not always consistently defined. The heavy metals are natural components of the earth's crust. Heavy metals cannot be degraded or destroyed. Heavy metals can enter our body through food, drinking water. As trace elements, some heavy metals are essential for the metabolism to maintain the human body. Heavy metals can cause poisoning from drinking water contamination (e.g.: lead pipes) high ambient air concentration near emission sources. Even though heavy metals have toxic effects, many heavy metals undergo excellent technological importance (e.g.) iron, zinc, tin, lead, copper, tungsten etc. Lately, for special chemical transformations various heavy metals involved in artificially designed "bio inorganic" catalyst as the central atom. If hazardous chemical elements released into the

environment, assemble in the soil and water body sediments. Pollution rises due to heavy metals on surface and underground water. When animals are exposed to contaminated water accumulate heavy metals in their tissues, and milk if lactating, in turn when humans consume these contaminated animals or their milk will result in various chemical disorders. So, suitable methods should be established to remove these toxic heavy metals from the water.

1. TYPES OF HEAVY METALS:

Heavy metals can be classified into two categories:

1. Essential heavy metals
2. non-Essential heavy metals

ESSENTIAL HEAVY METALS: These are required by living organisms for fundamental processes like growth, metabolism, and development of different organs. There are heavy metals like Cu, Fe, Mn, Zn, and Ni. Essential elements are often required in trace amounts in the level of 10-15 ppm. And they are known as Micro nutrients.

NON-ESSENTIAL HEAVY METALS: Non-essential heavy metals like Cd, Pb, Hg, Cr, and Al. These are not required by plants. Non-essential heavy metals have a risk to the human health and environment. Exposure to NEM's such as cadmium, lead and mercury leads to significant number of adverse health effects in human.

2. SOURCES OF HEAVY METALS:

The hazardous metals and minerals can cause an environmental pollution. It can rise from natural as well as anthropogenic sources.

NATURAL SOURCES: Seepage from rocks into water, volcanic activity, forest fires etc. Pollution also occurs from polluting elements between sedimentary rocks and their precursor sediments and water. The rapid industrialization and consumerist life style is due to the increase in this source of environmental pollution. Through food and water, the toxic elements can enter the body. In the lesser extent through inhalation of polluted air, by the use of cosmetics, drugs, poor quality herbal formulations and even items like toys, which have lead coating.

ANTHROPOGENIC SOURCES: Through uncontrolled extraction of huge quantities of metals, ores in open fires small number of heavy metals are released. In Industries, metals obtained from natural resources are processed from where heavy metals are moved on to the atmosphere. Likewise traces of heavy metals enter the environment through discharge of waste from domestic, agricultural and auto exhausts.

Several human activities through which heavy metals get into the environment are shown below: (i) Blending or processing of ores of metals (ii) Mining (iii) Burning of fossil fuels like

coal, petrol, kerosene oil (iv) Discharging agricultural waste, industrial waste and domestic waste (v) Discharge from auto exhausts (vi) Using pesticides containing compounds of heavy metals. Metals like iron, copper, zinc and some others with low concentrations are essential for organisms, they are called 'trace elements. On the contrary metals like lead, mercury, cadmium and some others are harmful to the organism above a definite concentration. In a large number of ways many industries process heavy metal pollution Some industries are more possibly to pollute then others.

3. TOXICITY OF HEAVY METALS:

When the heavy metal is not metabolised by the body, they become toxic and accumulate our soft tissues. Toxicity emerges from strong affinity of the heavy metal cations for the sulphur. Medicinal treatment for heavy metal poisoning is done by chelation therapy by regulating compounds known as chelates. Heavy metals poisoning can occur due to the lot of certain types of metals. It can cause you sick and affects the body. Heavy metals like arsenic, lead, mercury and others are around us. These are in the ground we walk on, or in the water we drink, and the products that are we use in every day. The high levels of most heavy metal can cause health problems. The poisoning can occur if you eat or drink something adulterated with heavy metals or if you breathe in contaminated dust particles. Mercury is more toxic than copper and zinc, which has similar toxicity levels. Common way of contamination for adults and children are respectively industrial exposure and ingestion.

Commonly encountered toxic heavy metals are:

- Arsenic
- Lead
- Mercury
- Cadmium
- Iron
- Aluminium

Table 1.1

METAL	ROUTE OF ENTRY	TOXICITY EFFECT
Arsenic	Inhalation and Ingestion	Irritation of respiratory system, liver and kidney damage, loss of appetite, nausea and vomiting
Cadmium	Inhalation and Ingestion	Lung, liver and kidney damage, Irritation of respiratory system
Aluminium	Inhalation, Ingestion and absorption through skin	Lung damage and irritation of respiratory system
Mercury	Inhalation, Ingestion and absorption through skin	Lung, liver and kidney damage
Lead	Inhalation and Ingestion	Lung and liver damage, loss of appetite, nausea etc.

4. SYMPTOMS:

ACUTE: This happens if you get a high dose at one time. Example: child intake a toy which is made with lead. The symptoms come quickly include sick, diarrhoea, abdominal pain, anaemia, feel confused, dehydration, kidney damage, liver damage, lung irritation, tingling, numbness, fluid in your lungs, premature labour, behavioural changes, brain problems, memory loss.

CHRONIC: It occurs after contact with a low dose over a long time. The symptoms come slowly includes headache, weakness and tiredness, achy joints and muscles, constipation. Contaminated drinking water (e.g., Lead) bring high risk especially for infants, so water test is requested.

DISEASE OCCURS DUE TO THEIR TOXICITY:

(i) Minamata disease- First discovered in Minamata city in Japan 1956. It occurs due to the release of methyl mercury from the industrial wastewater. When this highly toxic metal eaten by local population resulted in mercury poisoning. Deaths continued over more than 30 years.

(ii) Itai-itai disease – It caused due to cadmium poisoning in Toyama Prefecture in Japan. It is named after the occurrence of severe pain in the joints and spine. It occurs due to the mining companies in Japan released cadmium into the river.

Industrial Waste water from the industrial unit

5. SOURCES AND TOXIC EFFECTS OF HEAVY METALS:

Many metals are essential for life at right concentrations, but they can be poisonous in excess. Likewise, chronic low exposure to heavy metals can cause serious health problems in the long run. Heavy metals like lead, cadmium, arsenic and mercury are the main threat to human well-being that are targeted by international legislative bodies.

Table 1.2

METALS	COMMON SOURCES	EFFECTS
Lead (Pb)	PVC pipes in sanitation, agriculture, recycled PVC lead paints, jewellery, lead batteries, lunch boxes, etc	Penetrates through protective blood brain barrier (BBB) and is proving to be a risk factor for Alzheimer's disease and senile dementia, also leads to neuro-degenerative diseases, decreases intelligence quotient, kidney damage, decreases bone growth, behavioural issues, ataxia, hyper irritability and stupor

Cadmium (Cd)	Paints, pigments, electroplated parts, batteries, plastics, synthetic rubber, photographic and engraving process, photoconductors and photovoltaic cells	Renal toxicity, hypertension, weight loss, fatigue, microcytic hypochromic anaemia, lymphocytosis, pulmonary fibrosis, atherosclerosis, peripheral neuropathy, lung cancer, osteomalacia, osteoporosis and hyperuricemia.
Mercury (Hg)	Combustion of coal, municipal solid waste incineration and volcanic emissions	Impaired neurologic development, effects on digestive system, immune system, lungs, kidneys, skin and eyes. Minamata, acrodynia, increases salivation, hypotonia, hypertension
Arsenic (Ar)	Wooden electricity poles that are treated with arsenic-based preservatives, pesticides, fertilizers, release of untreated Waste water, oxidation of pyrite (FeS) and arseno pyrite (FeAsS)	Causes effects on central nervous system (CNS), peripheral nervous system (PNS), cardiovascular, pulmonary diseases, gastrointestinal tract (GI), genitourinary (GU), haemopoietic, dermatologic, foetal and teratogenic diseases, anorexia, brown pigmentation, hyper-pigmentation, localized oedema and skin cancer
Chromium (Cr)	Leather industry, tanning and chrome plating industries	Reproductive toxicity, embryotoxicity, teratogenicity, mutagenicity, carcinogenicity, lung cancer, dermatitis, skin ulcers, perforation of septum and irritant contact dermatitis
Silver (Ag)	Refining of copper, gold, nickel, zinc, jewellery and electroplating industries	Argyria, gastroenteritis, neuronal disorders, mental fatigue, rheumatism, knotting of cartilage, cytopathological effects in fibroblast, keratinocytes and mast cells
Zinc (Zn)	Soldering, cosmetics and pigments	Respiratory disorders, metal fume fever, bronchiolar leucocytes, neuronal

		disorder, prostate cancer risks, macular degeneration impotence
Copper (Cu)	Fertilizers, tanning and photovoltaic cells	Adreno-cortisol hyperactivity, allergies, anaemia, alopecia, arthritis, autism, cystic fibrosis, diabetes, haemorrhaging and kidney disorders

TABLE 1.3: ESTIMATE OF GLOBAL EMISSIONS OF HEAVY METALS TO THE ATMOSPHERE FROM INDUSTRIAL AND NATURAL SOURCES

INDUSTRIAL SOURCES (10 Kg/yr.)	Cd	Cr	Cu	Ni	Zn
Coal combustion	0.53	11	5.2	14	11
Oil combustion	0.14	1.4	1.9	27	1.4
Nonferrous metal production	5.5	-	23	8.7	72
Steel and iron manufacturing	0.16	16	1.5	3.7	20
Refuse incineration	0.75	0.84	1.6	0.36	5.9
Phosphate fertilizers	0.17	-	0.41	0.41	4.1
Cement production	0.27	-	-	0.49	9.8
Wood combustion	0.12	1.3	0.9	12	3.6
Others	-	-	-	-	3.2
Total	7.6	30.5	34.5	55.9	131.0

NATURAL SOURCES (10 kg/yr.)	Cd	Cr	Cu	Ni	Zn
Windblown dust	0.20	27	8	11	19
Sea salt	0.06	0.7	3.6	1.3	0.44
Volcano	0.82	15	9.4	14	9.6
Wild forest fires	0.11	0.09	3.8	2.3	7.6
Continental biosphere particulate	0.15	1.0	2.6	0.51	2.6
Continental biosphere- volatile	0.04	0.05	0.32	0.10	2.5
Marine biosphere- volatile	0.05	0.06	0.39	0.12	3.0
Total	1.43	43.9	28.9	29.333	44.7
Interference factor (IF)	5.34	0.69	1.23	1.90	2.93

6. CHROMIUM:

6.1. CHARACTERISTICS:

Chromium is far spread in the environment in many forms and it is a naturally occurring element. In the developing countries and to some other place chromium is described as one of the major pollutants, the 17th most abundant element on earth. Chromium has the atomic number of 24 and atomic weight of 52.01. Chromium was first introduced by the French chemist named Vauqnelin in 1798 in the Siberian red leas ore (crocoite). It is situated in the group VI-B of the periodic table and considered as a transition element with a ground – state electronic configuration of (Ar)3d 4s. It has a high melting point with the characteristic of steel -gray, lustrous, hard metal that takes a high polish. Chromium is odourless, tasteless and highly resistant to corrosion. Chromium is composed of three stables in nature: Cr^{52}, Cr^{53}, and Cr^{54} with Cr^{52} being the most significant (83.78% natural abundance).

Majority if the chromium occurs in two oxidation state Cr (III) and Cr (IV). Because of its toxicological effects on environment, it is very important to consider its oxidation state of Chromium compound and its solubility in aqueous solution. Trivalent state if chromium is abundantly available in nature and more stable, Cr (III) and Cr (VI) are also found in nature. Development of several oxide and hydroxide species are limited by the solubility of Cr (III) compounds. Cr (III) salts provides a green to blur colour to aqueous solution. Cr (III) is less toxic and is mainly remain bonded to organic matter in soil and aquatic environment. Cr (III) salts hydrolysis behaviour is hard and it make mononuclear species $CrOH^{2+}$, $Cr(OH)_2^{+1}$, $Cr(OH)_4^-$, neutral species $Cr(OH)_3^\circ$ and polynuclear species.

Hexavalent chromium compounds solubility equilibrium is complicated and pH dependent. Cr (VI) is more soluble under many environmental conditions compared to trivalent chromium compounds. Hexavalent chromium compounds are strong oxidizing agents in acid media. Cr (VI) is easily reduced to Cr (III) at low pH, however trivalent chromium is readily oxidized to the hexavalent state at high pH. The most common form of chromium is hexavalent. The National Toxicological Programme (NTP) estimated that hexavalent form is more toxic than trivalent form of chromium. Cr (VI) is human carcinogen as reported by International Agency for Research on Cancer (IARC). The Cr (VI) symbolised by chromates and dichromate show yellow colour to aqueous solution and strongly acidic.

The cationic, trivalent chromium hydroxide, $(Cr(OH))^{2+}$ is strongly consumed by suspended matters in nature. Mineral particulates of positively charged can able to absorb the anionic, hexavalent chromium, CrO_4^{2-}. The movement of hexavalent chromium in groundwater is notably greater than trivalent chromium.

6.2. SOURCES OF CHROMIUM:

Naturally happening element found in the volcanic-ash, volcanic-gases, soil and rock is chromium. Anthropogenic sources of chromium emission in the environment includes chrome plating, leather tanning, combustion of natural fuels (gas, oil, coal), cement industries, catalysts fertilizers, dye manufacturing industries, batteries making printers, emission from the cooling towers, air condensers and incineration of sewage sludge, municipal refuse and other solids wastes. As a result of industrial and manufacturing activities more than 1,70,000 metric tons of chromium wastes are emitted annually. The major problem for the heigh influx of chromium to biosphere is leather industry, therefore total industrial use accounting for 40% Chromium mostly exists in the trivalent form in food, air, water and soil. Substantial amounts of Cr (VI) are present in the environment as a result of human activities. Cr (VI) is quite soluble

and is leached from soil to ground water or surface water whereas Cr (VI) is comparatively insoluble.

6.3.APPLICATION OF CHROMIUM:

Chromium is used as alloys to produce products like Stainless steel, chrome plating silvery mirror coating was obtained in steel using chromium plating. For shiny finish and corrosion resistance chromium is used in metallurgy. Chromium is used to produce synthetic rubies and its salts give emerald green colour to glass. It acts as catalyst in dyeing, tanning of leather and to make moulds for the firing of bricks. Magnetic tape is manufactured by using Cr (VI) oxide.

TABLE 1.4: SOURCES OF THE CHROMIUM PRODUCTION

NATURAL SOURCES	Volcanic eruption		<1%
	Biological cycle	Extraction from soil by vegetation	14%
		Weathering of rocks and soil	15%
ANTHROPOGENIC SOURCES	Metallurgical operations		3%
	Fossil fuels combustions		7%
	Metal use and decompose activities		60%

TABLE 1.5: LEVEL OF CHROMIUM IN DIFFERENT KINDS OF WASTES OR WASTE WATER

CATEGORY	SPECIFIC SOURCE	CONCENTRATION (mg L)
INDUSTRIAL WASTE WATER	Leather tanning	10.0-50.0
	Textile mill	5.0-20.0
	Electroplating	140.0-180.0
ANIMAL WASTES	Cattle manure	20.0-31.0
	Poultry wastes	6.0
	Cow manure	56.0
FERTILIZER	Fertilizer	0.10-0.45

6.4. HEALTH EFFECTS OF CHROMIUM:

Chromium contains two effects they are beneficial and detrimental effects. Man can uptake the chromium through breathing, eating, drinking and adsorption through skin. The chromium present in the air mostly as fine dust particles which eventually settle over land and water. Chromium can strongly attach to soil but only a small amount of chromium can dissolve in water and move deeper in the soil underground water.

Even though Cr (III) is an essential nutrient for humans, its deficiency caused heart problems, disruptions of metabolism and diabetes. Health effects and instance skin rashes causes due to the excess intake Cr (III). Ulceration and perforation of nasal spectrum also occurs due to over exposure Cr (III). The lethal dose value for the Cr (III) is 10mg kg^{-1} of the body weight of animal. Cr (VI) is about 300 times more toxic than Cr (III) because of it is involved in the respiratory chain due to its capability to inhibit respiration by inactivating enzymes. Toxicity results because Cr (VI) is taken up via sulphate or thiosulphate transporter and oxidizes biological molecules. Cr (VI) also serves as carcinogenic and a potent sensitizer of skin and lung tumours. Severe diarrhoea, epigastric pain, dermatitis, kidney damage, liver damage, lung cancer and internal haemorrhage caused by compounds of Cr (VI).

Oxidation state and the chemical and physical form within the oxidation state determines the kinetics of chromium compounds. Cr (III) is harmless and immobile whereas Cr (VI) moves eagerly through soil and aquatic environments and it is being absorbed through the skin due to its strong oxidizing agent capacity. Most of the daily chromium is in the trivalent form and is ingested with food. Total intake of trivalent chromium absorbed by the body is about 0.5-0.3%. The gastro-intestinal absorption of Cr (VI) is 3-5 times more than Cr (III), however some of it is decreased by gastric juice.

6.5. BIOLOGICAL ROLE OF CHROMIUM:

Cr (III) belong to the trace elements, which is required for human and animal vital activity. Accumulation takes place when it enters the organism from digestive tract and is transported to the tissues. Disturbances in metabolite processes caused by the deficiency of Cr (III). The chief reaction of organism to Cr (III) deficiency included lowered tolerance of glucose (serve as Glucose Tolerance Factor), which is the outcome of changes in insulin affinity to its receptors on cells. The appreciable amount of Cr (III) showed in nucleic acid have influence on their metabolism, replication and transcription. The chromium ion lowers the content of corticosteroids in plasma and increases the functional activity of immune system of organism.

7. NICKEL:

7.1. CHARACTERISTICS:

Nickel is silver-white colour, hard, ductile, magnetic metal. Its atomic number is 28 and atomic mass is 58.69. Nickel is soluble in acids and insoluble in water. It forms mildly basic oxides and belongs to the iron me group. In meteorites and Ores combined with sulphur, antimony or arsenic nickel occur free. Naturally nickel does not occur in water. It is one of the ferromagnetic elements. The Oxidation state of nickel under environmental Conditions +2 but o, +1, +3 and +4 oxidation states are also observed. Among all existing elements nickel -62 is the most stable nuclide of all. It is abundant in the earth's crust at 0.018%. There are five naturally occurring isotopes of nickel it includes Ni-58(67.76%), Ni-60(26.16%), Ni-61(1.25%), Ni -62(3.66%), Ni-64(1.16%). It forms complexes with both inorganic and organic substances.

7.2. SOURCES OF NICKEL:

Through geothermal emissions, weathering of minerals and rocks, anthropogenic activities such as industrial and vehicular emissions nickel enters the environment. Various other activities such as phosphate fertilizer industry, metal finishing units, paint units, acid mine drainage, fossil fuel burn by power plants, automobiles and smelting processes etc releases nickel in the environment. Tea is a rich source of nickel ;1 kg of dried leaves contains 7.6 mg of nickel. The certain amount Waste water in waste water containing nickel from the industries like nickel-plating plants, silver refineries, zinc-based casting industries and storage batteries are introduced into the water bodies. Nickel is used for alloy preparations because of excellent corrosion resistance property. Small quantities of nickel are used as an additive in gasoline, ceramics, fungicides, pigment and in inorganic chemicals manufacturing industries etc. In recent years nickel are used in nuclear power plant, gas turbine engines, cryogenic container and pollution abatement equipment's. The water sources are polluted by the presence of nickel. The forging waste water contain up to 130 mg L of nickel, and nickel is also present in metal finishing, mine drainage, tableware plating. In certain industrial wastewaters the metal concentration may exceed 1000 mg L.

7.3. BIOLOGICAL ROLE OF NICKEL:

The one of the essential trace elements for humans' plants and animal is nickel. Nickel is Consistently present in RNA. The proteins, amino acids and serum albumins are the several biological substances in nickel. It also activates enzyme like arginase, trypsin, acetyl coenzyme-A, carboxylase and synthetase the main route of the human body where the nickel enter is from oral injection of food and drinking water. From breathing also can intake the

nickel to the body. Even though the essential element is nickel but it can cause toxicity to aquatic life at higher concentration of nickel.

7.4. HEALTH EFFECTS OF NICKEL:

Nickel is a compound that can found in the environment at very low levels and it act as a micro nutrient but it can also have toxic effects in the excessive quantities. human can intake a Nickel through by breathing drinking, eating or smoking. Skin also gets contact with Nickel through contaminated soil or water may also result in Nickel exposure. It is generally inhaled and then absorbed.

Ni - carbonyl highly volatile and is absorbed readily in lungs. Ni - carbonyl is in a lipid soluble and it penetrates in the blood brain barriers. At a higher concentration of Ni (II) can cause a cancer of lungs, nose and bone. The food stuffs also contain naturally a small amount of Nickel and in the chocolate and fats are also known to contain severely high quantities of Nickel. Nickel fumes are the respiratory irritants and it may cause pneumonitis. The exposure of Nickel and its compounds may result in the development of dermatitis known as " Nickel itch" in sensitized individuals. The first symptom is usually itching which occurs up to 7 days before skin eruption occurs. The erythematous or follicular these are the primary skin eruption. Which may be followed by skin ulceration. Nickel can react with DNA in the higher concentration and it can result in DNA damage as shown in vitro mutagenicity. Nickel and its compounds are irritants to conjunctive of the eye and the mucous membrane of upper respiratory tract. Nickel accumulates in aquatic food chain and biomagnification factor is 2000 - 40000 in algae and 40 in fresh water fish has been reported.

7.5. APPLICATIONS OF NICKEL:

The Nickel can also be used in the major preparation of alloys. The nickel alloys are characterized through the strength, ductility and resistance to corrosion and heat. Nickel forms alloys with copper, manganese, zinc iron and molybdenum. Stainless steel is the most widely used in Nickel alloy. Nickel-copper alloy has an excellent corrosion resistance property. About 65% of the Nickel are consume in the Western world and it is used to make stainless steel. Whose composition can vary but it's typically Iron with 18 % chromium and 8% nickel. 12% of all the Nickel consumed goes into super alloys. The remaining 23% of consumption are divided between alloy steels, rechargeable batteries, catalyst and other chemicals, coinage, foundry products and plating. On account of its permanence in air and inertness to oxidation, it is used in smaller coins for plating iron and brass for chemical apparatus and in certain alloys as German silver. The chemical, Marine, electrical, nuclear and aerospace applications are used by Nickel alloys. Nickel is easy to work and it can be drawn into wire. Even though it can resist

corrosion during at high temperature and for this reason it is used in gas turbines and rocked engines. Monel is an alloy of Nickel and copper. It is not only hard but it can resist corrosion by sea water, so that it is ideal for propeller shaft in boats and desalination plants. More than 40 percentage of Nickel is produced and is used in steel factories Nickel batteries and production of alloys. Which cost increasing burden of Ni (II) on the ecosystem and deterioration of water quality. Concentration range of Nickel present in the waste water is 3.40 to 900mgL-1

8. GENERAL FOR THE REMOVAL OF HEAVY METALS:

Chemical precipitation, lime coagulation, ion exchange, and reverse osmosis are all typical methods for removing metal ions from aqueous streams (Ahalya *et al.*, 2003). Each method's technique is described in detail below.

8.1. Reverse osmosis

Heavy metals are separated using a semi-permeable membrane at a pressure higher than the osmotic pressure induced by dissolved solids in wastewater in this procedure. This approach has the disadvantage of being costly.

8.2. Electrodialysis

The ionic components (heavy metals) are separated using semi-permeable ion selective membranes in this procedure. When an electrical potential is applied between the two electrodes, cations and anions migrate towards their respective electrodes. Cells of concentrated and dilute salts are created by the alternate spacing of cation and anion permeable membranes. The production of metal hydroxides, which block the membrane, is a drawback.

8.3. Ultrafiltration

They are pressure-driven membrane operations that remove heavy metals through porous membranes. The biggest drawback of this method is the production of sludge.

8.4. Ion exchange

Metal ions from dilute solutions are exchanged with ions retained on the exchange resin by electrostatic forces. The downsides include the high cost and incomplete ion elimination.

8.5. Chemical precipitation

Metal precipitation is accomplished by adding coagulants such alum, lime, iron salts, and other organic polymers. The biggest downside is the vast amount of sludge containing hazardous compounds created throughout the process.

8.6. Phytoremediation

Phytoremediation is the use of specific plants to clean up metal-contaminated soil, sediment, and water. The downsides include the fact that metal removal takes a long time and that plant regeneration for continued biosorption is difficult. As a result of drawbacks such as inadequate metal removal, high reagent and energy needs, and the production of toxic sludge or other waste products that must be carefully disposed of, a cost-effective treatment process capable of removing heavy metals from aqueous Waste water is required.

8.7. Biosorption of heavy metals

Chemical precipitation, lime coagulation, ion exchange, reverse osmosis, and solvent extraction are all common methods for extracting metal ions (table 1-2) from aqueous streams **(Rich and Cherry, 1987)**. As a result of drawbacks such as partial metal removal, expensive reagent and energy requirements, and the production of toxic sludge, researchers are looking for new low-cost solutions **(Ahalya *et al.*, 2003)**. Biosorption has received a lot of attention as a new technology for removing harmful metals from wastewaters. Biosorption is the ability of biological materials to absorb other biological materials metabolically collect heavy metals from wastewater. Uptake mechanisms that are mediated or physio-chemical **(Fourest and Roux,1992).** A sorption process involves a solid phase (sorbent) and a liquid phase (solvent). a dissolved species in a liquid phase (solvent, typically water)
be suffocated (sorbate, e.g., metal ions). As a result of the higher 'affinity' of the two. The sorbate species is pulled into the solid by the sorbent. Different systems bind you there. This procedure continues until the amount of sorbate bound to solids reaches equilibrium, species and the amount of it still in solution **(Volesky, 2006). Gupta and Rastogi (2007)** agreed that biosorption is the most important factor. Heavy metal ions are effectively removed using this approach. The root system of Sedum alfredii, a novel Zn-hyperaccumulator plant native to China, has been identified as the principal interface of material exchange between plants and their environment, and it plays a significant role in metal uptake and transport in plants **(Li *et al.*, 2006).**

Despite the fact that the literature contains papers that use bacteria such as *Staphylococcus saprophyticus* **(Ilhan *et al.*, 2004)**, algal cells **(Cossich *et al.*, 2005)**, filamentous fungi, *Aspergillus spp.* **(Sen and Ghosh Dastidar, 2007),** and other biomaterials **(Lister and Line, 2001),** Sang Yun and Volesky use waste crab-shells (Ucides cord (V). Tudury (2006) showed that eggshell can be used to bio absorb a variety of heavy metals such as Cd, Ni, Zn, and Co.

Table.1.6. Different technologies for the removal of heavy metals from the industrial wastewater:

Technology	Concentration Dependence	pH	Suspended solids	Waste water Concentration (mg/L)	Regeneration	Sludge Generation
Biosorption	Yes	Yes	Yes	<1	Yes	No
Hydroxide Precipitation	No	No	Yes	2-5	No	Yes
Sulphide Precipitation	No	No	Yes	<1	No	Yes
Ion Exchange	Yes	Some	No	<1	Yes	Yes
Evaporation	Yes	Yes	Yes	1-5	-----	No
Reverse Osmosis	Yes	Some	No	1-5	No	No
Adsorption	Yes	Some	Yes	1-5	Yes	No

9. Types of bio sorbents

Biomass must come from nature or even be a waste resource, according to the economics of environmental restoration **(Vieira and Volesky, 2000).** According **to Ilhan *et al.* (2003),** nonviable microbial biomass has a stronger affinity for metal ions than viable biomass, which is likely due to the absence of competing protons created during metabolism. **Cabuk *et al.* (2004)** point out that using dead cells has several advantages over using live cells: the metal removal system is not limited by toxicity, there is no need for growth media or nutrients, adsorbed metal ions can be easily desorbed and biomass can be reused, and dead cells can be used again. Traditional treatment methods can be used to biomass-based treatment systems. Adsorption models are now in use. As a result, the usage of dead fungal biomass has grown in popularity favoured in a number of investigations on hazardous metal ion biosorption from water-based solutions. Excreted metabolites, for example, are microbial by-products. Polysaccharides or components of cell walls have also been employed.

9.1. Chitosan-based adsorbents:

It comes under Adsorption – based separation. Chitosan has amino (–NH2) and hydroxyl (–OH) groups, that's why it is consider as natural adsorptive polymer which has a

great affinity towards wastewater pollutants. It makes the regeneration inefficient as it undergoes low mechanical strength and poor stability besides their unique feature. Due to its low porosity, low surface area, resistance to mass transfer and high crystallinity it is difficult to use chitosan in its powder or flake form. To reduce these defects structural and chemical modifications have been introduced. By linking polymer chains and the functional groups, supply strength to chitosan. This cross-linking chemical modification approach decreases the intake.

Another chemical modification method is Grafting that leads to a unique increase in the adsorption capacity which requires the covalent bonding of functional groups (such as amine and hydroxyl) on the backbone of chitosan. To improve chitosan's adsorption capability, mechanical strength and thermal stability combining chitosan with other adsorbent materials has also been suggested. To produce adsorbents which have high selectivity for target metal ions, imprinting method was followed. On the presence of the protonation or non-protonation of amine (–NH2) and phosphoric (H3PO4) groups the uptake of CS depends, which affect the pH value of the wastewater. CS based adsorbents exhibit low reusability in the absence of modifications. Strong bond (between metal ions and the adsorbent surface), low thermal/chemical strength, low mechanical, incomplete desorption, declination in the effective adsorbate-adsorbent interactions and unavailability of adsorption site might be assigned by this behaviour. Therefore, alternative regeneration techniques were suggested to improve the reusability of CSs.

9.2. Algae

The Bengal gramme husk Cicer arietinum has been used to remove Cr (VI) ions from aqueous solutions. The milling agro-waste Bengal gramme husk (Bengal Gram Husk) Cicer arietinum is abundant in a tropical country like India. Furthermore, the protein content of Bengal Gram Husk is less than 5%, which is preferable to protein-rich algal and fungal biomass, which is more likely to putrefy under damp conditions (**Ahalya *et al.*, 2005**). The dried biomass of several common brown sea algae species like Ascophyllum and Sargassum, which accumulate more than 30% of their dry weight in the metal, has been shown to successfully extract lead and cadmium from very dilute solutions (**Volesky and Holan, 1995**). Sargassum natant, a brown seaweed that isn't alive.

9.3. Yeast and fungi

Industrial steroid-transformation fungus mycelium Rhizopus and Absidia are efficient bio sorbents for lead, cadmium, copper, zinc, and uranium, and can bind up to 25% of the biomass dry weight in other heavy metals (**Volesky and Holan, 1995**). Fungal cell walls and

their components, as shown in figure (1-1), play an important role in biosorption and also take up suspended metal particles and colloids (**Ilhan *et al.*, 2004**). *Rhizopus arrhizus* is a filamentous fungus that uses chitin, a naturally occurring substance, and chitosan, a deacetylated derivative of chitin, as a cell wall component. It was discovered that they had good metal-sequestering characteristics (**Sag and Aktay, 2002**). **Yan and Vira Raghavan (2000)** looked into the influence of pre-treatment on heavy metal adsorption in Mucor rouxii. The effect of pre-treatment of *Aspergillus niger* biomass on lead, cadmium, copper, and nickel biosorption was investigated by Kapoor and **Viraraghavan (1999).** *Saccharomyces cerevisiae* non-living samples sequestered uranium, zinc, and cadmium to a larger level than living biomass. *Rhizopus arrhizus* dead fungal biomass had the maximum uranium uptake, with 180 mg U/g dry biomass, whereas *Saccharomyces cerevisiae* dead yeast biomass had 157 mg U/g dry biomass (**Kuyucak and Volesky, 1988**).

9.4. Bacteria

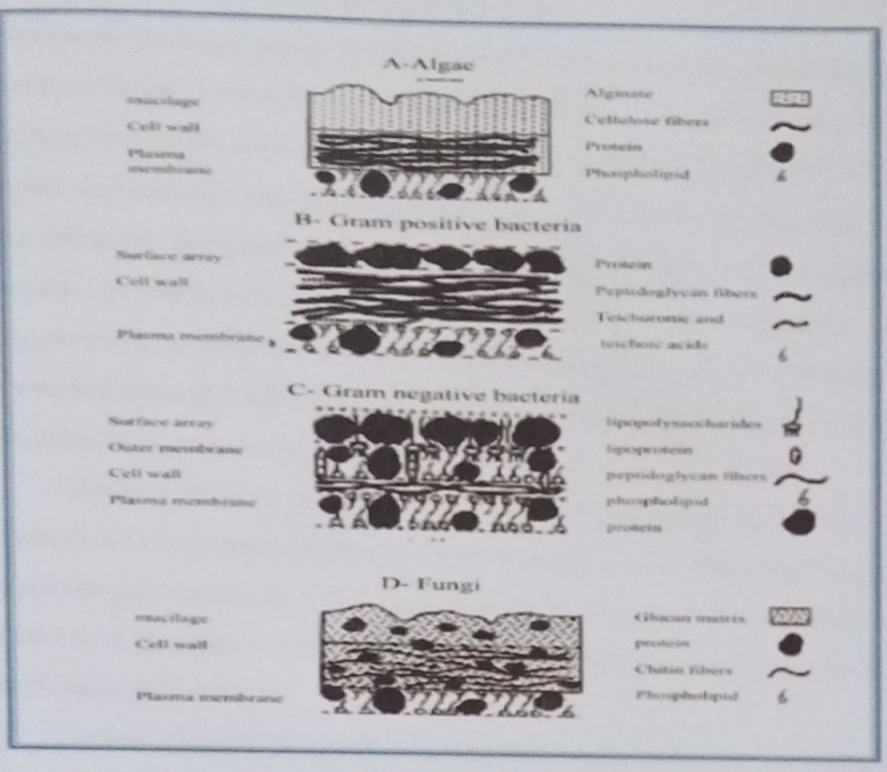
Gram positive walls are made up of a variety of hetero- and homopolymers that together produce an electronegative charge density. Bacterial surfaces are typically anionic and interact with metal cations. According to **Strandberg *et al.* (1981),** uranium builds up by Pseudomonas aeruginosa occurred intracellularly and was exceedingly quick (less than 10 seconds), with no environmental parameters found. The carboxyl groups of peptidoglycans in Bacillus subtilis cell walls are the principal sites of divalent metal complexation, according to **Matthews and Doyle (1979). In Hussein *et al* (2004).**'s study of heavy metal biosorption by several Pseudomonas species, the majority of metal ions were rapidly sequestered from solutions during the first 10 minutes, with essentially little rise in the quantity of bound metals after this time interval. It is important to note that heavy metals can be absorbed and accumulated by both living and dead cells. Dead biomass is preferable over living biomass for the following reasons:

(1) The biosorption procedure is frequently carried out in adverse settings.

(2) There is no need for maintenance or nutrition, and the bio sorbents can be stored for an extended period of time without losing their effectiveness.

(3) Because metals have no harmful impact on deceased bacteria, their sorptive capacity is unaffected.

(4) In many circumstances, dead biomass sorption is more efficient than living biomass sorption (**Tsezos, 1990**).



Cell wall structures of some microbial bio sorbent

9.5. Biosorption and bioaccumulation

Biosorption and bioaccumulation are two terms that are used interchangeably. Microorganisms have evolved a variety of ways for removing and tolerating heavy metals from their environment. Biosorption and bioaccumulation are the two ways of metal uptake. Microbial biomass has the ability to bind huge amounts of metal(s) passively. **Tsezos and Volesky (1982); Macaskie and Dean (1985)** coined the term "biosorption." Both living and non-living biomass can be used for biosorption, however only living biomass can be used for bioaccumulation **(Garnham *et al.*, 1992).** Only live biomass can cause bioaccumulation **(Garnham *et al.*, 1992).** Biosorption is a fast-acting process of non-growing biomass sequestering metals. Cell surface complexation, ion exchange, and micro precipitation are the primary components of biosorption **(Volesky, 1987).**

Heavy metal uptake by biomass is usually divided into three categories:

(1) Binding to the cell surface.

(2) Accumulation within cells.

(3) Extracellular enlargement.

Because cell surface binding is metabolism-independent, it can occur in both living and inactivated bacteria, whereas intracellular and extracellular metal build-up are often energy-driven processes that can only occur in living cells **(Ilhan *et al.*, 2004).**

The affinity of different microorganisms for distinct heavy metal(s) has been discovered to vary, and therefore their metal binding capabilities.

Some biomass (es) have a predilection for specific heavy metal(s), whereas others have a broad spectrum of binding **(Greene and Darnall, 1990)**.The initial step appears to be a stoichiometric interaction between the metal and the reactive chemical groups in the cell wall, followed by an inorganic deposition of larger amounts of metal (s)**.Crist *et al.* (1981)** found that carboxylate, amine, imidazole, phosphate, sulfhydryl, sulphate, and hydroxyl are the possible metal binds in groups in photoautotrophs (eukaryotic algae cells). When protonated, amines and imidazole's become positively charged, and they can form negatively charged metal complexes **(Greene and Darnall, 1990).** Gram-negative bacteria have thinner cell walls that are also less cross-linked than gram-positive bacteria.

They have an outer membrane made up of a lipopolysaccharide (LPS) outer layer, phospholipids, and proteins. **(Remacle, 1990) Gourdon *et al.* (1990)** evaluated gram-positive and gram-negative bacteria's Cd2+ biosorption capabilities. Glycoprotein, which is found on the outside of gram-positive bacterial cell walls, is thought to have more Cd2+ binding sites than phospholipids and LPS.

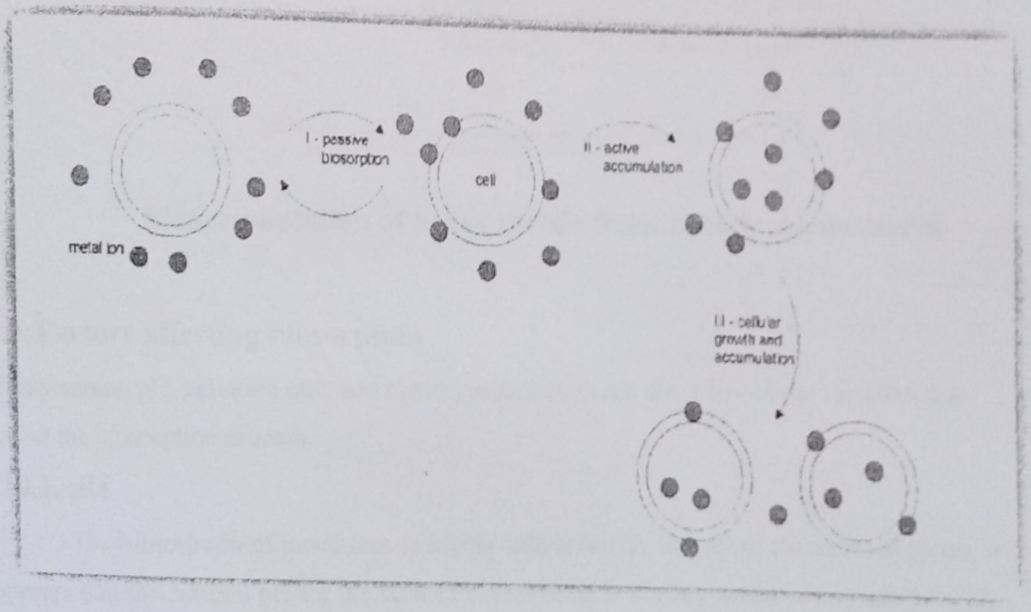
Teichoic acid and teichuronic acid were shown to be the primary sites for metal binding in *Bacillus subtilis* and *Bacillus licheniformis*, respectively. In the *E. coli* outer membrane, the phosphoryl groups of LPS and phospholipids have been shown to be the most likely metal cation binding sites **(Ferris and Beveridge, 1984).**

A very unusual metal uptake mechanism has been discovered in Citrobacter sp. In a glycerol 2-phosphate-supplemented solution, this species eliminated a lot of U6+, Cd2+, Cu2+, and Pb2+. The method of absorption was the phosphatase-mediated breakdown of glycerol 2-phosphate to form HPO4 2, which precipitated metal on the surface as insoluble metal phosphate **(Macaskie and Dean, 1985).** One of the ways bacteria take metals from liquids is through extracellular accumulation/precipitation. (II) Cell surface sorption or complexation. accumulation within cells (III). Process (I) may be aided by the use of viable microorganisms, while process (II) can proceed with alive or dead microorganisms, and process (III) necessitates microbial activity. **Muraleedharan and colleagues (1991).**

Sulfonate groups and alginate were investigated in biosorption by a dry biomass of Sargassum fluitans by **Fourest and Volesky (1990).** Simple adsorption, ion exchange, electrostatic contact, complexation, precipitation, and crystallisation may be the modes of interaction between metal species and microbial cell components. **(Crist and colleagues, 1994).**

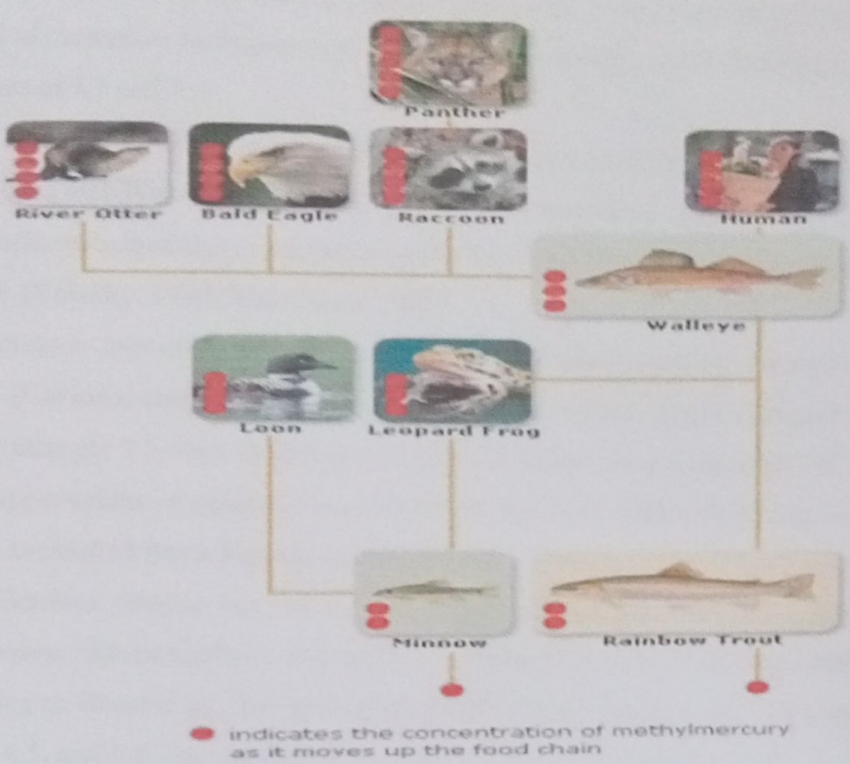
Sargassum sp., a brown seaweed composed primarily of the polysaccharide alginate, commonly calcium and sodium alginates, has a significant propensity for heavy metal build-up when compared to other algae species **(Da Costa and de Franca, 1996).**

Metal is taken up by microorganisms either actively (bioaccumulation) or passively (biosorption) **(Shumate and Strandberg, 1985)** Because living systems (active uptake) frequently require the addition of nutrients and hence increase biological oxygen demand (BOD) or chemical oxygen demand (COD) in the Waste water, biosorptive processes are more suitable than bio accumulative processes. Furthermore, metal toxicity and other unfavourable environmental variables make maintaining a healthy microbial population challenging. (1982, Brown and Lester). It is widely known that marine algal biomass contains carboxyl groups capable of cation-exchange binding of Ni (II) **(Holan and Volesky, 1994)** In the case of Ecklonia biomass, the functional group involved in proton-metal ion interactions is a carboxyl group.



The stages of bioaccumulation

Bioaccumulation is the sum of two processes: biomagnification and bioconcentration. Bioconcentration occurs when living organisms directly intake a substance from the medium (e.g., water) via skin, lungs and gills. Fish that filter huge quantities of water by their gills are subject to higher bioconcentration. Biomagnification occurs from dietary intake, commonly takes place in predatory organisms. Therefore, the heavy metals from prey are transferred to predators.



Bioaccumulation of heavy metals from aquatic environment

10. Factors affecting biosorption

Temperature, pH, agitation rate, and metal concentration are just a few of the variables that affect the biosorption process.

10.1. pH

The biosorption of metal ions is highly influenced by the pH of the aqueous phase; it appears that the optimal pH for the bulk of bio sorbents is slightly acidic to neutral (5.0-7.0). **(Kiff and Little, 1986).** pH has an impact on metal or cell wall chemistry, according to a study by **Guibal *et al.* (1992)** on uranium biosorption by Mucor miehei. Ganoderma lucidum has a substantially higher biosorption capacity at pH 6 than at pH 4. **(Matheickal *et al.*, 1991)**. **Tsezos** and **Volesky (1981)** hypothesised that an acid pH in solution would reduce metal uptake by biomass due to competition at the cellular level. Even though biosorption occurred above pH 4, Brady *et al.* (1994) found that the best pH for biosorption of Zn on Saccharomyces cerevisiae biomass was 7.5. *Penicillium spinulosum* removal of copper was reduced at lower pH, according to **Ross and Townsley (1986).** At pH values below 3, Rhizopus nigricans showed a considerably poor lead sorption capability; at pH values above 4, higher lead

biosorption was expected (**Zhang *et al.*, 1998**). Furthermore, it was discovered that at higher pH levels, insoluble lead hydroxide began to precipitate. Significant precipitation effects in the removal of chromium by Mucor meihi were also observed by **Tobin and Roux (1998)** at initial pH values of 5.5 and 7.0.

The pH of a solution has an effect on both the ionic forms of metals and the ligands used to bind metals to the cell surface. Which has a significant impact on biosorption, and it is widely acknowledged that many bio sorbents have high heavy metal absorption capabilities at high pH. **(Volesky, 1990; Kaewsarn, 2000).**

Phanerochaete chrysosporium had the highest biosorption capability for both $Ni2+$ and $Pb2+$ at pH 4 **(Ceribasi and Yetis, 2000)**. Ulva reticulata copper uptake increased with rising pH, peaking near pH 5.5, then declining at higher pH values. At pH less than 2.0, there was little or no copper uptake in general. To avoid the formation of copper hydroxide, working over pH 6.0 was avoided **(Vijaya Raghavan *et al.*, 2004)**.

Bacillus subtilis had the highest nickel adsorption at pH 8.0, while *Enterobacter agglomerans* had the highest value at pH 7.0. **(Kaewchai and Prasertsan, 2002).**

According to **Ilhan *et al.*,** the optimal pH values for chromium, lead, and copper biosorption are 2.0, 4.5, and 3.5, respectively.

The initial pH of the solution influenced uranium accumulation significantly. The largest amount of uranium accumulation was obtained at pH 6.5 (86 percent within the first 5 minutes), while the quantity of uranium accumulation was less at higher and lower pH. **(Malekzadeh *et al*, 1996).** Cr (VI) biosorption by *Trichoderma viride* was pH dependent, with the highest adsorption occurring at pH 2.0. **(Bishnoi *et al.*, 2007).**

10.2. Temperature

Temperature has a significant impact on biosorption. Lowering the temperature below 24 degrees Celsius inhibited fungal growth and enzymatic activity.

Furthermore, lowering the temperature to around 40° C reduced fungal development and, as a result, the amount of chromium removed. The greatest biomass growth and chromium removal rate were achieved at 30°C, according to Nouri **Sepehr *et al.*, (2005).** The results showed that when the temperature rose, the adsorption of metal ions by the Caladium bicolour biomass increased, which is characteristic of the biosorption of most metal ions from their solution **(Ho, 2003)**. According to **Singleton and Simmons (1995)**, increasing the temperature from 4 to 55 degrees Celsius reduced silver biosorption by *Saccharomyces cerevisiae*. According to **Horsfall *et al.*, (2005),** the adsorption of metal ions by the Caladium bicolour biomass increased with increasing temperature.

Metal concentration at start

Ceribasi and Yetis (2000) found that the biosorption capabilities of Phanerochaete chrysosporium for Ni and Pb increased as the initial metal concentration increased. At initial concentrations of 193.66 mg Cr2+ /l, 100 mg Pb2+ /l, and 105 mg Cu2+ /l, the maximum adsorption was observed, and the biosorption values were found to be 88.66 mg Cr6+/l, 100 mg Pb2+ /l, and 44.94 mg Cu2+ /l, respectively **(Ilhan *et al.*, 2004).**

It was discovered that when the concentration of uranium grew, the amount of uranium taken up by the cells increased.

There is no growth in Uranium uptake was found at values of more than 200 mg/L. **(Malek Zadeh *et al.*, 1996).**

Organics may obstruct the biosorption process due to their presence and concentration. Saccharomyces cerevisiae, for example, was ineffective in removing chromium from tannery wastewater, most likely due to chromium interacting with organics such as proteins, bacteria, or tannins in solution **(Brady *et al.*, 1994).** As a result, it's possible that some bio sorbents can be used in wastewater with low organics concentrations. **Padmavathy *et al.*, (2002)** demonstrated that raising the initial metal concentration boosted the nickel sorption capacity of baker yeast.

10.3. Physiological state

According to **Safarikova *et al.*, (2004),** yeast cells that are first magnetically manipulated and then heated have a better adsorption capacity than yeast cells that are heated and then magnetically changed. **Cabuk *et al.* (2004)** discovered that *Aspergillus versicolor* live biomass had the highest bio sorbent activity for lead ions among the species studied, including *Metarhizium anisopliae var. anisopliae* and *Penicillium verrucosum* live biomass. **Cabuk *et al.*, (2004)** also discovered that pre-treatment increased lead ion biosorption, possibly due to the exposure of active metal binding sites embedded in the cell wall or chemical changes of cell wall components.

10.4. Agitation rate

Copper uptake by *Sargassum sp.*, rises with shaking rate (q = 2.7 mg/g in the absence of agitation and 3.8 mg/g at 50 rpm), whereas the algae's adsorption capacity remained constant at 4.3 mg/g for agitation rates greater than 100 rpm **(Antunes *et al.*, 2003).**

This parameter was changed between 50 and 250 rpm to investigate the influence of shaking velocity on *Aspergillus oryzae* chromium removal rate. At 150 rpm, the maximum quantity of biomass growth and chromium elimination occurred. Biomass growth remained constant as shaking velocity increased **(Nouri Sepehr *et al.*, 2005).**

10.5. Time of biosorption

According to **Ahalya *et al.*, (2006)**, the sorption of iron by Bengal gramme husk biomass was highly quick, with equilibrium reached in 15 minutes. According to **Bishnoi *et al.* (2007)**, the adsorption equilibrium for Cr (VI) with *Trichoderma viride* was reached in 90 minutes. According to **Park *et al.* (2005)**, the concentration of Cr (VI) dropped as contact duration increased.

11. Mechanisms of biosorption

Ion exchange, coordination, complexation, chelation, adsorption, and microprecipitation are some of the mechanisms involved in the process **(Guibal *et al.*, 1992; Fourest and Roux, 1992)**. These can happen even when cells are metabolically dormant, as when they are killed by physical or chemical means **(Brady *et al.*, 1994)**. The method does not require an active membrane transport mechanism or metabolic energy. The process is dominated by an undirected physicochemical reaction **(Gadd, 1992)**.

Metal binding to cell surface components and intracellular accumulation have been identified as two pathways in live microorganism metal biosorption **(Gadd and Grifiths, 1978)**. The accumulation of substances within cells or metabolic processes can lead to the accumulation of substances. Although these reactions produce enormous amounts of metal, they are sluggish and largely dependent on nutritional and ambient conditions **(Brierley *et al.*, 1985)**. Surface and wall binding occurs in both live and dead biomass and is a passive process. This non-metabolic surface binding is quite fast, taking only a few minutes in most cases **(Khovrychev, 1973)**. This type of metal uptake happens through ion exchange mechanisms involving specific chemical sites on the cell wall, **(Hancock, 1986)**.

Paton and Budd (1972) discovered that zinc uptake by living *Neocosmospora vasinfícta* has two phases: a rapid established phase of adsorption to negatively charged groups in the hyphal surface membrane and a slowly developed phase of transfer into the cytoplasm.

According to **Khalid *et al.* (1993)**, two phase adsorption phenomena were observed in the biosorption of uranium on living Trichoderma harzianum.

The first was caused by quick physical sorption, while the second was caused by structural changes and surface alteration. Copper biosorption in living yeast Saccharomyces cerevisiae was likewise discovered to be biphasic, with an initial, rapid surface binding followed by a later intracellular uptake. Copper uptake within cells could account for 23% of total copper uptake **(Huang *et al.*, 1990)**.

Muraleedharan and Venkobachar (1994) found that metal uptake by Ganoderma lucidum occurred at the cell wall, with structural polysaccharides likely serving as the primary

interface. According to **Kiff and Little (1986),** surface adsorption accounted for the majority of cadmium biosorption on live Aspergillus oryzae, while intracellular accumulation was minor.

Tsezos and Volesky (1982) proposed that surface binding was important for the quick development of uptake equilibrium in dead Rhizopus arrhizus biosorption of uranium and thorium. **Huang *et al.* (1990)** discovered that copper biosorption by the dead yeast Saccharomyces cerevisiae was accomplished solely through surface binding, and that cadmium and lead biosorption by the live yeast was also accomplished solely through surface binding.

The uptake of Cu, Cd, and Zn by Penicillium spinulosum and Aspergillus niger mycelium was suggested to be a non-metabolic process by **Ross and Townsley (1986).** It is likely that extensive binding of metal ions to external parts of cells would mask the metabolism-dependent transport of smaller amounts of metals into the cells under some conditions. However, **Paton and Budd (1972)** found that zinc uptake by *Neocosmospora vasinfectum* mycelium was metabolism-dependent, as was copper biosorption by P. ochro-chlorensis protoplasts **(Gadd and white, 1985).** Rhizopus arrhizus biosorption was found to be independent of ionic charge or electrostatic strength, and was controlled linearly by both. The radius of ionisation **(Tobin *et al.*, 1984).** The ionic radius, on the other hand, was inversely related to Penicillium biomass biosorption **(Galun *et al.*, 1987). Huang *et al.* (1988)** found that adsorption rather than precipitation was responsible for cadmium removal in the pH range less than 8.

The binding of uranium and thorium by Rhizopus arrhizus was not shown to be effective. The chitin component of fungal biomass has been identified as a substantial contributor to metal removal capacity **(Strangberg, 1981).**

Tien and Huang (1988) found that the polysaccharide and protein components were equally important in metal adsorption.

Chitin concentration varied little amongst fungal species, averaging 30% (w/w) on average **(Huang *et al.*, 1988).**

Surface or cell wall binding, as previously stated, involves specific chemical locations on the cell wall. Because the cell walls are ripped open, more surface binding sites are exposed in dead biomass **(Gadd, 1990).** Carboxylate, amine, phosphate, hydroxyl, sulfhydryl, and other functional groups are all possible ligands in biomass **(Beveridge and Koval, 1981).**

Amino, phosphate, sulfhydryl, carboxyl, or hydroxyl groups, according to **Sigg (1987),** are important potential metal ion adsorption sites on microbial surfaces. The coordination of the

functional groups in the biomass to the metal ions may account for a large amount of the metal absorption.

Tobin *et al.* (1984) proposed that any site could have numerous distinct functional groups involved in metal ion binding to varying degrees. Heavy metal biosorption on certain fungus may involve. To differing degrees, different functional categories **(Kapoor and Viraraghavan, 1998).**

Proteins, carbohydrates, nucleic acid, and lipids make up the polymeric structure of biomass surfaces, which has a negative charge due to the ionisation of organic groups like carboxylic, aliphatic, aromatic, and amino groups, as well as inorganic groups like hydroxyl and sulphate groups **(Hughes and Poole, 1989)**. According to **Bux and Kasan (1994),** the net negative surface charge of the biomass is the primary driving force for metal ion biosorption. The stronger the attraction and adsorption of heavy metal ions, the higher the biomass electronegativity. As a result, the performance of these functional groups will be affected by pH. **Tobin *et al.* (1984)** discovered that at pH 4, Rhizopus arrhizus is capable of metal binding. Many carboxylate groups would be neutral; thus, they would not interact with metal ions. Above pH 3.0, most phosphate groups have a negative charge, therefore food binding continues **(Beveridge and Murray, 1980).** Because carboxylate and phosphate ligands are weak bases and can form weak interactions with metal ions, most metal binding sites contain carboxylate or phosphate ligands, or both **(Tobin *et al.*, 1984).**

Few functional groups in the biomass have negative charges at pH 4. **Hunt (1986)** suggested that increasing the hydroxide concentration between pH 6.0 and 7.0 could activate additional a1 binding sites in the hyphal walls, such as sugar phosphate monoesters or phospholipids. According to Kapoor and **Viraraghavan (1997),** a carboxylate chitosan monomer's group **(Zhang *et al.*, 1998).** According to **Farkas (1980),** amino- or non-amino-polysaccharides made up up to 90% of dry fungal biomass. The amino group acts as a strong Lewis base to coordinate metal ions since it has an electron pair available for coordination. The ion exchange process controls the function of various chemical sites. In other words, ion exchange was thought to be a primary metal uptake mechanism (see figure 1.2). Ca and H ions were liberated during copper biosorption on *Gunudenna Iuciduni* **(Muraleedharan and Venkobachar, 1994).** Biosorption of metal ions on Aspergillus niger released K, Cd, H, and Mg ions, according to **Kapoor and Viraraghavan (1998),** indicating an ion exchange process.

AIM AND OBJECTIVES

1.To investigate the feasibility of reducing heavy metal contamination in the Waste water from the waste water

2. Isolation and identification microorganism from different environments to be used in heavy metals (nickel, chromium, lead and zinc) biosorption experiments.

3. Studying the ability of different isolates in heavy metals (nickel, chromium, lead and zinc) biosorption and select the efficient isolate for this purpose. - Studying the optimum conditions for heavy metals biosorption by the selected isolate.

4. Characterization of bio sorbent such as Effect of pH, Bio sorbent, Dosage, Contact time

5. To determine the presence of chemical functional groups in bio sorbent and water sample using FTIR, SEM.

REVIEW OF LITERATURE

Duruibe j.o *et. al* (2007) explained that the bio toxic effect of heavy metal. It requires adequate occupational hygiene in handling them. The need to prevent heavy metal pollution and control the subsequent human poisoning.

N.K Srivastava, C. B. majumder (2005) described that the heavy metal redaction is done by certain microbial cloning. This helps in the process of removing heavy metal contamination by bio filters.

Shahla nigar *et. al* (2021) stated that the amount of Pb in water affect the physiological behaviour of fish and affect their diversity. There is a consistent need to determine the toxicity of heavy metal because the consumption of heavy metals in the form of food may impair the health.

M. A. Barakat et .al (2010) reported the suitable treatment of removing heavy metal compare to other technologies. There is basic parameters such as pH, initial metal concentration, overall treatment efficiencies and economic parameter should be taken in consideration in selecting most effective treatment.

Marlin koller and Hossam M. Saleh (2018) illuminated that phytoremediation can also be used cure water and successfully removed the arsenic cadmium, chromium, lead and nickel. The heavy metal properties and nature are the main source.

Sandhya babel *et al* (2002) mentioned that low- cost adsorbents usage is found highly efficient for heavy metal removal. This the use of low- cost adsorbents may contribute to the sustainability of the surrounding environment.

Ebtesam El Bestway *et.al* (2012) concluded that the approach resulted in adaptation of the system where sludge microbes acquired and developed natural resistance enhancement of both organic matter and heavy metals removal.

Patricia H. Clarke, S.T. lowan (1952) illuminated the advantages of this methods are simple, clear- cut results, use of complex media small number of cells required to carry out a series of tests, rapidity & reproducibility.

R. Morgan grittin (2002) stated that heavy metal poisoning is rare. Not all heavy metal is poisoning some are useful in body building. It can be divided into acute and chronic poisoning.

Yunusa thairu *et al* (2014) described that gram stain is a deceptively simple procedure staining can be performed quickly and easily. Gram stain results of direct smears are more reliable and useful when sample are from sterile sites.

Morri makowitz (1999) concluded that heavy metal substrates may be toxic when present in excessive

K. Chojnacka (2009) reported the stages of bioaccumulation of metal. This preparation is suitable for heavy metal water treatment.

N.A.A. Qasem *et. al* (2021) mentioned the key factors of the operation cost, initial concentration of the metal ions, environmental impact, pH values, chemicals added, removal efficiency, & economic feasibility.

Mohammed mizanur Rahman, Nurun N lata, sunzida H Rimu, Adib H, chisty (2020). "Simultaneous determination of heavy metal and cationic dyes from industrial Waste water by prawn shell derived chitosan -g. poly acrylic acid bio composite. Pg: 252 - 262

Feng Lian Fu, Qi hlang (2011) illustrated that the heavy water pollution of wastewater is one of the most significant problems throughout the world. To meet the increased more and more stringent environmental regulations, a wide range of treatment technologies such as chemical precipitation, coagulation- flocculation, flotation, ion – exchange & membrane filtration have been developed for heavy metal removal from wastewater

Maretaingtias dwi ariani (et) 2009 described that chitosan can be used as a material for heavy metal reduction. chitosan did not have any toxic effect. the spectrophotometer is used to check the concentration of heavy metal.

M.A. Baraket et (2010) concluded that chemical precipitation, adsorption, membrane filtration, electrodialysis and photocatalysis had been highly developed for heavy metal removal from contaminated wastewater. The more effective and inexpensive treatment to protect the environment totally is based on basic parameter such as Ph, initial metal concentration.

Sewvandi, G.A *et al* (2011) stated that chitosan is a promising agent for the heavy metal adsorption from waste water. FTIR spectrum of chitosan shown that optimum Cr6+ adsorption. According to this chitosan was a good element for heavy metal removal.

Abhrajyoti taratdar & Gargi Biswas (2013), "extraction of chitosan from prawn shell wastes and examination of its viable commercial applications" volume – 2 concluded that chitosan produced by deacetylation of chitin was observed to have many important properties like antibacterial, antifungal and radical scavenging activity.

Rayane Santa Cruz martins de Quiroz Antonino *et al* (2017) "preparation & characterization of chitosan obtained from sells of shrimp (litopenaeus vannamci Boone". Described that chitosan samples with physical &chemical properties are suitable for pharmaceutical applications.

Mahi pal Singh Santhal *et al* (2016) "heavy metals contamination in water & their hazardous effect on human health. A review "volume -5(10) concluded that the practice of trace element detection should be continued to avoid possible consumption of contaminated

eatables. The bioaccumulation of heavy metals may pose great hazard to health of humans & animals that rely on the water bodies.

Rashmi yerma & Pratima Dwivedi (2013) "heavy metal water population – A case study" volume-5(5) pp:98-99 concluded that heavy metal poisoning should be clinically diagnosed & medically treated the best option is to prevent heavy metal pollution & the subsequent human poisoning.

Md. Hatezur Rahman *et al* (2021) concluded that the composite is potentially enough for the adsorption of heavy metals especially for the removal of cd ions from aqueous solution.

A.A. Zynudheen *et al* (2009) demonstrated that chitosan derived from the shell waste of *F. indicus* was found to be effective in 10mperison so other chelating agents in removing heavy metals. Retention of the heavy metals after treatment with chitosan's in the water samples was well below the maximum allowable concentration of water to be used.

Sonia Hossain, Md koushic u din (2020) explained the process of deacetylation, demineralization & deproteinization and successfully extracted chitosan from shrimp waste by laboratory techniques.

Hazrat Ali (2019) illuminated that the heavy metals are considered hazardous due to three characteristics which include persistence bioaccumulation, and toxicity (PBT) A comprehensive study of the environmental chemistry and ecotoxicology of hazardous heavy metals & metalloids shows that steps should be taken to minimize the impact of these elements on human health & the environmental.

Lateef Ahmad malik *et al* (2019) stated that heavy metal ion toxicity has been reported to cause many health issues to living beings. Many methods have been adopted earlier for these heavy metal reductions. Effective, environmentally friendly and cheap method are explained.

Joseph baby *et al* (2010) concluded that the ecosystem contamination from heavy metal pollution may damage marine organism at the cellular level and possibly effect the ecological balance. Non at the end of food chain suffers from heavy metal pollution.

Gunathilake S.K. (2015) elucidated that biological treatment of heavy metal are eco - friendly rather than of physical and chemical methods of treatment.

Ravindra k. Gautang *et al* (2014) pointed that heavy metal degradation from aqueous solution have gained tremendous popularity among the scientific community to treat industrial water. Removal technique have been developed and applied for the treatment.

M.canli, R.W. Furness *et.al* (1992) corroborated that toxicity of organic and inorganic mercury, cadmium, copper, zinc, lead was found. In these, organic chemical is accumulated in higher concentration than inorganic one.

Shanta pokhrel.*et al* (2016) elucidated that molecular weight of the commercial chitosan was observed higher than the synthesized chitosan. FTIR spectra of chitosan suggested that the formation of chitosan from chitin. SFM of chitin powder revealed the typical cellular structures of the animal skeleton.

Robert S. Boyd (2010) concluded that combinations of potentially toxic materials may act additively, synergistically or antagonistically. Many chemical ecology studies pointed that population, community and ecosystem scales is important for ecological effects.

F. Nessa (2010) illuminated that the quality and physiochemical properties of chitosan vary widely with crustacean species and methods of preparation. The temperature was a very important factor that should be monitored constantly.

Manju Bhargavi Gump *et al* (2015) pointed the limits, sources and effects of various heavy metal ion contamination, toxicity of mechanism of heavy metals.

Shiny martis B *et.al* (2019)) Heavy metal tolerance of klebsiella pneumoniae kpn555 isolated from coffee pulp waste. This review explained that klebsiella pneumonia kpn555 is used to reduce the concentration of heavy metal such as cd, Hg and Li. But klebsiella are pathogenic so the klebsiella pneumonia kpn555 is corroborated to use as a removal of these heavy metals from the polluted sites.

Bei li *et.al* 2014 pointed that K. pneumoniae is an opportunistic pathogen it can cause life threatening invasive infections. Additionally, K. pneumoniae virulence factors include LPS, fimbriae, outer membrane, proteins and nitrogen source utilization. At last, the most important virulent factor is CPS.

Guoying wang *et.al* (2020) it mentioned that K. pneumoniae has recently become a notorious virulent factor. It is believed that many infection meds including pneumonia, liver abscess, GI intestinal colonization as well as different strains, may be better known for these pathogens. There is a study of the biology, physiology and interactions with host of K. pneumoniae it will provide that important cell to flight K. pneumoniae infection.

Nermin hande Avcioglu and Isil seyis bilkay (2016) this review pointed that K. pneumoniae is used as a strain in the treatment of cyanogen wastes in the presence of different ions which are important pollutants besides cyanide in waste. Like waste waters, and soils.

Nadia G. kandile *et.al* (2018) mentioned that we can take a chitosan in the different degrees of deacetylation from the deacetylation of chitin chitosan possesses the highest thermal stability and crystallinity. And its morphology pointed the crystals on its smooth surface.

Stephen O. majekadunmi (2016) explained that the extraction, evaluation, characteristics and properties of chitosan has been described that the chitosan plays a important role in medicine and pharmaceutical industries. chitosan remains are of the most important polymer, in the chemical extraction of chitosan from chitin the high temperature. Should be better avoided because it can destroy the physiochemical properties of the polymer.

Ang li *et.al* (2016) descried the cross – linked chitosan aerogel as an environment – friendly absorbed was obtained by a simple method involving cross – linked process and

freeze-drying technique. The adsorption capacity for heavy metal was also considerable and have maximum adsorption capacity.

Ayaid khadem zgair.*et.al* (2019) concluded that S. aureus is the best planktonic bacteria isolate to decrease the heavy metals. The presence of functional groups in the cell wall and the chemical configuration of the metal plays an important role in biosorption of heavy metals.

Hillel. S (1996) described that S. aureus uptake is followed by intracellular immobilization of Pb (11). Crystal is deposited as the non – toxic, negligibly soluble phosphate.

Shamim Naser radhi (2012) illuminated that S. aureus bacteria can tolerate some of heavy metals, while the different pH values had not any clear effect also this isolate had an ability of removing heavy metals from water and can applicable this at industrial level for big scale treatment of waste water before discharge in the environmental.

Ashok Kumar *et.al* (2010) presented that staphylococcus sp uptake the lead in very significant amount, it was reduced by staphylococcus sp.

Nomanbhay S.M and palanisamy. K that detailed the preparation of the bio sorbent, characterization and adoption studies dominant sorption mechanisms are ionic interaction and complexation.

W.F. yap *et.al* (2011) presented the thin film of chitosan crosslinked with glutaraldehyde and studied by XPS and AFM. The XPS section confirmed the presence of carbon, oxygen and nitrogen. The AFM measurement confirmed the successful formation of crosslinked chitosan thin film on the substrate.

Mandy Mende *et.al* (2018) described that chitosan with a degree of deacetylation of 90% is a good adsorbent for heavy metal ions in water. Increasing the initial metal salt concentration, the adsorption capacity increase.

W.S. Wan Ngah *et.al* (2010) illuminated that adsorption wing chitosan composition is becoming a promising alternative to replace conventional adsorbent in removing dyes and heavy metal ions.

MATERIALS AND METHODS

1. Sample Collection

The waste water was collected from the power plant industry situated in Thoothukudi, was chosen as the site of sample collection. The untreated Waste water and the water from Waste water discharged area was taken as samples. The samples were collected in plastics cans respectively and brought to the analytical laboratory for further analysis.

1.2. Characteristics of the Waste water

The waste water collected from the power plant industry was observed for the following features.

1.3. pH

The pH of the Waste water was recorded by using pH indicator strips.

1.4. Colour:

The colour of the Waste water was observed visually.

1.5. Odour:

The odour of the Waste water was observed through nasal inhalation and recorded.

1.6. Heavy metals present:

The heavy metals contaminants in samples were determined by the statements of the industrialists. The results for the characteristics of the Waste water are given in Table 2.

2. Isolation of Organisms from the water sample:

Sample Preparation:

1ml of water were added to 99 ml of distilled water and serially diluted.
The diluted sample were plated on nutrient agar medium and incubated for 24-48 hours.
Biosorption of Heavy Metals 25

2.1. Identification of the isolated organisms:

Isolated organisms from waste water sample were identified according to their morphological, physiological characteristics and biochemical tests as follows:

2.2. Morphology and Biochemical Characterization of Microorganisms:

The identification of bacteria was performed on the basis of microscopic and macroscopic examination and biochemical test according to Berkey's manual of determinative bacteriology.

3. Macroscopic Observation

3.1. Colony morphology

Bacterial isolates were streaked aseptically on Nutrient agar medium, EMB agar and Mannitol salt agar medium. Plates were prepared and pH of the medium was adjusted to 6.5. Plates were incubated at 37°C for 48 hours. After incubation, the colony morphology was observed and recorded.

4. Microscopic Observation

4.1. Gram's Staining

➢ The bacterial isolates were identified morphologically based on their shape using Gram's staining and the isolation were categorized as either gram positive (+ve) or gram negative (-ve) organisms.

➢ The glass slide was cleaned. A thin smear of the given culture was streaked in the slide. The smear was air dried and heat (**Yunusa Thairu *et al.*, 2014).**

4.2. Motility

➢ Place a very small drop of culture in the centre of a cover slip; a small drop of Vaseline at each corner of the depression side of the cavity slide.

➢ Insert the slide over cover slip, hanging drop is suspended in the well. The slide was focused with 10 X objective.

5. Biochemical Characterization

The isolated organisms were subjected to various biochemical tests such as:(Patricia. H *et al.*, 1952).

Biosorption of Heavy Metals 26

5.1 Indole Production Test

➢ The indole production was performed to bacterial isolates to determine the ability of the organisms to split indole from the amino acid tryptophan.

➢ A loop full of isolated bacterial culture was inoculated aseptically into the tryptone broth and incubated for 24 hours at 37°C after incubation 1ml of Kovac's reagent was added and the result were observed.

5.2. Methyl Red Test:

➢ Methyl Red also called C.I. Acid red is an indicated dye turns red in acidic solution It is an azo dye, pH indicator, MR-VP medium was prepared and 5 ml of medium was transformed in test tube and sterilized.

➢ One loopful of the culture was inoculated in MR-VP medium and inoculated at 37°C for 24 hours. After incubation methyl red reagent was added and the results were observed.

5.3. Voges-Proskauer Test

➢ Voges-Proskauer test is used to detect acetone in a bacterial broth culture. The test is performed by alpha naphthol and potassium hydroxide to the Voges Proskauer broth which has been inoculated with bacterial culture.

➢ The isolates were inoculated in MR-VP broth and the tubes were incubated for 24 hours at 37°C.

➢ After incubation, 1ml of 4% potassium hydroxide and 3ml of 5% of naphthol solution in absolute ethanol was added and the result were observed.

5.4. Citrate utilization Test

➢ Citrate Utilization test is used to detect the ability of bacterial to utilize citrate as a carbon source. The Simmons citrate agar was prepared and inoculated with the isolated culture and incubated at 37°C for 24 hours.

➢ After incubation the result were observed.

5.5 Catalase Test

➢ Catalase test is used to detect the availability of catalase production by the organisms. Transfer small quantity of culture to glass slide.

Biosorption of Heavy Metals 27

➢ Add one drop of 3% Hydrogen Peroxide (H_2O_2) on the culture with the help of Pasteur pipette and observed for bubble formation.

6. Acclimatization of the cultures (Ebtesam EI *et al.*, 2013)

6.1 Nickel Acclimatization

➢ The isolated Klebsiella sp., Staphylococcus sp. culture was acclimatized in nickel sulphate salt

along with nutrient broth.

➢ They were allowed to grow at 10 mg/100 ml concentration of nickel salt in nutrient broth for 24 hours.

➢ Subsequently they were transferred to 20 mg/ml and so on up to 60 mg/ml.

6.2 Chromium Acclimatization

➢ The isolated Klebsiella sp., Staphylococcus sp. culture were acclimatized in potassium

dichromate salt added to the nutrient broth.

➢ They were allowed to grow at 10 mg/100 ml concentration of chromium salt in nutrient broth for 24 hours.

➢ Subsequently they were transferred to 20 mg/ ml and so on up to 100 mg/ ml.

Biosorption of Heavy Metals 28

7. Preparation of Standard graph

7.1 Nickel Standard Graph: (Dimethyl glyoxime colorimetric method)

Approximate volume of nickel working solutions were placed, ranges up to 150 mg in a series of flasks for the preparation of a standard curve.

➢ Flask containing only distilled water served as the blank.

➢ 20 ml of 0.5 N HCL was added to the blank and standards.

➢ The following reagents were then added in the order as given with a homogeneous mixing after each addition.

1. 10ml of sodium citrate,
2. 2ml of iodine solution,
3. 4ml of dimethyl glyoxime solution.

The solutions were made up to 50 ml with distilled water and allowed to stand for 20 minutes. They were estimated photometrically using a spectrophotometer at 470 nm. The calibration curve was prepared and the microgram nickel was calculated from the observed optical density.

7.2 Chromium Standard Graph: (Diphenyl carbazide Colorimetric method)

Approximate volumes of chromium working solutions were placed, ranging from 2 mg, 4 mg, 6 mg up to 20 mg in a series of flasks for the preparation of a standard curve.

➢ Flasks containing only distilled water served as the blank.

➢ All the samples were made up to 50 ml with distilled water.

➢ 2 ml of diphenyl carbazide was added to all the flasks including blank.

All the samples were allowed to stand for 5 min for the colour development and the absorbance was taken at 540 nm.

By using the chromium concentration and the optical density, the standard curve was prepared.

Biosorption of Heavy Metals 29

8. Estimation of Initial Metal Level in the Waste water:

1ml of the Waste water was taken as a sample and the amount of nickel and chromium was estimated by using the standard method and the initial number of metals were calculated by calibrating the concentration against the standard graph.

8.1. Effect of biomass concentration:

To regulate the effect of biomass concentration on nickel and chromium biosorption, concentration of chromium and nickel was kept constant, as the biomass concentration was varied to 0.25,0.5,0.75,1.0,1.25 and 1.5 (g/l). The optimum biomass concentration was used in the following experiments.

8.2. Effect of pH:

To regulate the optimum pH for chromium and nickel biosorption, chromium and nickel solutions were adjusted to different pH values (4,5,6,7,8,9)

8.3. Effect of temperature:

This is performed to determine the effect of temperature on chromium and nickel biosorption with optimal pH and biomass concentration. The incubation temperatures used were 25,30,35,40,45,50°C.

8.4. Effect of initial concentration:

For metal sorption, various metal concentrations were used to regulate the capacity of biomass. The biomass concentration was kept constant while chromium and nickel concentrations were (10,20,40,60) mg/L.

9.Controls:

Many controls were maintained to make sure that there was no uptake of metal in any other source other than *Staphylococcus sp., Klebsiella sp.*, as given below.

9.1. Nutrient broth(100ml)

9.2. Nutrient broth and Waste water

100 ml of nutrient broth and 1 ml of Waste water was taken.

9.3. Nutrient broth, Waste water and organisms

1 ml of Waste water, 99 ml of nutrient broth with 1ml of 24 hrs *Klebsiella sp., and Staphylococcus sp., culture.*

9.4. Reduction of Metals in the Waste water

100 ml of nutrient broth was taken to which 1ml of Waste water was added, the pH was adjusted to 4,5,6,7,8 and 9. The broth was sterilised and cooled to which *Klebsiella sp.*, and *Staphylococcus sp.*, were added and allowed to stand for 24 hrs and 48 hrs.

9.5. Effect of contacting time

Biomass concentration and metal concentration kept constant for optimal pH, as the contacting times were varied (24,28,62,86,110) hrs.

9.6. Statistical analysis:

Chi square test describes the discrepancy between theory and observation. Chi square was applied to test the hypothesis that **"the reduction of chromium and nickel**

10. Collection of samples:

Prawn shell was collected from a prawn industry

10.1. Sample preparation:

The shells were obtained from local market (places). Then the samples were washed with tap water and dried under sunlight. Dried samples were subjected to size reduction and sieve analysis. The sample size ranges from 0.3 mm to 0.8 mm.

10.2. Deproteinization:

The samples were treated with 4% NaOH solution at room temperature for 24 hours with constant stirring to achieve demineralization. Demineralized samples were washed with distilled water until pH becomes neutral.

10.3. Demineralization:

Deproteinized shells were treated with 4% HCl solution at ambient conditions. The solution was stirred for 12 hours to remove the minerals. pH of the solution was increased to 7 by treating demineralized samples with distilled water to get raw chitin. Raw chitin was then dried at room temperature.

10.4. Purification of chitin:

Chitin obtained was further treated with 2% NaOH and 1% HCl solutions and then washed with distilled water to get pure chitin.

10.5. Decolourization:

Decolourization was carried out by treating chitin with 1% potassium permanganate solution about an hour followed by 1% oxalic acid for 30 minutes with stirring.

10.6. Deacetylation:

Pure chitin sample were subjected to deacetylation to get chitosan by treating with 65% NaOH solution at ambient conditions with stirring for 72 hours. Then alkali samples were washed with distilled water until pH reached neutral value. Further chitosan samples were dried at room temperature. The chitosan obtained will be in a creamy white form.

11. Optimization of Heavy metals using chitosan:

50 ml of 3mg/ chromium and nickel solution was taken and 50mg of 150µm particle size chitosan was added. Then the mixture was continuously stirred using magnetic stirrer for 6 hours at room temperature (30°C). After that solution was filtered and filtrate and 3mg/l chromium and nickel solution were analysed using atomic adsorption spectroscopy to determine amount of chromium and nickel absorbed by chitosan. Similar effect of temperature was studied by changing only reaction temperature to 50 °C and keeping other parameters as constant.

Effect of particle size of chitosan powder on amount of metal take up was studied by increasing the particle size of powder to 355µm and repeated in the above procedure.

pH of chromium and nickel solution was set to 5.7 by using 1M NaOH solution.

Finally, intake of chromium and nickel by amine groups ($-NH_2$) on chitosan was investigated using FTIR spectroscopy in the range of 400 to 4000 cm^{-1}.

12. Optimization of Heavy metals using organism:

10,20,40,60 mg/L of chromium solution and nickel solution was prepared and isolated microorganisms were inoculated. Then they are incubated and the effect of organism on amount of metal uptake was studied by continuous checking of the incubated sample by taking OD value for different days.

13.Chitosan Characterization: (Rashmi *et al.*,2016)

13.1. Moisture content determination

Moisture content of the samples were determined on wet basis. The samples were kept in an oven for 1 hour at 100 °C. The percentage moisture content was the difference between the weights of the wet and oven dried samples and expressed as:

$$\text{Moisture content (\%)} = \frac{\text{(wet weight – dry weight)}}{\text{Wet weight}} \times 100$$

13.2. Ash content determination

Ash content of the chitosan was determined by combustion using a constant weight crucible 1.0 g of chitosan sample was combusted in the crucible in an oven at 550 ° C ± 20 ° C for 3 hours until constant weight was achieved.

Ash content was then calculated in percentage as

$$\text{Ash content (\%)} = \frac{\text{(initial weight – final weight)}}{\text{Final weight}} \times 100$$

13.3 pH

The pH measurement of chitosan solution were carried out using pH meter and pH paper.

13.4. Viscosity

Viscosity was determined using Ostwald's viscometer by dissolving the chitosan samples in 1% acetic acid

13.5. FTIR Spectroscopy:

Aim:

Fourier Transform Infrared Spectroscopy (FTIR) produces an infrared absorption spectrum by identifying chemical bonds in a molecule. To screen and scan samples of many different components this spectrum is used, that produce a profile of the sample, a distinctive molecular fingerprint.

Principle:

It acts on the principle that when infrared radiation (IR) progress through a sample, some of the radiation is absorbed. The radiation which passes through the sample is recorded. Due to their different structures of different molecules produce different spectra, the spectra is used to identify and distinguish between molecules. FTIR is the preferred process of infrared spectroscopy for various reasons. First, it does not destroy the sample. Second, it is notably faster than older techniques. Third, it is very much sensitive and precise. FTIR analysis can be used to identify unknown materials, additives within polymers, surface contamination on a material and more.

Procedure:

Step 1: Place sample in FTIR spectrometer. The spectrometer directs beams of IR at the sample and measures what quantity of the beam and at which frequencies the sample absorbs the infrared radiation. The sample has to to be thin enough for the infrared radiation to transmit through, or a skinny slice of the fabric must be removed. Reflectance techniques is used on some samples and no damage is finished to the sample. Samples conducive to reflectance are residues, stains or films on a reasonably flat reflective surface or somewhat pliable materials that are thin enough to suit under the microscope using the attenuated total reflectance attachment to the microscope.

Step 2: The reference database houses thousands of spectra, so samples are often identified. The molecular identities may be determined through this process.

13.6. SEM

Aim:

A Scanning electron microscope (SEMs) creates an image over a surface by scanning a focused stream of electrons. It produces various signals by interacting between the electrons in the beam and the sample therefore it can be used to obtain information about surface's topography and composition.

Principle:

Scanning electron microscopes (SEMs) involves a resolution down to the nanometre scale by using an electron beam to image samples. The electrons are released from a filament brought parallel into a beam in the electron source. By a set of lenses in the electron column, the beam is then focused on the sample.

Procedure:

Fixation with Aldehyde (Proteins)

↓

Fixation with osmium tetroxide (Lipids)

↓

Dehydration series with solvent

(Ethanol or Acetone)

↓

8.Drying with low surface tension agents:

HMDS solution (cells)

Liquid CO_2 in a critical point dryer (Tissues)

RESULT AND DISCUSSION

Industrial Waste water pollute soil and groundwater and have a variety of negative consequences on plant, animal, and human health. Because of the negative environmental effects of toxic metals, several studies and techniques were involved in the treatment of industrial wastes before they are discharged, including the use of bacteria to remove metal ions from Waste water, which has a lot of potential **(Kasan and Baicker, 1988).**

The Waste water's distinguishing characteristics are presented in (Table 1)

In this investigation, the bacteria population in the sample ranged from 100 to 200 colonies per millilitre. A total of ten bacterial colonies that can resist heavy metal Waste water were identified. Based on dehydration and resistance capabilities, the two best isolates were chosen. Bacteria have metal binding abilities and have been observed to show tolerance as well as dehydration. **(Mohammed Umar Mustapha2015)**

Microbiological study was performed on the wastewater collected. These isolates were to grow on EMB agar and mannitol salt agar were identified and suspected as *Klebsiella* and *Staphylococcus*. (Plate 1 and 2) The further identified according to their cultural and morphological characteristics. The different morphological characteristics, microscopic examination of *klebsiella spp* are gram negative, non-spore forming, capsulated, non-motile and arranged single or pairs **(Mona Mohammed Aly *et.al* 2014)** and *Staphylococcus spp* are gram positive, spread assay non spore forming, capsulated. **(Konuka *et.al* 2022).**

Biochemical test of *klebsiella spp* gave positive reaction for catalase test, Voges Proskauer test, citrate test and urease test. Meanwhile the isolates were negative for indole, oxidase and methyl red test. *Staphylococcus spp* showed negative results for indole, methyl red, Voges-Proskauer reaction, hydrogen sulphide, gelatin liquefaction, and oxidase test and showed positive results with citrate, lactose, sucrose, glucose, lipid hydrolysis, catalase, and starch hydrolysis test (table 1.8 and 1.9)

In bioremediation studies, *Klebsiella spp.* and *Staphylococcus sp.* play an important role. They adapt to any environment quickly and achieve maximum growth efficiency

Heavy metal concentrations in India's industrial areas are far greater than the "World Health Organization allowed "'a limit (WHO). Metals in the environment cause a variety of diseases and disrupt metabolic activities in humans **(Tyagi *et al.*, 1999).**

Nickel-containing enzyme, causes kidney stone development and pyelonephritis in ureolytic infection. Urinary colitis and hepatic shock as a result of elevated pH and excessive ammonia levels **(Hausinger RP, 1987).**

Nickel appears to be relatively non-toxic to mammals, with only modest toxicity, but at 1.6 mg/ml, it causes a 50% drop in fecundity and survival **(Pickering, 1974).**

In this present study, Ni concentrations have ranged from 0.5 to 5 mg/kg **(Monika Das *et al.*, 2011).**

The decrease of nickel in waste water through dehydration employing *Staphylococcus spp, Klebsiella spp,* and chitosan is demonstrated in this work under various pH and time intervals (Table 2.4 -2.7 & 3.2 -3.5)

The initial nickel reduction concentration was estimated to be 53 mg/ml. There is a significant level of reduction at varied nickel concentrations of 10mg, 20mg, 40mg, and 60mg, as well as varying pH and time intervals in various temperatures. *Staphylococcus spp.* was found to be capable of reducing nickel by 35%. There was a 25% reduction in *Klebsiella spp* infections.

The reduction of nickel by *Staphylococcus spp.* and *Klebsiella spp.* under different pH and time intervals is shown in the graph.

The current research also shows that *Staphylococcus spp* can perform a more efficient degradation rate. This could be attributed to acidic isolates that have been acclimatized in acidic environments (i.e., Nutrient broth with nickel sulfate salt).

Chromium is an important element in trace levels, but it is harmful when consumed in excess **(Sreenath *et al.*, 2003).**

The harmful effects of Cr on humans are mostly associated with its hexavalent form, according to **Athar and Vohora (1995)**. Chromium toxicity includes liver necrosis, nephritis, and gastrointestinal irritation. The various controls used in *Staphylococcus spp.* chromium adsorption studies.

In this present study the Cr content varied from 1.16 to 3.85 µg/L,).

The different concentrations of nickel which 10mg ,20mg,40mg,60mg along with different pH with proper time interval in various temperature has considerable amount of reduction.

The reduction of chromium through *Staphylococcus spp* and *klebsiella spp* under varied pH and time intervals are depicted in (Table.2.0-2.3 & 2.8-3.1) The initial amount of chromium (Cr) was estimated as 5 mg/m.

There was a considerable reduction at varied pH. Efficient reduction of chromium was recorded by *Staphylococcus spp* was *25%* Klebsiella *sp.* was 25 %.

The current research also shows that *Staphylococcus spp* can perform a more efficient degradation rate.

Chitosan is a polycationic, biodegradable, nontoxic bio polysaccharide that occurs naturally **(Y. Shigemasa *et.al* 1996).** Chitosan is abundant in nature and may be easily extracted from shrimp shells that contain chitin, a N-acetyl glucosamine polymer **(S. Ahmed et. Al 2014).** Alkaline de acetylation with NaOH converts the N-acetyl glucosamine polymer to glucosamine **(X. Fci Luc 2001).** Because of its NH2 and -OH groups, chitosan has been used as an excellent adsorbent to remove heavy metals **(F. C. Wu et. Al 2001).**

Chitosan was isolated from waste prawn shells using the Rashid *et al.* technique (2012). Chitosan is made from prawn shell materials after they have been treated with dilute HCL and NaOH.

The amount of chitosan in various sources is calculated. All functional groups in chitosan macromolecules were elucidated in the experimentally synthesized chitosan; the hands were more pronounced than in the normal one, indicating that the former had a higher degree of morphological arrangement (higher degree of crystalline order). At room temperature, the protein was extracted with 4% NaOH. HCl solution was used to extract minerals from deproteinized shell samples. **(Trung Ts and colleagues, 2006).**

Decolorization of chitin with 1% potassium permanganate and 1% oxalic acid yields purified chitin.

To obtain chitosan, pure chitin was deacetylated. Chitosan should be white when obtained **(Muzzarelli RAA *et.al* 1985).** Chitosan had a moisture content of roughly 4.1 percent (Table 3.6). The efficiency of the procedure employed for prawn shell deacetylation is determined by the ash concentration of chitosan. Chitosan's pH value is neutral, ranging from 6.5 to 8.0.

At 50 degrees Celsius, chitosan was soluble in 1% acetic acid. The purity is determined by the solubility. All functional groups in chitosan macromolecules are revealed via FTIR analysis **(Rashmi S.H *et.al* 2016).**

The FTIR result shows a major adsorption in the range of 3438 cm to 1075 cm, which corresponds to the free amino group ($-NH_2$) at the C_2 part of glucosamine, a main component of chitosan. Chitosan is a better absorbent than the Waste water resistant organisms.

The SEM results shows the micrograph of prawn shell. The bio sorbent is shown in different magnification in figure. The organic material containing high proportion of heavy metal that binds to the bio sorbent surface, the dark region is rich in dehydration because it has high proportions of Alkanes and Alcohols.

The purified form of chitosan magnification of SEM micrograph of 20μm in figure 6.7 represent the micrograph of heavy metal loaded bio sorbent.

The nickel dehydrated chitosan magnification of SEM micrograph at 5μm are shown in figure 6.8 represent the micrograph the image of dehydrated chitosan.

The chromium dehydrated chitosan magnification of SEM micrograph 5μm are shown in figure 6.9 represent the chitosan after dehydrated.

The result shows the presence of different functional groups present in the absorption surface of bio sorbent. Different functional group such as alcohols, alkenes, amine are potential site for absorption and uptake the metal depends on various factors such as abundance of sites, their accessibility, chemical state and affinity between absorbent site and metal. The prawn shell is potential to adsorption.

The analysis of FTIR spectra of chitosan has broadened peak around 3450 .77 cm^{-1} of O_H bonding and more intense characteristics peaks around 2926 cm^{-1} due to the stretching bending motion of C-H indicate that the surface modification was successful. The further adsorption peaks are shown at 1649.19,1629.9,1556.61 ,1421.58 cm^{-1}. This shows the confirmation of chitosan.

Future studies could look into the degree of dehydration caused by chitosan in different industrial waste water.

CONCLUSION

From the wastewater *Staphylococcus spp* and *klebsiella spp* were isolated and analysed for the removal of nickel and chromium from the heavy metal contaminated water.

Chitosan, a low-cost adsorbent was used in this present study for the removal of heavy metal from the waste water.

Chitosan act as good absorbent and shows more reduction of nickel and chromium (i.e.) almost 50% of the initial metals. Among resistant organisms *Staphylococcus* spp shows 35% reduction of nickel 25% reduction of chromium. The reduction of metal was confirmed through optical density with carried out under different pH and temperature. There were different controls maintained to make sure that there was only adsorption of metals by organisms

The enhancement and removal of metal ions by both chitosan organisms suggests that they could be used in retreatment. Further research and studies into heavy metal reduction by chitosan could have a significant impact on lowering pollution and health impacts.

SUMMARY

Klebsiella pneumonia and Staphylococcus sp. were isolated and analysed for the removal of nickel and chromium from thermal power plant waste water the bacterial species and Chitosan were used for this study. Bacterial species and Chitosan result in a larger decrease of nickel and chromium (almost 50% of the starting metals). Chitosan and bacterial species regeneration were used to reduce nickel and chromium even further. The reduction of metal was confirmed by bacterial species and chitosan biosorption. Different controls were used to ensure that only bacterial species and chitosan were involved in the bio adsorption of metals. The ability of bacterial species and chitosan to enhance and remove metal ions suggests that these organisms could be used in wastewater treatment. It's called biosorption.

Tables:

Table-1.7
CHARACTERISTICS OF THE WASTE WATER:

1.	pH	Neutral
2.	Colour	Colourless
3.	Odour	Odourless
4.	Heavy metals present	Nickel, Chromium

Table-1.8
BIOCHEMICAL CHARACTERIZATON:

S. No	Biochemical Tests	*Staphylococcus sp.,*
1.	Indole	-ve
2.	Methylene Red	+ve
3.	Voges Proskauer	-ve
4.	Simmon Citrate	+ve
5.	Triple Sugar Iron	+ve
6.	Catalase	+ve
7.	Urease	+ve

Table-1.9

S. No	Biochemical Tests	*Klebsiella sp.,*
1.	Indole	-ve
2.	Methyl Red	-ve
3.	Voges Proskauer	+ve
4.	Simmon Citrate	+ve
5.	Triple Sugar Iron	-ve
6.	Catalase	+ve
7.	Urease	+ve

Table-2.0 REDUCTON OF NICKEL 10% BY *Staphylococcus sp.,*

Culture	pH	Concentration	Temperature	Contact time Hrs	O. D
Staphylococcus sp.,	4	10	25	24	0.824
Staphylococcus sp.,	5	10	30	48	1.006
Staphylococcus sp.,	6	10	35	24	1.467
Staphylococcus sp.,	7	10	40	48	1.645
Staphylococcus sp.,	8	10	45	24	1.253
Staphylococcus sp.,	9	10	50	48	0.795

Table-2.1 REDUCTION OF NICKEL BY 20% OF *Staphylococcus sp.,*

Culture	pH	Temperature	Concentration	Contact time Hrs	O. D
Staphylococcus sp.,	4	25	20	24	0.807
Staphylococcus sp.,	5	30	20	48	1.006
Staphylococcus sp.,	6	35	20	24	1.0486
Staphylococcus sp.,	7	40	20	48	1.0453
Staphylococcus sp.,	8	45	20	24	1.237
Staphylococcus sp.,	9	50	20	48	0.713

Table-2.2 REDUCTION OF NICKEL BY 40% OF *Staphylococcus sp*

Culture	pH	Concentration	Temperature	Contact time Hrs	O. D
Staphylococcus sp.,	4	40	25	24	0.824
Staphylococcus sp.,	5	40	30	48	1.747
Staphylococcus sp.,	6	40	35	24	1.477
Staphylococcus sp.,	7	40	40	48	1.309
Staphylococcus sp.,	8	40	45	24	1.245
Staphylococcus sp.,	9	40	50	48	0.934

Table-2.3 REDUCTION OF NICKEL BY 60% OF *Staphylococcus sp.,*

Culture	pH	Concentration	Temperature	Contact time Hrs	O. D
Staphylococcus sp.,	4	60	25	24	0.825
Staphylococcus sp.,	5	60	30	48	1.008
Staphylococcus sp.,	6	60	35	24	1.493
Staphylococcus sp.,	7	60	40	48	1.394
Staphylococcus sp.,	8	60	45	24	1.125
Staphylococcus sp.,	9	60	50	48	0.813

Table-2.4 REDUCTION OF NICKEL BY 10% OF *Klebsiella sp.,*

Culture	pH	Concentration %	Temperature	Contact time Hrs	O. D
Klebsiella sp.,	4	10	25	24	0.835
Klebsiella sp.,	5	10	30	48	1.001
Klebsiella sp.,	6	10	35	24	1.474
Klebsiella sp.,	7	10	40	48	1.326
Klebsiella sp.,	8	10	45	24	1.245
Klebsiella sp.,	9	10	50	48	0.975

Table-2.5 REDUCTION OF NICKEL BY 20% OF *Klebsiella sp.,*

Culture	pH	Concentration %	Temperature	Contact time Hrs	O. D
Klebsiella sp.,	4	20	25	24	0.818
Klebsiella sp.,	5	20	30	48	1.006
Klebsiella sp.,	6	20	35	24	1.486
Klebsiella sp.,	7	20	40	48	1.345
Klebsiella sp.,	8	20	45	24	1.209
Klebsiella sp.,	9	20	50	48	1.135

Table-2.6 REDUCTION OF NICKEL BY 40% OF *Klebsiella sp.,*

Culture	pH	Concentration	Temperature	Contact time Hrs	O. D
Klebsiella sp.,	4	40	25	24	0.819
Klebsiella sp.,	5	40	30	48	1.001
Klebsiella sp.,	6	40	35	24	1.478
Klebsiella sp.,	7	40	40	48	1.326
Klebsiella sp.,	8	40	45	24	1.154
Klebsiella sp.,	9	40	50	48	0.945

Table-2.7 REDUCTION OF NICKEL BY 60% OF *Klebsiella sp.,*

Culture	pH	Concentration	Temperature	Contact time Hrs	O. D
Klebsiella sp.,	4	60	25	24	0.840
Klebsiella sp.,	5	60	30	48	1.005
Klebsiella sp.,	6	60	35	24	1.432
Klebsiella sp.,	7	60	40	48	1.326
Klebsiella sp.,	8	60	45	24	1.245
Klebsiella sp.,	9	60	50	48	1.124

Table-3.0 REDUCTION OF CHROMIUM BY 10 % of *Staphylococcus sp.,*

Culture	pH	Temperature	Concentration (%)	Contact time hrs	O. D
Staphylococcus sp.,	4	25	10	24	0.827
Staphylococcus sp.,	5	30	10	48	1.645
Staphylococcus sp.,	6	35	10	24	1.476
Staphylococcus sp.,	7	40	10	48	1.004
Staphylococcus sp.,	8	45	10	24	0.805
Staphylococcus sp.,	9	50	10	48	0.801

Table-3.1 REDUCTION OF CHROMIUM BY 20% of Staphylococcus sp.,

Culture	pH	Temperature	Concentration	Contact time Hrs	O. D
Staphylococcus sp.,	4	25	20	24	0.818
Staphylococcus sp.,	5	30	20	48	1.006
Staphylococcus sp.,	6	35	20	24	1.864
Staphylococcus sp.,	7	40	20	48	1.476
Staphylococcus sp.,	8	45	20	24	1.355
Staphylococcus sp.,	9	50	20	48	0.803

Table-3.2 REDUCTION OF CHROMIUM BY 40% of *Staphylococcus sp.,*

Culture	pH	Concentration %	Temperature	Contact time Hrs	O. D
Staphylococcus sp.,	4	40	25	24	0.812
Staphylococcus sp.,	5	40	30	48	1.008
Staphylococcus sp.,	6	40	35	24	1.478
Staphylococcus sp.,	7	40	40	48	1.284
Staphylococcus sp.,	8	40	45	24	1.156
Staphylococcus sp.,	9	40	50	48	1.125

Table-3.3 REDUCTION OF CHROMIUM BY 60% of *Staphylococcus sp.,*

Culture	pH	Concentration %	Temperature	Contact time Hrs	O. D
Staphylococcus sp.,	4	60	25	24	0.840
Staphylococcus sp.,	5	60	30	48	1.005
Staphylococcus sp.,	6	60	35	24	1.394
Staphylococcus sp.,	7	60	40	48	1.637
Staphylococcus sp.,	8	60	45	24	1.207
Staphylococcus sp.,	9	60	50	48	1.115

Table-3.4 REDUCTION OF CHROMIUM BY 10% OF *Klebsiella sp.,*

Culture	pH	Concentration	Temperature	Contact time Hrs	O. D
Klebsiella sp.,	4	10	25	24	0.834
Klebsiella sp.,	5	10	30	48	1.001
Klebsiella sp.,	6	10	35	24	1.468
Klebsiella sp.,	7	10	40	48	1.354
Klebsiella sp.,	8	10	45	24	1.274
Klebsiella sp.,	9	10	50	48	1.156

Table-3.5 REDUCTION OF CHROMIUM BY 20% OF *Klebsiella sp.,*

Culture	pH	Concentration	Temperature	Contact time Hrs	O. D
Klebsiella sp.,	4	20	25	24	0.818
Klebsiella sp.,	5	20	30	48	1.006
Klebsiella sp.,	6	20	35	24	1.466
Klebsiella sp.,	7	20	40	48	1.375
Klebsiella sp.,	8	20	45	24	1.284
Klebsiella sp.,	9	20	50	48	1.112

Table-3.6 REDUCTION OF CHROMIUM BY 40% OF *Klebsiella sp.*,

Culture	pH	Concentration	Temperature	Contact time Hrs	O. D
Klebsiella sp.,	4	40	25	24	0.819
Klebsiella sp.,	5	40	30	48	1.008
Klebsiella sp.,	6	40	35	24	1.477
Klebsiella sp.,	7	40	40	48	1.356
Klebsiella sp.,	8	40	45	24	1.240
Klebsiella sp.,	9	40	50	48	1.101

Table-3.7 REDUCTION OF CHROMIUM BY 60% OF *Klebsiella sp.*,

Culture	pH	Concentration	Temperature	Contact time Hrs	O. D
Klebsiella sp.,	4	60	25	24	0.827
Klebsiella sp.,	5	60	30	48	1.005
Klebsiella sp.,	6	60	35	24	1.493
Klebsiella sp.,	7	60	40	48	1.384
Klebsiella sp.,	8	60	45	24	1.207
Klebsiella sp.,	9	60	50	48	1.125

Table-3.6 CHITOSAN CHARACTERIZATION

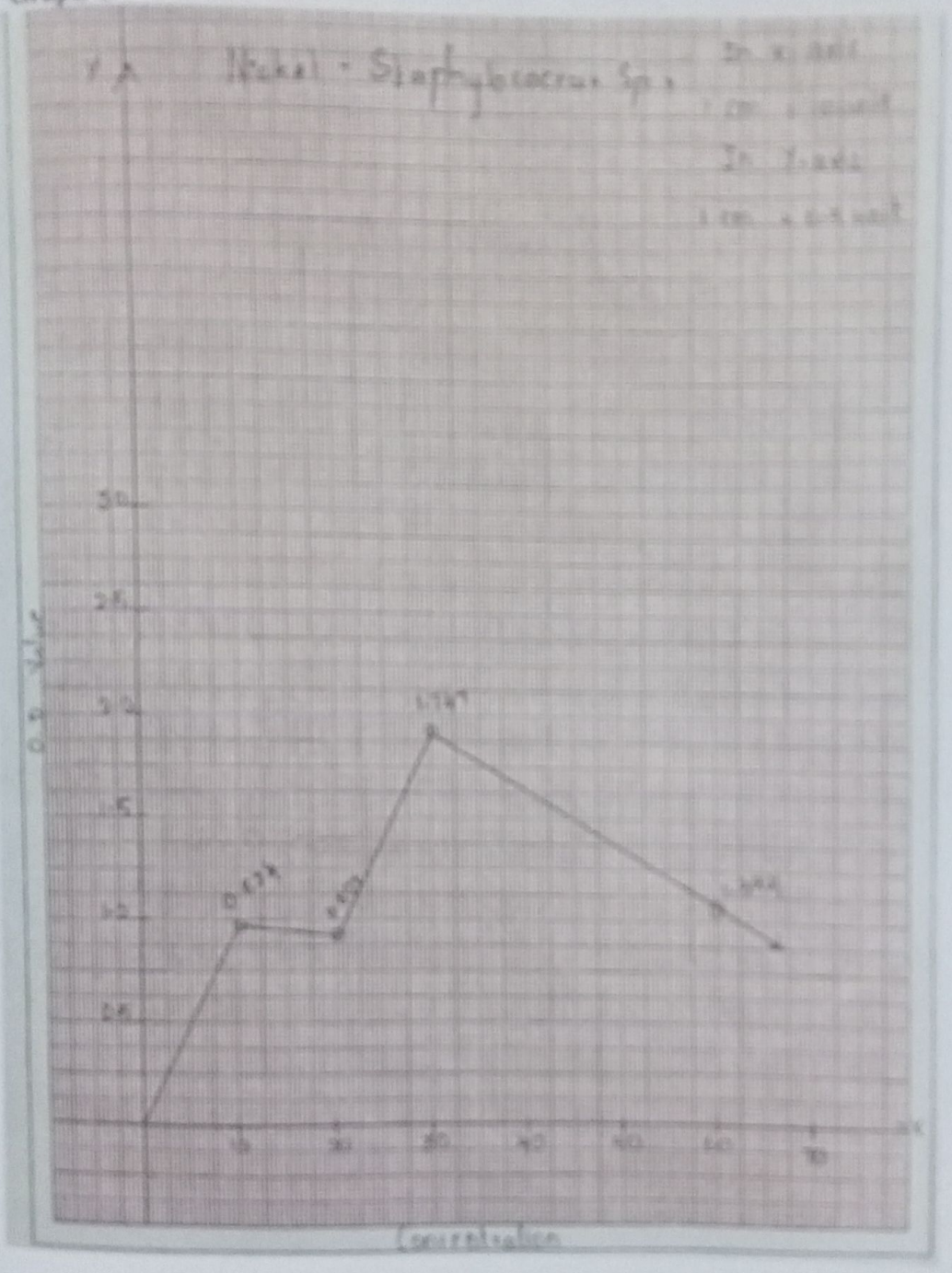
S. No	Characteristics of Chitosan	Valve
1.	Moisture content	4.1%
2.	Ash content	1.20%
3.	pH	Neutral
4.	Viscosity	Low viscosity
5.	FTIR	3438 cm 1075 cm

Table-3.7 FTIR

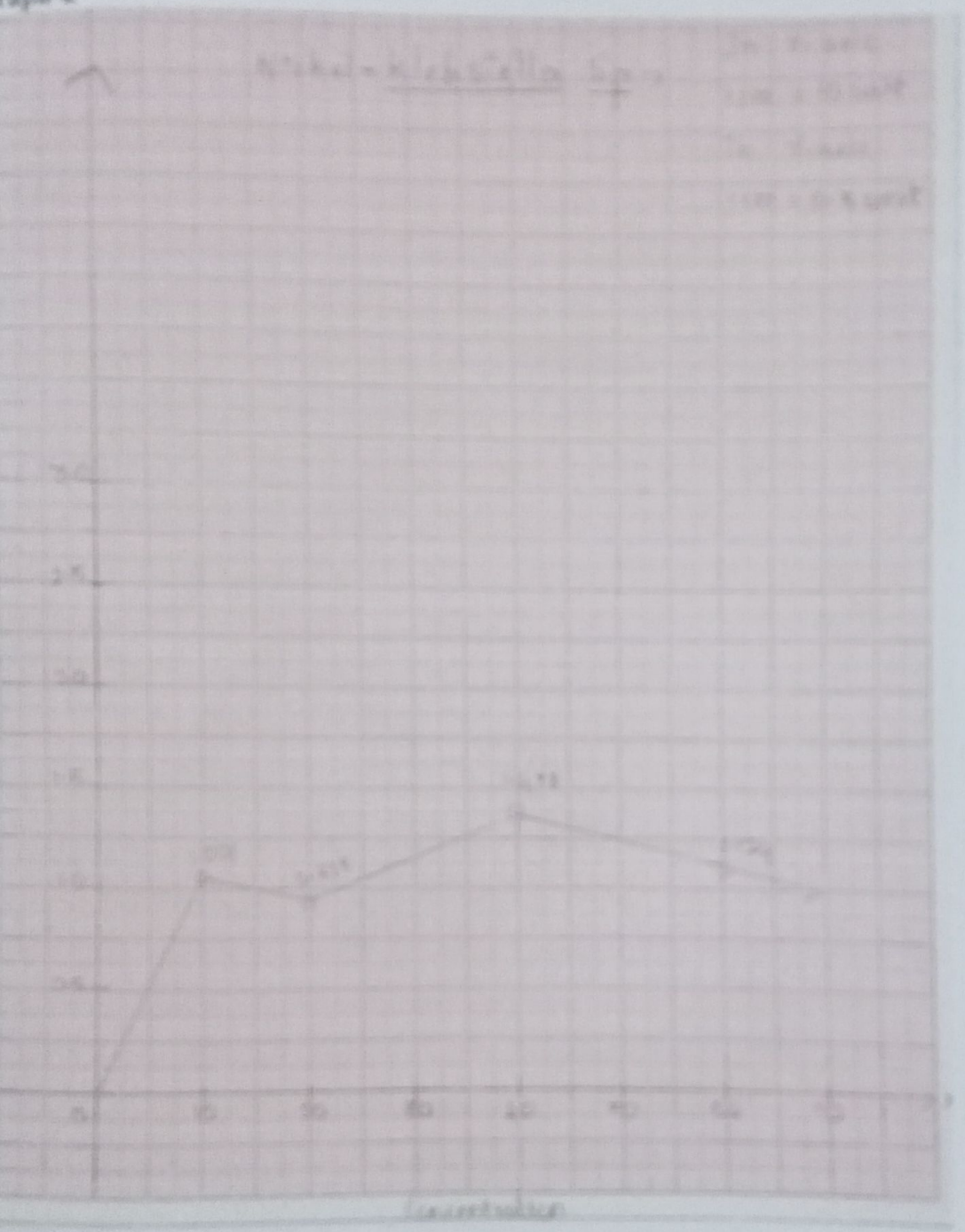
FREQUENCY RANGE	FUNCTIONAL GROUPS
3450.77	O -H
2926.11	C-H
1649.19	C=C
1629.9	N-H

STANDARD GRAPH

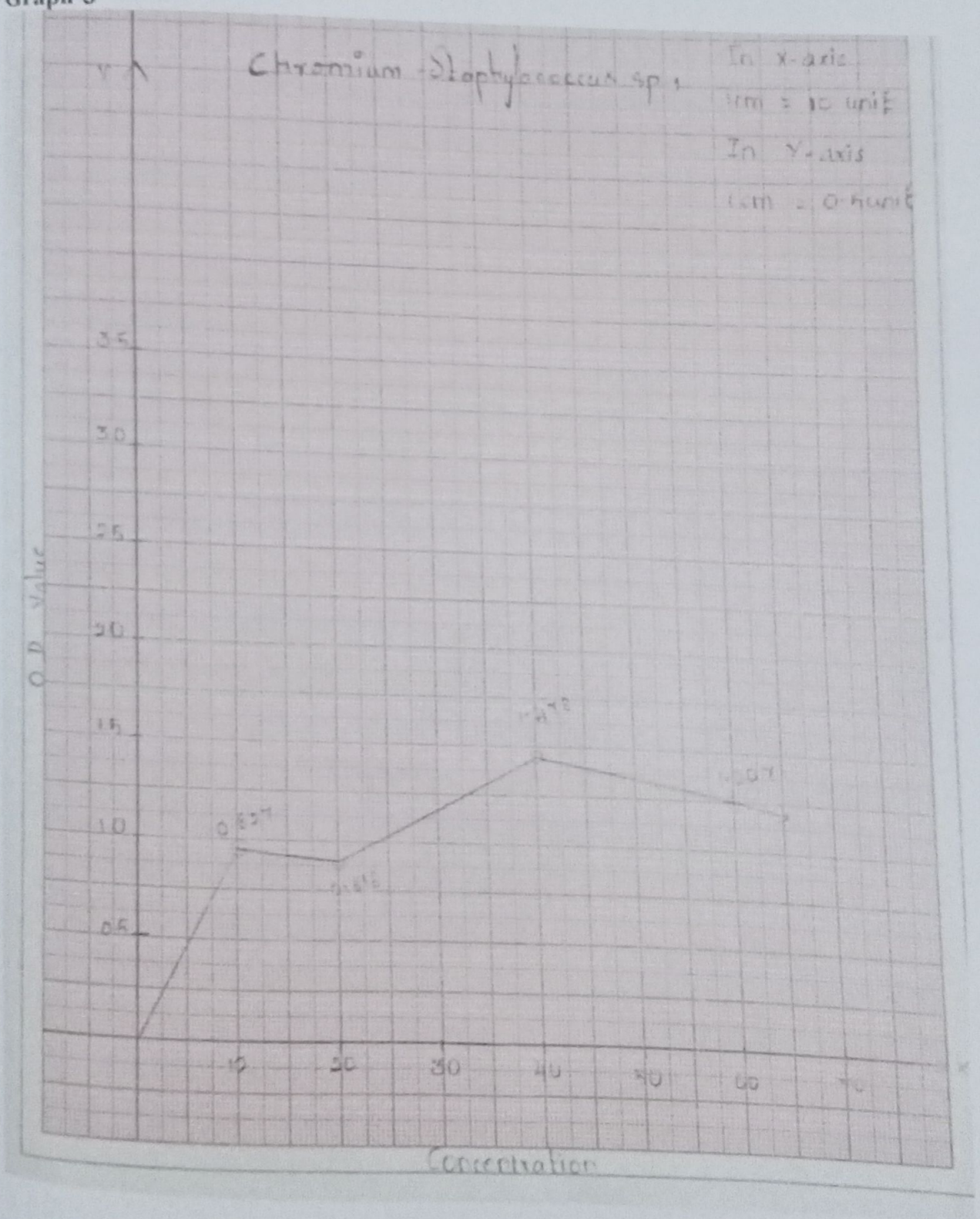
Graph-1



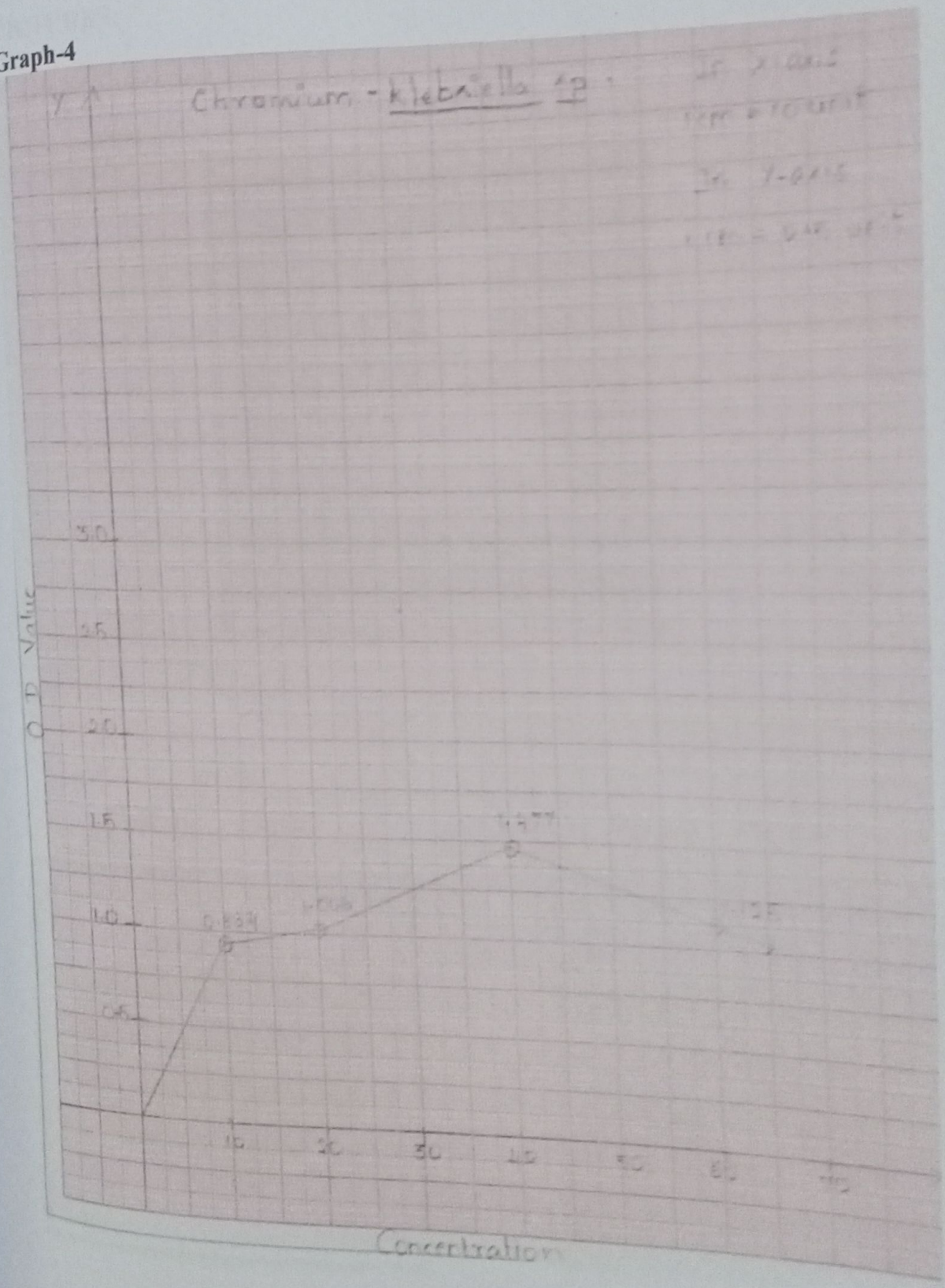
raph-2



Graph-3


Chromium - Staphylococcus sp.
In X-axis
1cm = 10 unit
In Y-axis
1cm = 0 unit
OD Value
35
30
25
20
15
10
05
10
20
30
40
50
60
Concentration

Graph-4


Chromium - Klebsiella sp.
Y
O.D Value
3.0
2.5
2.0
1.5
1.0
0.5
10
20
30
40
50
60
70
Concentration

PICTURES:

1. Sample Collection

Fig-1

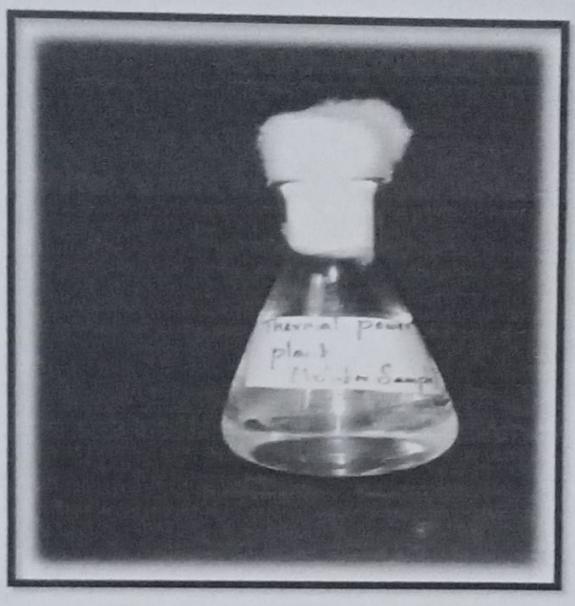
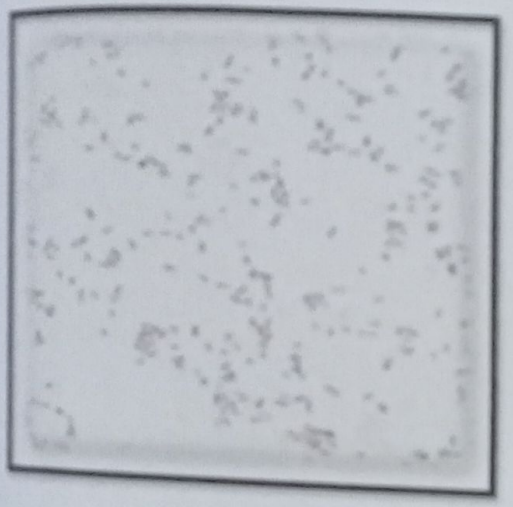


Plate-1 Fig-2.1

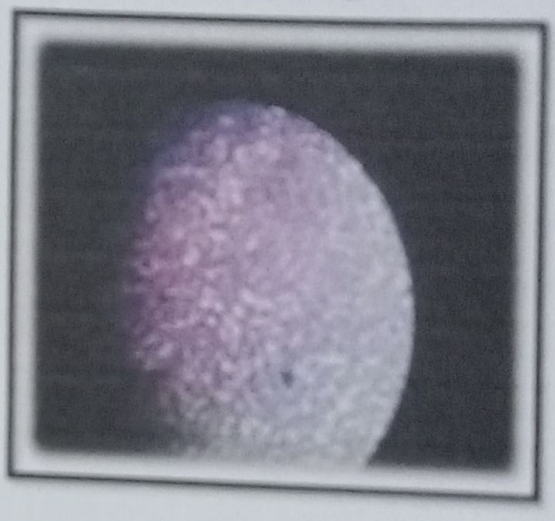
Plate-2 Fig-2.2

Microscopic Examination

Klebsiella sp., Fig-3.1



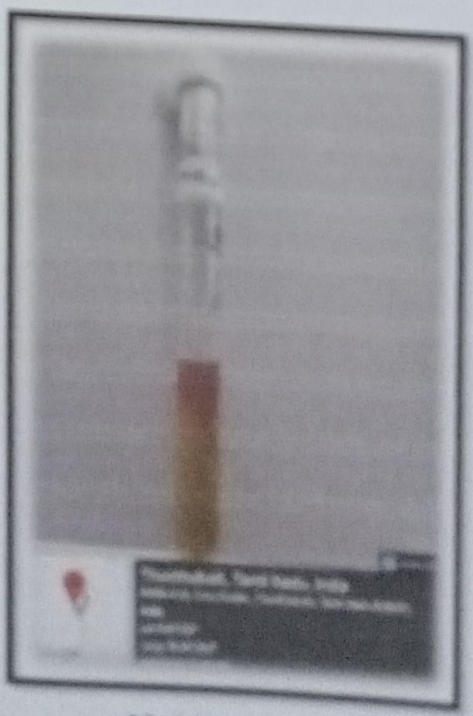
Staphylococcus sp., Fig-3.2



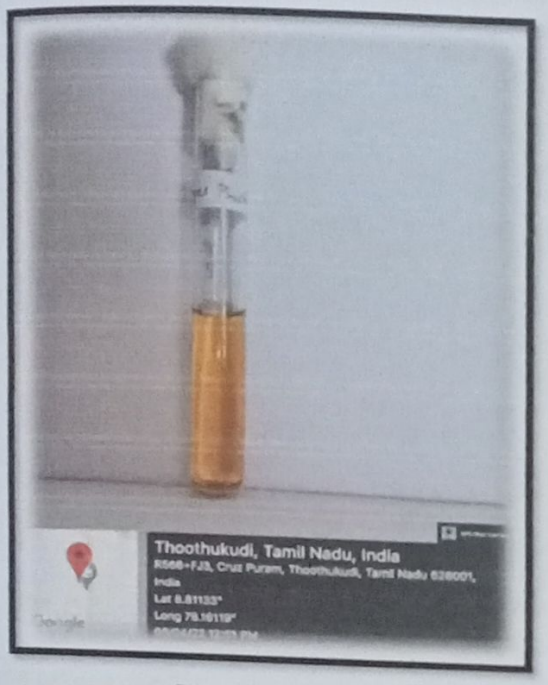
Biochemical Characteristics
Fig-4

(i) *Staphylococcus sp.,*

Indole



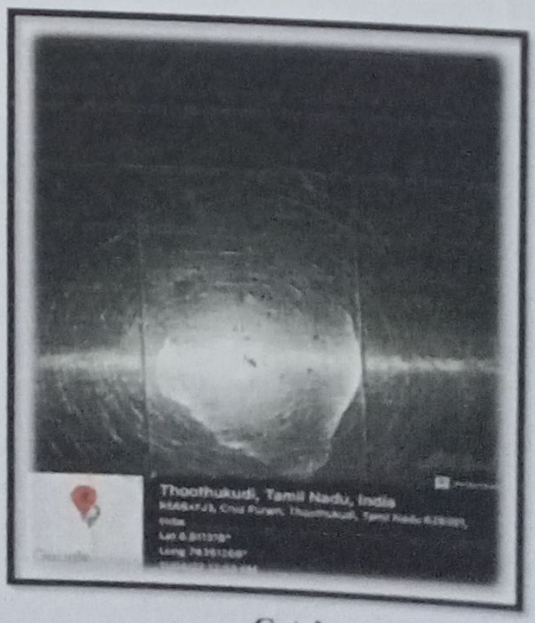
Methylene Red



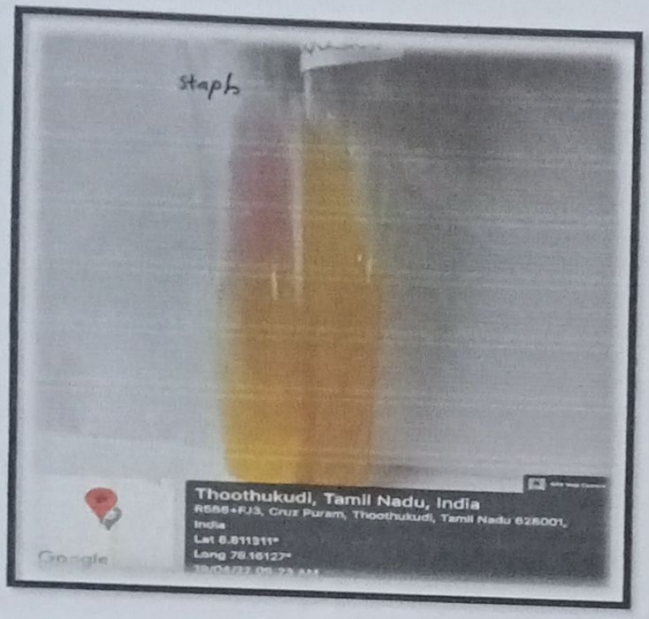
Voges Proskauer

Simmon Citrate

Triple Sugar Iron



Catalase

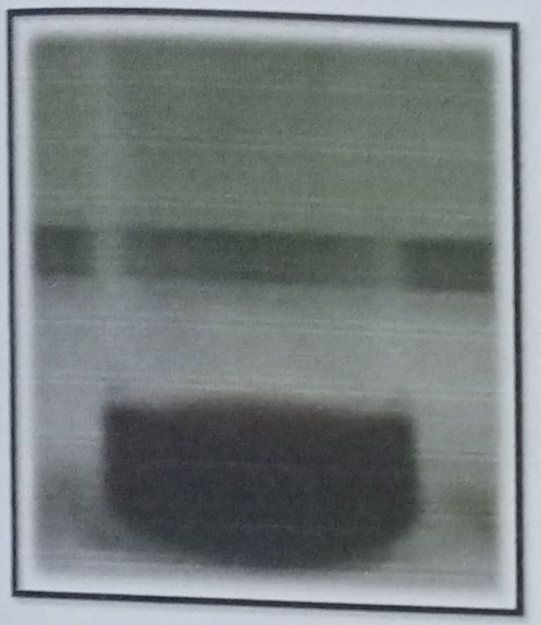


Urease

(ii) ***Klebsiella sp.,***

Indole

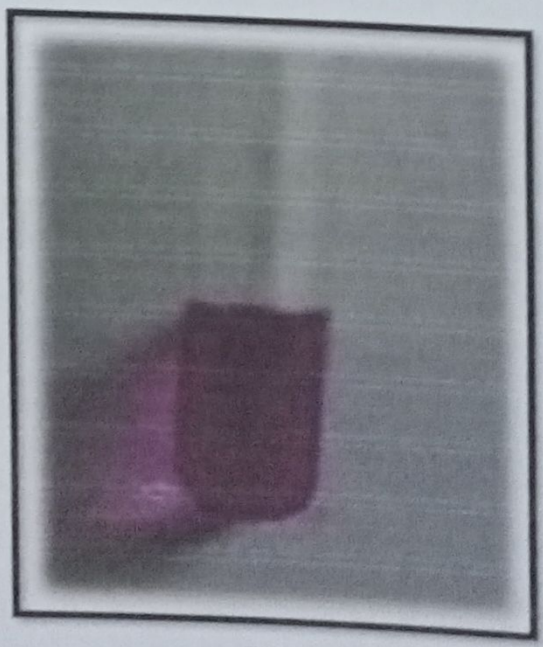
Methylene Red



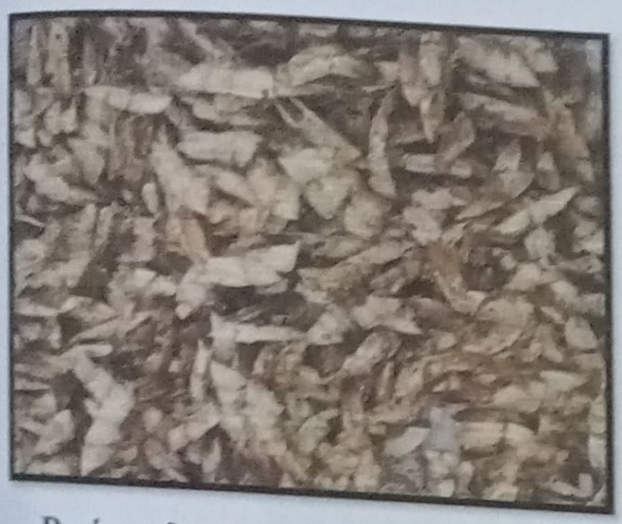
Voges Proskauer

Simmon Citrate

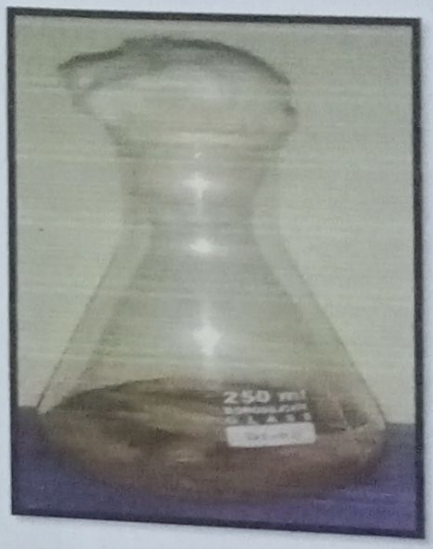
Triple Sugar Iron



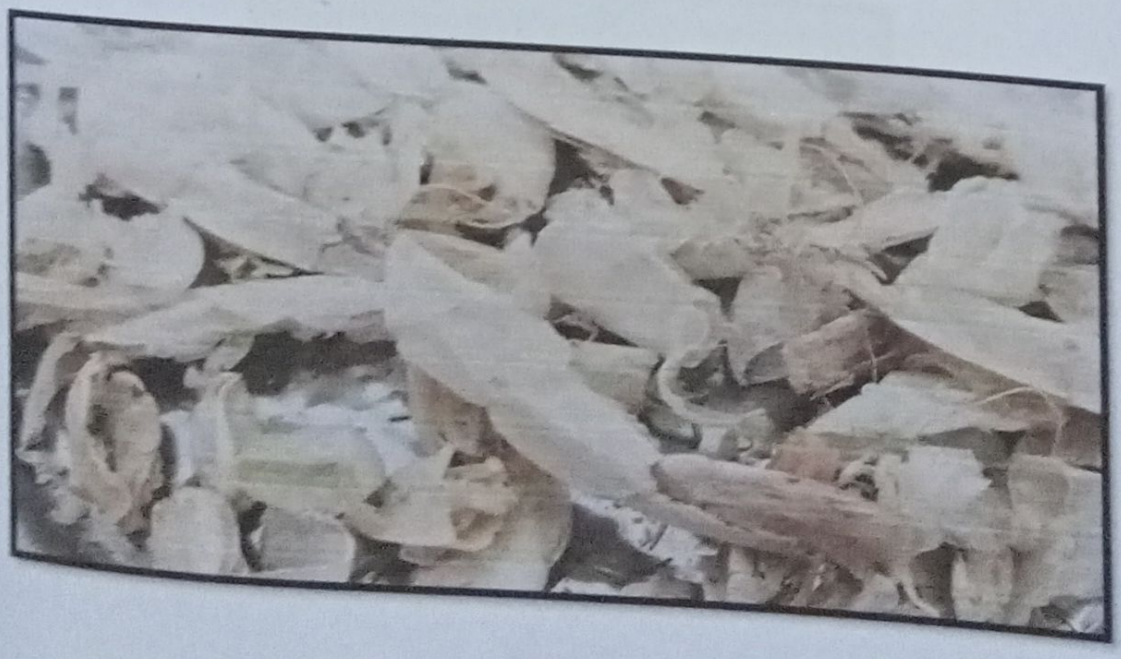
Urease



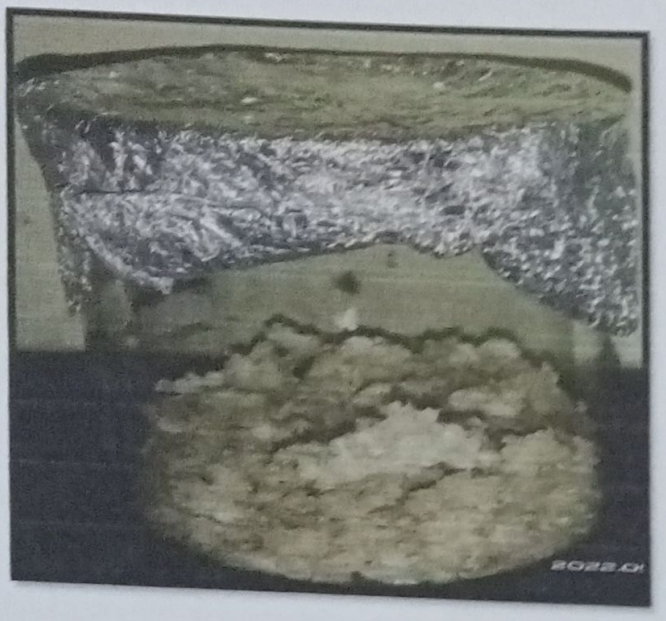
Drying of prawn shell
Fig-6.1



Prawn shell soaked in
NaOH solution
Fig-6.2

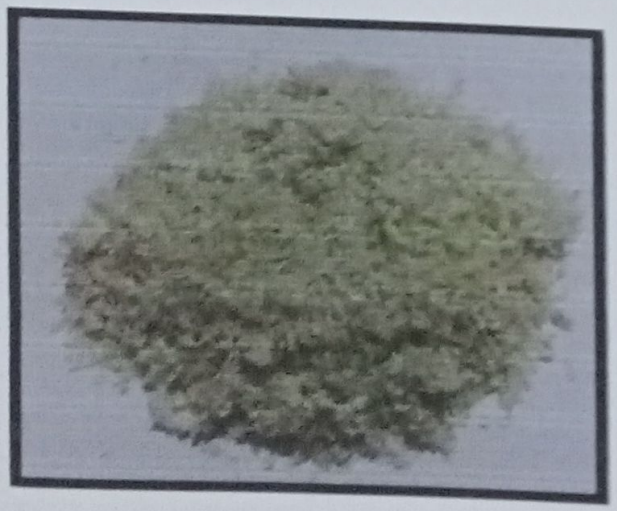


Dried prawn shell
Fig-6.3



After demineralization

Fig-6.4



Deacetylation

Fig-6.5

SHIMADZU

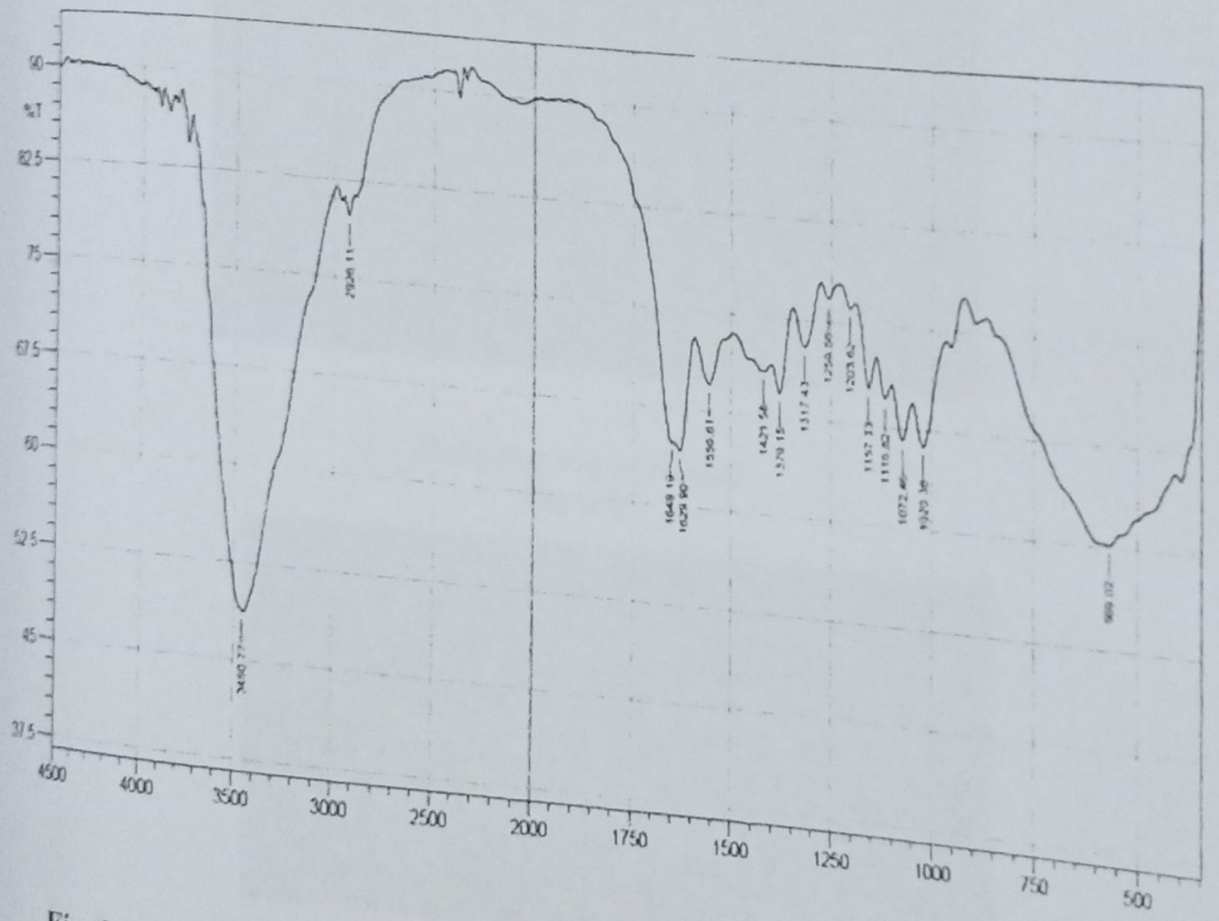


Fig-6.6

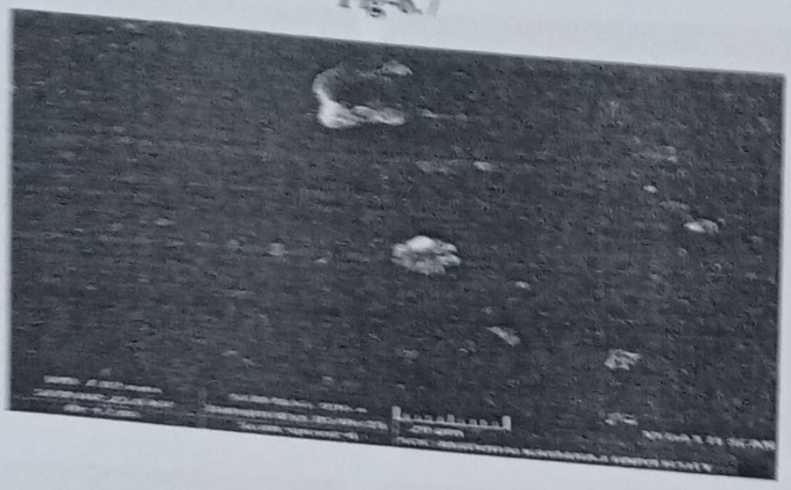
FT-IR SPECTROSCOPY

USIC-MKU

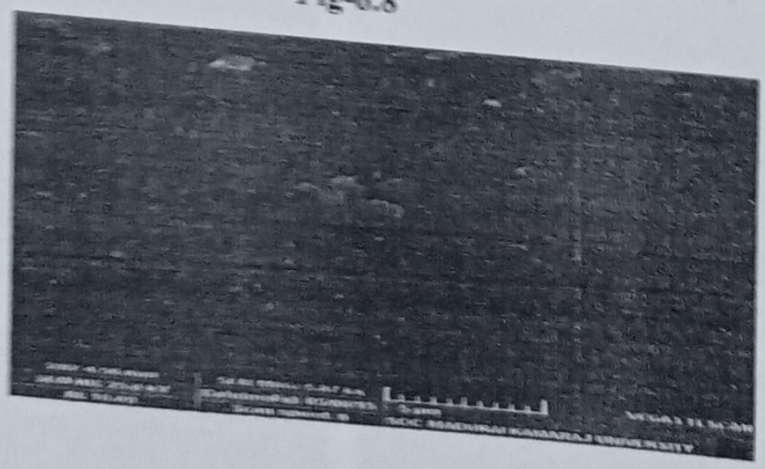
SHIMADZU

	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
1	969.02	60.266	0.349	584.45	540.09	9.667	0.085
2	1029.36	66.316	4.807	1047.38	996.37	12.734	1.123
3	1072.46	66.654	3.443	1099.46	1047.38	8.603	0.574
4	1110.82	69.648	1.964	1138.04	1099.46	5.811	0.338
5	1152.33	70.371	3.786	1190.12	1138.04	7.121	0.507
6	1203.42	76.165	0.918	1230.83	1190.12	4.615	0.081
7	1253.59	76.801	1.146	1276.92	1230.83	5.139	0.126
8	1317.43	72.967	3.817	1340.36	1276.92	6.705	0.802
9	1370.15	69.348	3.895	1400.37	1340.36	7.727	0.965
10	1421.58	70.926	0.099	1425.44	1400.37	3.71	0.013
11	1550.61	69.547	3.484	1593.25	1523.82	10.356	0.781
12	1629.9	64.38	3.06	1645.33	1666.16	8.693	0.623
13	1649.19	64.907	0.364	1836.29	1647.26	18.091	4.506
14	2926.11	76.843	1.466	2948.26	2601.02	4.608	0.181
15	3450.77	46.797	26.742	3641.73	2989.76	133.414	60.54

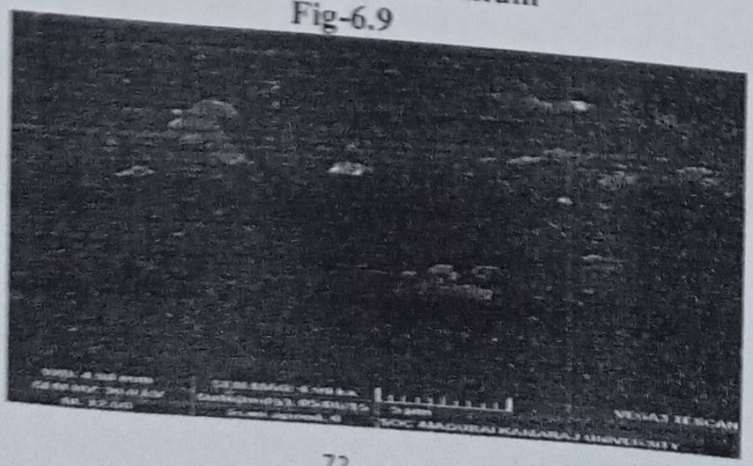
Chitosan
Fig-6.7

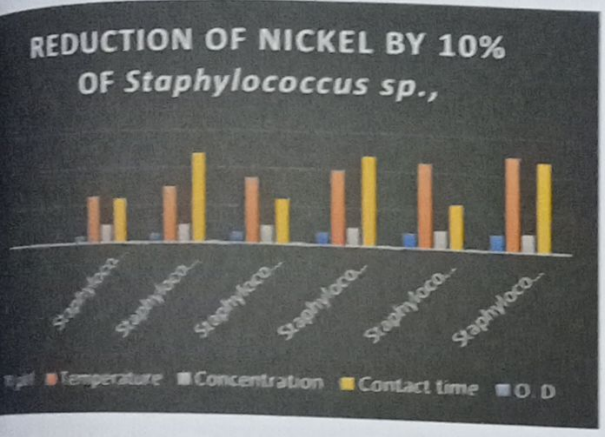


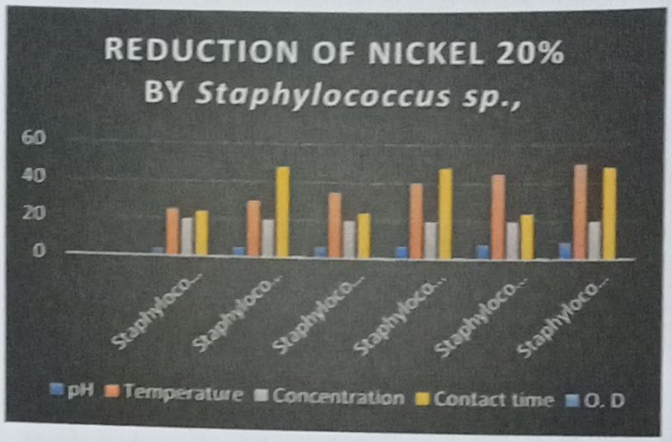
Dehydration of Nickel
Fig-6.8

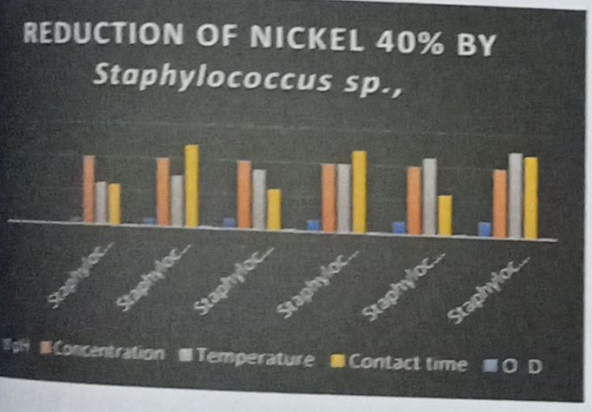


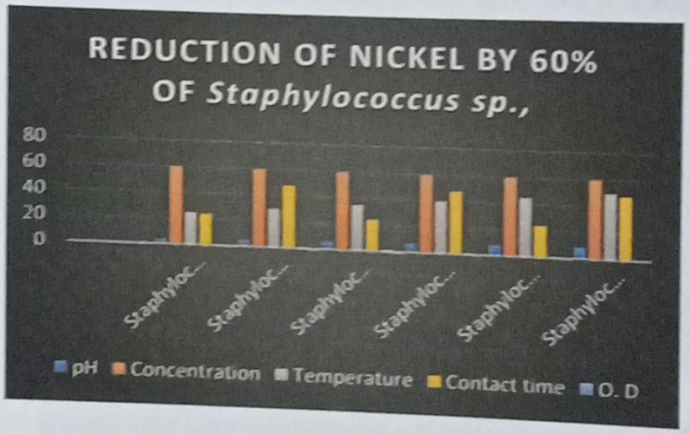
Dehydration of Chromium
Fig-6.9

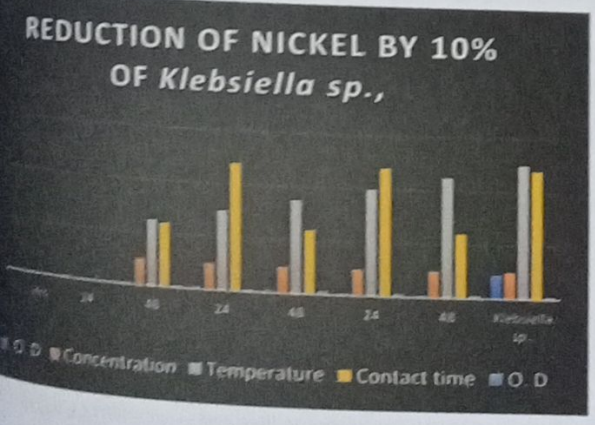


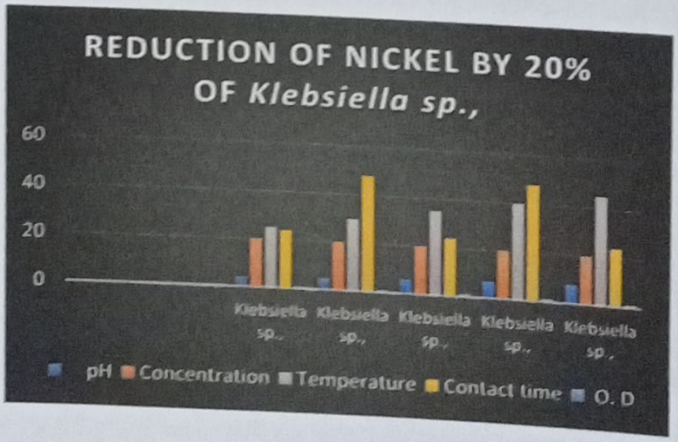

REDUCTION OF NICKEL BY 10%
OF Staphylococcus sp.,
Temperature
Concentration
Contact time
O. D

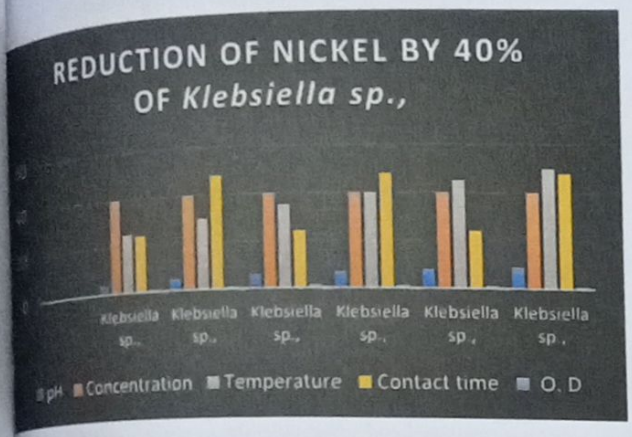

REDUCTION OF NICKEL 20%
BY Staphylococcus sp.,
60
40
20
0
pH
Temperature
Concentration
Contact time
O. D


REDUCTION OF NICKEL 40% BY
Staphylococcus sp.,
Concentration
Temperature
Contact time
O. D


REDUCTION OF NICKEL BY 60%
OF Staphylococcus sp.,
80
60
40
20
0
pH
Concentration
Temperature
Contact time
O. D


REDUCTION OF NICKEL BY 10%
OF Klebsiella sp.,
Concentration
Temperature
Contact time
O. D


REDUCTION OF NICKEL BY 20%
OF Klebsiella sp.,
60
40
20
0
pH
Concentration
Temperature
Contact time
O. D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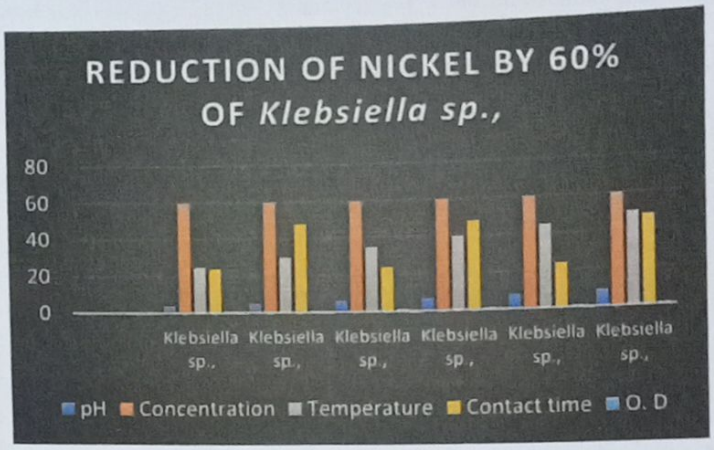
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OF Klebsiella sp.,
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Klebsiella sp.,
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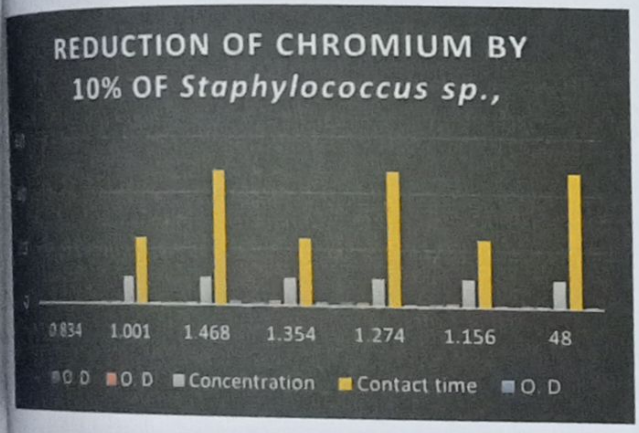
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48
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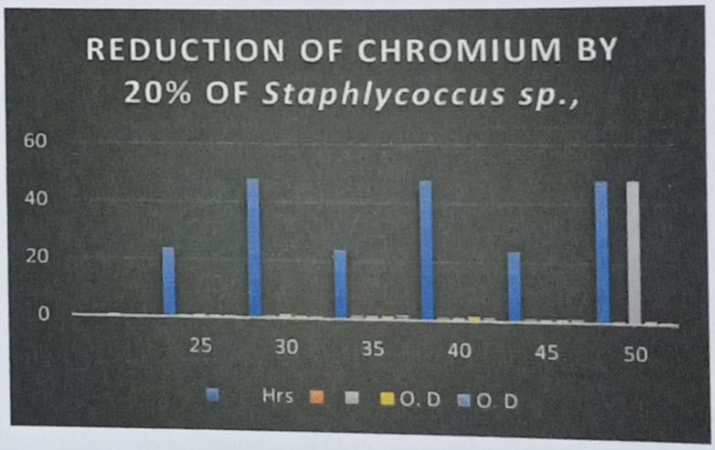
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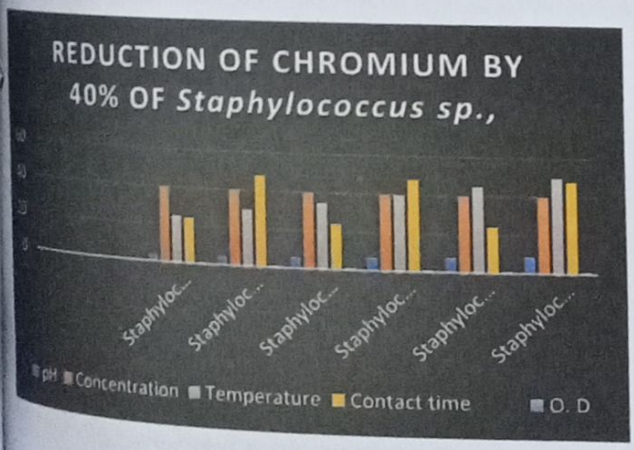
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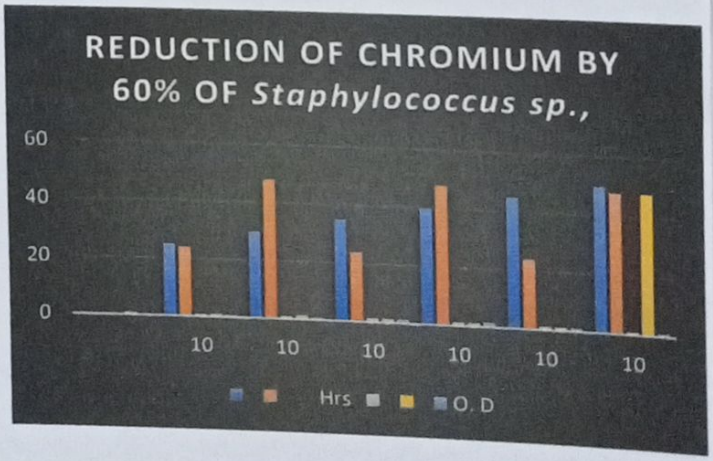
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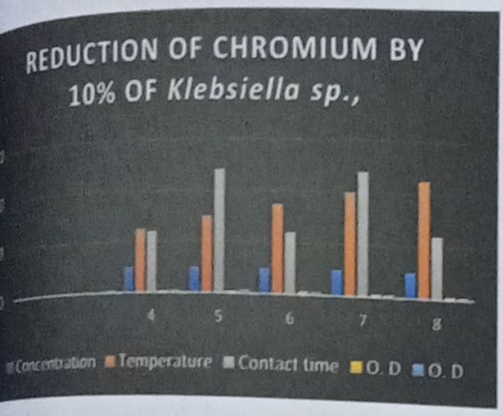
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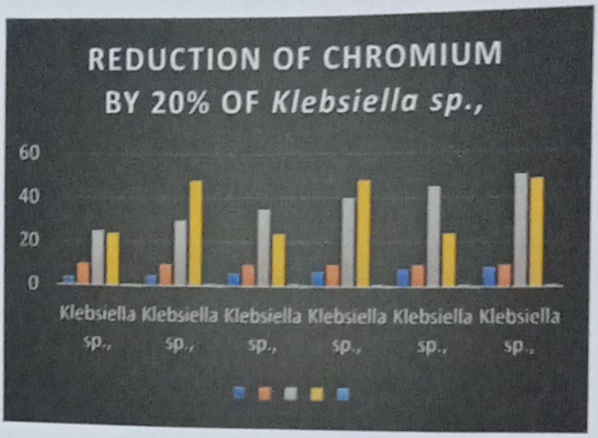
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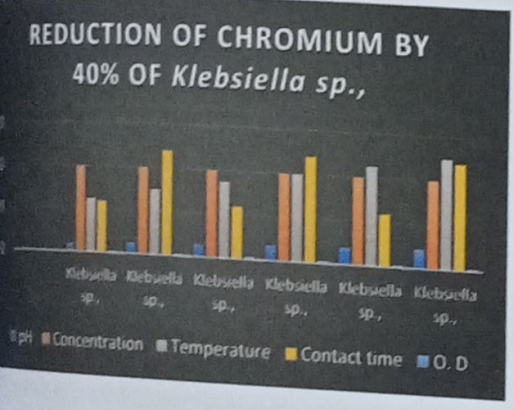
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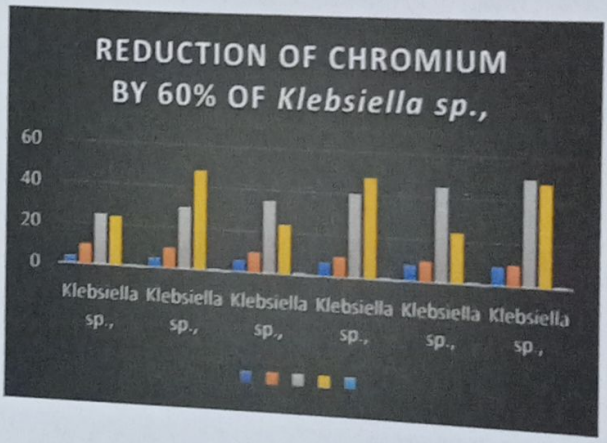
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In partial fulfilment of the requirements for the award of the degree of

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P.JEYA PRABHA (19SUMB14)

D.JOSELIN STARINA (19SUMB15)

B.JOTHI LAKSHMI (19SUMB16)

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Under the Guidance of

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MAY 2022

BONAFIDE CERTIFICATE

This is to certify that this dissertation work **entitled " ISOLATION AND CHARACTERISATION OF EXOPOLYSACCHARIDE FROM MARINE BACTERIA"** is a bonafide record of the original work completed by **D. JENITTA SUGIRTHAM , P. JEYA PRABHA, D.JOSELIN STARINA, B.JOTHI LAKSHMI, K.LIZA ESWARI, M. MAHESWARI (Reg.no 19SUMB13-19SUMB18)**, during the academic year 2021-2022 in St. Mary's College (Autonomous), Thoothukudi, and submitted as a partial fulfilment of requirements for the award of the degree of Bachelor of Science in Microbiology prescribed by the university of Manonmaniam Sundaranar.

Signature of the Guide

Signature of the H.O.D
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Signature of the Director
Director
Self Supporting Courses
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Thoothukudi - 628 001.

Signature of the Principal
Principal
St. Mary's College (Autonomous)
Thoothukudi - 628 001.

Signature of the External Examiner

DECLARATION

We hereby declare that the dissertation work entitled " **ISOLATION AND CHARACTERISATION OF EXOPOLYSACCHARIDE FROM MARINE BACTERIA** " is a bonafide record of the work completed by us during the academic year 2021-2022 in St. Mary's college (Autonomous), Thoothukudi and submitted as a fulfillment of requirements for the award of the degree of Bachelor of Science in Microbiology prescribed by the Manonmaniam Sundaranar University. We affirm that this is a original work done by us under the supervision of our guide Mr.C.Edward M.Sc.,M.Phil.,Assistant Professor of department of Microbiology, St. Mary's College (Autonomous), Thoothukudi.

D. Jenitta sugirtham.
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Signature of the Students

Signature of the Guide

Place: Thoothukudi

Date : 25.05.2022

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ABBREVIATION

Ml	Millilitre
Gm	Gram
Hrs	Hour
%	Percentage
Min	Minutes
FTIR	Fourier Transform Infrared Spectroscopy
DF	Dilution factor
CFU	Colony forming unit
H_2S0_4	Sulfuric acid
NaCl	Sodium chloride
ZMA	Zobell Marine Agar
Mg	Milli gram
EPS	Exopolysaccharides
YMG	Yeast Mannitol Glucose agar

INTRODUCTION

Isolation and characterisation of Exopolysaccharides from marine bacteria

INTRODUCTION

Marine microorganisms have unique properties since they have to adapt to extreme marine environment conditions such as high or low temperature, alkaline or acidic water, high pressure and limited substrate in the deep-sea water. These distinctive characteristics have attracted many researchers to explore in depth since there is potential of marine microorganisms used in industry. Research into natural products from the marine environment, including microorganisms, has rapidly increased over the past two decades. Despite the enormous difficulty in isolating and harvesting marine bacteria, microbial metabolites are increasingly attractive to science because of their broad-ranging pharmacological activities (*(Nidhi Vijayan et.al.,2012)*

Exopolysaccharides (EPSs) are high molecular weight carbohydrate polymers that make up a substantial component of the extracellular polymers surrounding most microbial cells in the marine environment. They constitute a large fraction of the reduced carbon reservoir in the ocean and enhance the survival of marine bacteria influencing the physicochemical environment in proximity of the bacterial cell. Moreover, they assist the microbial communities to endure extremes of temperature, salinity, and nutrient availability. In recent years the increased demand for natural polymers for pharmaceutical, food and other industrial applications has led to a remarkable interest in polysaccharides produced by microorganisms. *(Annarita poli et.al.,2010)*.

Bacteria release EPS in the environment in the form of capsules or slime to help these microorganisms cope with adverse environmental conditions as desiccation prevention and adhesions by forming biofilms. (Wijesekara et al., 2011) water soluble polysaccharides could be ionic or nonionic polymers (Freitas et al., 2011).

EPSs are divided into two groups: homopolysaccharides and heteropolysaccharides. Homopolysaccharides are made up of a single type of monosaccharide, like dextran or levan. Heteropolysaccharides are made up of several types like xanthans or gellans, have complex structures and are usually synthesized inside the cell in the form of repeating units. EPS biosynthesis can be divided into three main steps: (i) assimilation of a carbon substrate, (ii) intracellular synthesis of the polysaccharides and (iii) EPS exudation out of the cell (F.Donot et.al 2012).

Bacterial exopolysaccharides include dextran, alginate, xanthan, curdlan, cellulose, succinoglycan, glucuronan, colanic acid etc. Bacteria producing exopolysaccharides are *Acetobacter* spp., *Rhizobium meliloti*, *Pseudomonas aeruginosa*, *Lactobacillus helveticus*, *Lactobacillus rhamnosus*, *Xanthomonas* spp., *Shigella* spp., *Escherichia coli*, *Salmonella* spp., *Enterobacter* spp.etc .Several bacteria such as *Geobacillus thermodenitrificans*, *Bacillus thermantarcticus* etc are isolated from intense environments: deep-sea hydrothermal vents, geothermal springs, saline lakes and Antarctic ecosystems have been studied as possible sources of Exopolysaccharides (Freitas *et al.*, 2011).

Synthesis of exopolysaccharide is an intracellular process involving nucleoside diphosphate sugars. This constitutes a cassette of genes, the products of which are responsible for acylation and the addition of individual sugars to isoprenoid lipid acceptors. The repeating units are polymerized on the carrier lipids and excreted into the extracellular environment (Anita Suresh kumar et.al 2007) Exopolysaccharides shows different properties like, thickening, gelling, emulsifying, etc., *(Nowdo et .al., 2012)*.

EPS-producing bacteria are widely present in *marine* ecosystems and can be isolated from the water column, sediments, animals, etc. Bacteria producing polymers with novel structures and innovative properties have been isolated in atypical environments (Mancuso-Nichols et al., 2005). Several factors and parameters influence the production of EPS, among these are the composition of the medium, especially carbon and nitrogen sources, pH, temperature, and incubation time. (Vincent et al., 1994).

Applications of EPS

The EPS functional features have found many applications in industries such as cosmetics and pharmaceutics (Delbarre-Ladrat *et al.*, 2017). Potentially, EPS have proved various physiological activities in human beings as antitumor, antiviral and anti-inflammation agents, as well as being inducers for interferon, platelet aggregation inhibition, and colony stimulating factor synthesis. (de Godoi *et al.*, 2014).Indeed, the genus *Halomonas* has received increasing interest as several species are able to produce significant quantities of EPS with high surface activity and/or rheological properties (Martinez-Checa *et al.*, 2002 and Pepi *et al.*, 2005).

Antibacterial and fungal properties of EPS was found due to the many possible linkages and configurations. PolysaccharideB1 from the marine bacterium, *Pseudomonas* sp., was found to be more cytotoxically active to the central nervous system and lung cancer cell lines since the EPS induced apoptosis in the cells. It has been found that the EPS produced by marine bacteria have strong affinity for heavy metals and thus EPS can be used for bioremediation of heavy metals from the environment. Intriguing properties of the EPS derived from *Halomonas* species, such as emulsification activity, appear to be worthwhile for an ample range of products and application.

Commercial Applications of Exopolysaccharide: (Mariam Zaheer et.al 2019)

EPSs	**Source**	**Application**
Gelrite or Kelcogel	*Sphingomonas paucimobilis*	Used in foods as stabilizing, suspending and gelling agent
Xanthan	*Xanthomonas compestris*	Used in oil recovery and various foods as viscosifying agent
Emulsan	*Pseudomonas fluorescence*	Used in various food as an emulsifying agent
Dextran	*Leuconostoc mesenteroides, Streptococcus mutans*	Used to purify different molecules such as Sephadex
Curdlan	*Agrobacterium* and *Rhizobium* spp	Used in biomedical applications such as antithrombotic activity etc
BioFill	*Acetobacter xylinum*	Used in general plastic surgery as Implantable material

Health benefits of Exopolysaccharide:

Lactobacillus casei	Activated mouse acrophages
Bifidobacterium bifidum	Antiulcer activity
Bacillus licheniformis	Antiviral and immunostimulatory activities
Lactobacillus kefiranofaciens	Increased gut mucosal immunity
Lactobacillus plantarum	Antimutagenic activity
Bacillus coagulans RK–02	Antioxidant and Antihyperglycemic activities

Several EPSs produced by microorganisms from extreme habitats show biotechnological promise. By examining their structure and chemical-physical characteristics, it is possible to gain insight into their commercial application and they are employed in several industries ranging from pharmaceutical to food-processing fields, through to the detoxification capability of polluted areas from petrochemical oils. Considering the microbial biodiversity of marine eco-systems an attempt was made in this study to isolate and characterize EPS producing bacteria from the interior part of the Thoothukudi ocean .

AIM AND OBJECTIVE

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- To isolate Exopolysaccharide producing marine bacterium from Thoothukudi ocean.
- To identify EPS producing bacterium based on morphological, biochemical and growth on selective media.
- To extract and quantify Exopolysaccharide from the selected bacterium.
- To characterise the EPS by FTIR method.

REVIEW OF LITERATURE

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Microbial polysaccharides are polymers that consist principally of carbohydrates and are excreted by some bacteria and fungi on to the outside of their cell walls .They may be either homo or hetero polysaccharide and may also contain a number of different organic and inorganic constituents. (Sutherland, et al.,1996).

The optimum temperature, pH, and Bacto-casitone concentration for exopolysaccharide production were 38°C, and 30g/liter, respectively, with apredicted yield of 295mg of exopolysaccharide/liter. The actual yield under these conditions was 354 mg of exopolysaccharide/liter, which was within the 95% confidence interval (217 to 374 mg of exopolysaccharide/liter). (Stacy A.Kimmei et al.,1997) .

Microbial polysaccharides are characteristics by considerable diversity in their composition and structure. Most polysaccharide of from microbial origin are heteropolysaccharides. This means there is a wide range of possible structures and differences in the properties of exopolysaccharides due to the many possible linkages and configurations. Microbial polysaccharides which are capable of interacting with the immune system to up regulate or down regulate specific aspects of the host response can be classified as immunomodulators or biologic response modifiers.
(Tziambos et al.,2000).

The EPS that released by bacteria have been shown to serve multiple functions including the promotion of the initial attachment of cells to solid surfaces. The formation and maintenance of micro colonies and mature biofilm structure; the durable biofilm structure; water retention; the retention ; the absorption of exogenous organic compounds for the accumulation of nutrients from the environment and enhanced biofilm resistance to environmental stresses and disinfectants. (Rittmann et al.,2002).

Structural polysaccharides that are components of the cell structures such as lipopolysaccharides and trichoic acids present as integral components of cell walls and extracellular polysaccharides referred to as exopolysaccharides (EPS). (Anita Iyer et al.,2005).

These monosaccharide components are negatively charged at sea water pH, give the EPS on sticky quality and may influence the availability of trace metals such as iron.
(Carol Mancuso Nichols et al.,2005).

EPSs producing microbes are present in a variety of ecological niches. High C/N ratio medium contain these microbes such as effluents from the industries of sugar, paper or food and sewage treatment plants (Morin, 1998). They are also present in terrestrial and marine hot springs. There are many microorganisms that produce EPSs as isolated slime in the nearby environment or as attached capsular material (Bajaj et al., 2007).

Most EPSs produced by marine bacteria are heteropolysaccharides containing three or four different monosaccharides arranged in groups of 10 or less to form repeating units .Components most commonly found in marine EPS are monosaccharide such as pentoses (as D-arabinose, D-Ribose, D-Xylose), hexoses (D-Glucose, D-Galactose, D-Mannose, D-Allose, L-Rhamnose, L-Fucose), amino sugars (D-Glucosamine and D-Galactosamine) or uronic acids (D-Glucuronic acids, D-Galacturonic acids).(Annarita Poli et al 2010)

Some EPS are neutral macromolecules, but the majority of them are polyanionic for the presence of uronic acids or ketal-linked pyruvate or inorganic residues such as phosphate or sulphate. EPS forming a layer surrounding the cells provide an effective protection against high or low temperature and salinity or against possible predators. (Barbara Nicolaus et al.,2010).

In solid state fermentation high cell mass and high EPS production were obtained. It is suggested that high energy generation with the high sugar concentration and the relatively low and moderate amounts of nitrogen (low C/N ratio) of sugarcane juice resulted high EPS production and relatively high cell mass production. (Phisitseesuriyachan et al.,2010).

These polysaccharides have several important applications in nature and industry. They form part of biofilm, where they function as ion exchanger antibacterial agents. Bacterial polysaccharide is

also used as bioflocculants. bioabsorbents and for drug delivery. (Lin et al.,2010).

Capsule EPS are produced mainly during the log phase of bacterial growth and slime EPS produced during the stationary phase. The structure of polysaccharide is relatively simple, comprising of homo polysaccharides or hetero polysaccharides (containing more than two types of monosaccharide units). (Muthusamy Ashok Kumar et al., 2011).

Bacterial exopolysaccharides have found wide range of applications in food, pharmaceutical, petroleum, and textile. Ceramics, paper, ink, adhesives and other industries, owing to the various functions of EPSs in marine ecosystem such as adhesion of bacteria stabilization of biofilms and maintenance of symbiotic association with different species.
(Al-Nahas, M.O et al.,2011).

Microorganism synthesize large spectrum multifunctional polysaccharides including intracellular polysaccharides, structural polysaccharides, extracellular polysaccharides. Exopolysaccharides generally consist of monosaccharide's and some non carbohydrate substituent's . (Mohamed Orsod et al., 2012).

Few microbial polysaccharides like xanthan, sphingan, cellulose etc., gain commercial importance, more interest have developed among researchers, to isolate exocellular substances with characteristic composition and properties. They yield of exopolysaccharides varies in different strains the production largely depends on the substrate composition and environmental conditions. (Rabha, et al.,2012).

Exopolysaccharide shows different properties like thickening, gelling, emulsifying etc., and in view of these pharmaceutical, other industries (Nowdo et al.,2012).

The production of exopolysaccharides is a significant energy cost to bacteria, and yet direct observation of bacterial cells in a wide variety of natural and industrial environments show, unequivocally, that all such cells are surrounded by structured exopolysaccharides and that many

produce very large amounts of extracellular glycocalyx material. (M.I. AbouDobara et al.,2013).

Most of these biofilm forming bacteria are resistant characteristics and produce bioactive compounds including EPS with unique structures. The extracellular materials such as polysaccharides, lipids, glycoprotein's and lipopolysaccharides can be used as stabilizers, crystallizing agents, adhesives, solidifying agents, emulsifying agents, flocculants and flushing agents in various industries. (P. Nisha et al.,2014).

Exopolysaccharides produced by lactic acid bacteria have important applications including as thickeners, stabilizers, emulsifiers, bodying and gelling agents in food, pharmaceutical and chemical products. (Phayungsak Manochai et al.,2014).

Natural bioactive molecules attract many interests in the search for new therapeutic drugs. Marine environment shields a high diversity of natural products and can be indeed a treasure chest for industrial and pharmaceutical purposes. Due to their functions in survival and competitiveness of marine bacteria in low nutrients and adverse environments, the EPS are ubiquitous in the marine environment. (Christine DelbarreLadrat et al., 2014) .

Exopolysaccharide protects the bacterial cell from harsh environment such as desiccation, involve in the biofilms formation and bioremediation activity.(Parthiban Karuppiah et al., 2014).

Exopolysaccharide is a complex mixture of macromolecular electrolyte contained on the outside of the bacterial cell is excreted as mucus that contributes to soil aggregation as an adhesive. (Hazarin Subair et al.,2015).

Bacterial polysaccharides have diverse unique properties for food applications and are used as viscosifiers, stabilizers, emulsifiers, or gelling agents. Due to these valuable properties several studies were performed to genetically engineer the producing organisms in order to generate novel polysaccharides variants and to improve production. (Jochen Schmid et al., 2015)

Exopolysaccharides have health beneficiaries for the immunomodulatory activities hypoglycemic, hypolipidemic, anti tumor and so forth. Due to the high option pharmaceutical values, nowadays it is also used as foods and beverage supplement. (Abin Mani et al.,2015).

Microbial Exopolysaccharides have found outstanding medical application of EPS and their derivatives are well suited- potentially non toxic, bio degradable drug carriers. The numerous potential application still wait to be developed into commercial pharmaceuticals and medical devices. (Misunn Moscovici., 2015).

Bacterial polysaccharides have diverse unique properties for food applications and are used as viscosifiers, emulsifiers, or gelling agents. Due to these valuable properties several studies were performed to genetically engineer the producing organisms in order to generate novel polysaccharides variants and to improve production. (Jochen Schmid et al., 2015)

Some biological activities such as antitumor, antioxidant, anti-inflammation,immuno formulating antianemies and so on, which make them have potential applications in the fields of health products and drugs. (Huiru Zhang et al.,2016)

Majorly they provide protection to the cells from environmental adversities such as desiccation, predation, and the effects of antibiotics. Bacterial exopolysaccharides mainly participate in such processes through microbial aggregation, surface attachment, biofilm formation, plant-microbe symbiosis, and environmental bioremediation. Furthermore, bacterial exopolysaccharides are emerging as a viable source of polymeric materials. (Sheetal Sonawdekar et al.,2016).

Polysaccharide extracted from cordyceps mycelium constitute the main bioactive agents and exhibit multiple pharmacological activities including antitumor, anti-inflammatory, immunopotentiation, hypoglycemic and hypocholesterolemic effects and antioxidant effects. (Nandhini Gautam et al., 2016).

For the cells, EPSs are thought to play a role in protection against desiccation, toxic compounds, bacteriophages, osmotic stress, permit adhesion to solid surfaces and helps in biofilm formation. In recent years the increased demand of natural polymers for the industrial applications has led to the remarkable attention to EPS. By studying their structure and physico-chemical properties, it is possible to gain insight into their commercial applications. (Shailesh R Dave et al.,2016).

Popularity of polysaccharides as components of edible items is mainly due to their strong water binding and water retention capability in addition to their immense swelling and gelatin potential, Bacterial polysaccharides are currently being used as as adjuvant in skin care regimens preferable because of their positive effects on skin hydration and also their positive effects on skin hydration and also their capability in stimulating cell renewal and biosynthesis of several skin constituents. (Dhruti Avlani et al., 2017).

Glucose in culture media was studied to select the medium that gives a maximum production of exopolysaccharide by *Penicillium* sp., Exopolysaccharide was isolated by ethanol precipitation. The medium which contains glucose had been selected to get the heights production of exopolysaccharide. Fermentation conditions were further investigated to optimize exopolysaccharide production by *Penicillium* sp.,(Alaa Jabbar et al.,2017) .

Exopolysaccharide are isolated, purified and characterized from *S.commune*.Mushroom polysaccharides have attracted attention in food and pharmacology fields because of their many biological activities. The Exopolysaccharides have characterized by the Anti-inflammatery activities. It is indicated as significant anti-inflammatory effects, which showed that exopolysaccharide might be exploited as an effective anti-inflammatory agent for application in no-related disorders such as inflammation and cancer. (Bin du et al.,2017).

Bacteria release EPS in the environment in the form of capsules or slime to help these microorganisms cope with adverse environmental conditions as desiccation prevention and adhesions by forming biofilms (Salma M Abdelnasser et al., 2017).

Exopolysaccharides are polymers of carbohydrates secreted by some bacteria and fungi outside their cell walls. Marine microorganisms have significant osmotic tolerance and leading to produce exopolysaccharide even at higher salt concentration. The exopolysaccharide producing bacteria were isolated from marine environment. (Rahul Chaudari et al.,2017).

Corrosion process causes great economic losses in various industries, ship building, jewelry, archaeological monuments, railway, water channels, and all countries of the world. For handling this problem are normally applied different physical and chemical methods, but they often prove toxic. A perspective in this regard can be the application example of exopolysaccharides produced by the good bacteria probiotics. (Ivanova.T et al., 2017).

Production of EPS in marine *B. subtilis* SH1 was inoculated in 50 ml aliquot of nutrient broth medium dispensed in 250 ml Erlenmeyer flask, the flask was incubated at 37°C under shake condition (120 rpm) for overnight. After overnight incubation, 500 µl was transferred to 50 ml of a fresh production media in a conical flask. Above step was carried out in an aseptic manner, the flask . (Sahar W.M.Hassan et al .,2017) .

Many marine bacteria produce exopolysaccharides as a strategy for growth, binding to the Sub-stratum, to survive unfavorable conditions and also to intra and inter specific communication and competition. (Dhanya BE et al.,2018).

Isolates were cultivated on YMG at 30^0C for 24 hours having congo red. Dye solution was sterilized separately and added to the medium after autoclaving. This dye is more sensitive than calcofluor white dye binds only with externally secreted EPS in the medium whereas congo red dye increases the mucoidness of the colonies.(Prashaka J.Shukla et al., 2018).

The marine environment is the largest aquatic ecosystem on earth and it harbours microorganisms responsible for more than 50% of total biomass of prokaryotes in the world. All these microorganisms produce extracellular polymers that often in the form of exopolysaccharides (EPS).(Angela Casillo et al., 2018).

Various types of inorganic and organic substituent's (sulphates, phosphates, acetates, ethers, amino acids, lactates, and pyruvate) can decorate the polysaccharide backbone that in turn can be linear or branched. (Rosa Lanzetta et al., 2018).

Most marine bacteria produce maximum EPSs either in stationary or exponential phase, under nutrient (e.g. nitrogen, phosphate, sulphate, potassium etc) depletion conditions or in response to other environmental stresses. These stresses are characterized by osmotic pressure, temperature, salinity, against possible predators as well as under high levels of heavy metals. EPSs have several functions including protection from desiccation, cryoprotection, stabilization of enzymes by buffering pH, salinity fluctuation, nutrient storage and in temperature changes. (Prashakha J. Shukla et al., 2018).

Exopolysaccharides from microorganisms with boundaries chemical and functional varieties. Pretty much 30,000 regesion items have been segregated from marine organisms, and a few of the medication competitiors. (Mohsen S Asker et al., 2018).

Bacterial exopolysaccharides (EPS) for produced by polluted soil. Bacteria have found their most valuable application in the improvement of the rheology, texture, stability and sensor properties of fermented foods. (Ravi Gangalla et al., 2018).

Polysaccharides and their, derivatives have been widely used in industries, such as food process, pharmaceutical cosmetic and health care. (Chunlei Wang et al., 2018).

Recently, synthesis and stabilization of metal nanoparticles have been of interest because their usefulness for many biomedical applications, such as antimicrobials, anticancer drugs, antioxidants, drug delivery systems, chemical sensors, contrast agents, and as catalysts. In this context, bacterial EPSs

have been explored as agents to aid in a greener production of a myriad of metal . (Augusto Vazquez-Rodriguez et al.,2018).

Exopolysaccharides improves appearance, flavor, viscosity and the organoleptic properties of fermented milk products such as cheese and yoghurt. It was also reported that exopolysaccharides can be used as functional starter cultures in yoghurt. Structural defects occurred in reduced-fat dairy products may be reduced .(Ibrahim Altun et al.,2018).

The characteristics of Bacterial EPSs are identified by their molecular structure, chemical composition, average molecular weight, conformation of single molecules and their assemblies in case of aggregation process and gel systems .(Mariam Zaheer et al.,2019) .

Exopolysaccharides are essential metabolites synthesized and excreted by certain microorganisms in response to extreme condition of P^H, temperature, salinity, osmotic stress and other contaminants for survival in such adverse environment (I.G. Nwosu et al.,2019).

The exopolysaccharide was insoluble in water with high moisture-retention ability and produced viscous solutions and gels. The exopolysaccharide contained 40.3% glucose, 34.4% galactose, 22.2% mannose and 3.1% of arabinose monomers revealed by GC. The exopolysaccharide showed emulsifying, flocculating and in-vitro antioxidant activity similar to standard exopolysaccharides like dextran and xanthan. This EPS can be of potential use in food, cosmetic, pharmaceutical and biomedical fields . (Hema Chandran et al.,2019).

Polysaccharides produced by lactic acid bacteria can be used to alter rheological properties, acting in processes involving viscosity, emulsification, and flocculation.some beneficial properties of exopolysaccharides (EPS) produced by *Lactobacillus plantarum* that have not been commercially explored . (Liliane Andrade Silva et al.,2019).

More recently, biological activities of exopolysaccharide have been reported, with a number of recent studies documenting their immune stimulatory, antiviral, antioxidative, anti-tumor and antibacterial properties. (Aadil Ahmad Aullybux et al.,2019).

In certain scenarios, the nutritional conditions can also influence the molecular weight and the EPS osidic composition. (D. Sangeetha et al.,2019).

Exopolysaccharides are polymers of carbohydrates secreted outside the cell walls by certain fungi and bacteria. Marine microorganisms have considerable osmotic resistance and cause exopolysaccharides to be produced also in salt concentration. (Anooj E.S et al.,2020).

Exopolysaccharides which form slime layer loosely attached to cell surface or secreted in to environment. (for example, Xanthan, Sphingan, Alginate, Cellulose etc).
(Ahmed Farag et al.,2020).

For the string test *K.pneumoniae* isolates were grown on mackonkey agar plates and touched with an inoculation loop. Strains were classified as hypermucoviscous if a string longer than 5mm was observed,otherwise strains were classified as mucoid .(Bruno Douradinha et al.,2020).

Marine microbial polysaccharides are characterized by unique properties making them or good lower of bioactive agents that can be used on many fields as anti-tumor, antioxidant, and antiviral activities. . (Manal S. Selim et al.,2020).

The solubility of EPSs was tested in diverse solvents by previously reported method. According to this method, diminutive EPSs and different solvents (distilled water, methanol and chloroform) were mixed in 2 mL tubes, vortexed for 1 min and pellet dissolution was checked.(Babak Rahmani et al.,2020) .

The FTIR spectra of EPSS2 was performed with KBr pellets, according to Brock-Neely (1957). While, Uronic acids were determined at 525 nm by the m-hydroxybiphenyl colorimetric method.Sulfate was determined using the turbidly method.The monosaccharide composition was determined equipped with Aminex carbohydrate HP-87C column with water deionized as the mobile phase at 0.5 mL/min(Mervat G. Hassan et al., 2020).

Isolated organisms were used for production of EPS. The bacterial isolates were maintained in TSA slants. Production was carried out in 250ml conical flasks having 50ml TSA broth. Media were sterilized at 121 0C for 15mins. After cooling of the media, the loopful of organism was inoculated. The flasks were incubated at rotary shaker at room temperature for 72h. After incubation, cells were harvested by centrifugation for 20min at 10000rpm. After centrifugation, ice-cold isopropanol (10ml) was added and stored at 4 0C. The precipitated material was collected and centrifuged for 20min at 10,000rpm. Pellets were dried at 100 0C and weight the dried EPS. (Das M et al.,2020).

The biocompatibility and functional properties of EPSs are important factors that promote their use in various biomedical applications, such as scaffolds, drug delivery systems, coating materials for medical devices, and surgical sealants. An EPS can be used in its native structure, cross-linked, or tailored with various bioactive materials. (Masrina Mohd Nadzir et al., 2021).

MATERIALS AND METHODS

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Sample collection:

The marine water sample for the present study was collected from the deepest part of the Thoothukudi ocean and brought to the lab .

Isolation of marine bacterial cultures:

Isolation of bacteria from the marine water was done by pour plate technique.1ml of marine water is added to 99ml of distilled water and then sample was serially diluted upto 10^{-6} dilution. The dilution were plated on sterile petri plates containing Zobell marine agar and plates were incubated at 30°c for 24 hours. Morphologically different colonies with mucoid surface were picked and sub culture for future use.

Morphological, Colonial and Biochemical characterisation of bacterial Isolate:

Simple Staining:

The bacterial culture was smeared with a simple stain. A thin smear of pure isolate colony was made on a clean glass slide dried in air and fixed by passing through flame of a burner. Commonly used basic dyes were added such as methylene blue, and then examined under the compound microscope.

Gram staining:

Isolated bacterial strain were identified by performing gram staining. A thin smear of pure isolate colony was made on the glass slide, dried in air and fixed by passing through flame of burner. The smear was covered with crystal violet kept for one minute. The slide was again washed with water, and covered with gram iodine stand for one minute .The slide was again washed with water, Decolourized with alcohol ,was achieved by rocking the slide gently for twenty second till the violet colour comes of the slide and then washed with water immediately .This counterstained with safranin for twenty second . Washed with water, blot dried and then examined under the microscope.

Motility test:

The isolated cultures was tested for motility by hanging drop method and the result was observed.

Indole Test:

Peptone water medium was prepared in the test tube and the culture was inoculated into the medium, and the tubes were incubated at 37°c for 24 hours, after incubation Kovac's reagent was added and the result was observed.

Methyl Red Test:

MR-VP broth medium was prepared in the test tubes and the culture was incubated at 37 °c for 24 hours, after incubation few drops of methyl red indicator was added and the result was observed .

Voges Proskauer Test:

MR -VP broth medium was prepared in the test tubes and the culture was inoculated into medium, and the tubes were incubated at 37 °c for 24 hours, after incubation 0.5ml of alpha naphthol ,0.2ml of KOH were added and the result was observed .

Citrate Test:

Simmon citrate Agar was prepared and the culture was inoculated as a single streak on the agar slant surface, and the tubes were incubated at 37 °c for 24 - 48 hours, after incubation the result was observed .

Starch Hydrolysis:

Starch agar plates were prepared and the culture was single streaked on the surface and the plates were incubated at 37 °c for 24 hours and the result was observed .

Urease Test:

Christensen's urea agar slants were prepared and culture was inoculated on the medium as zigzag streak, and the plates were incubated at 37 °c for 18 hours and the result was observed.

Casein Test:

Skim milk medium or casein medium, was prepared and organism was isolated into the plate as single line streak, then plates were incubated at 37 °c for 24 hours and then the result was observed .

Catalase Test:

The catalase test was performed by slide method, on a slide, few drops of hydrogen peroxide were placed to which the culture was added and the result was observed.

Growth on selective media:

After biochemical confirmation the suspected mucoid coloniès were plated on selective media like Zobell Marine agar.

Confirmation of EPS producing marine Bacteria:

EPS producing distinct bacterial isolates obtained from the above selective media was further confirmed by the following methods.

Congo Red Agar Plate Assay :

Screening for EPSs production was carried out by using Congo red agar plate assay. Isolates were cultivated on YMG at 30° c for 24 hours having Congo red (with 250µg /L dye concentration). Dye solution was sterilized and added to the medium after autoclaving. This dye is more sensitive and increases the mucoidness of the colonies and the result was observed.

String test :

Mucoid colonies have a glistening and slimy appearance on agar plates were the primary criteria for selection of bacteria The colonies when extended with wire loop form a long filament, EPSs production was confirmed visually or through the string test (Fang et al., 2004) by formation of a string (>5 mm) upon lifting of the loop indicates positive result.

Extraction of EPS:

The isolate obtained as above was quantified for EPSs production using method as mentioned below.

Precipitation:

50ml of YMG medium was dispensed in 250 ml Erlenmeyer flask and sterilized at 121^{0}C for 15 minutes. After sterilization, the medium was inoculated with 1ml of inoculum. (OD-1.0 at A600 SHIMADZU UV-Spectrophotometer) and incubated on an environmental shaker. (Thermo-Scientific MaxQ) at 30 ^{0}C for 24 hours. After incubation, the broth culture was inactivated at 100 ^{0}C for 20 min. The cells were harvested by centrifugation at 10,000 rpm for 20 min and the supernatant was subjected for deproteination using sevag method. The polysaccharides were mixed with sevag reagent, 5:1 (V:V) $CHCL_3$: n-BuOH and stirred for 30 mins. Then, the mixture was centrifuged and the

upper polysaccharide solution was collected. The polysaccharide solution was further deproteinized with Sevag reagent for 5 times until the absence of white layer between polysaccharide solutions, ensuring that the polysaccharide was free from proteins. After removal of the Sevag reagent, the resulting polysaccharide solution was precipitated by adding double volume of chilled ethanol and the final mixture was kept at 4^0C overnight. After incubation, the precipitates were collected by centrifugation at 10,000 rpm for 20 min and the precipitated EPSs were quantified by phenol sulphuric acid assay after drying for three days at 80 ^{0}C.

Quantification of EPs by Phenol - sulphuric acid method:

The carbohydrate content of EPS was measured using a modified phenol-sulfuric acid method and glucose standards (Dubois et al., 1951). Briefly, the lyophilized EPS was suspended in Milli-Q water at a concentration of 1 mg/ml. We mixed 1 ml of this suspension (and glucose standards separately) with 0.5 ml of phenol, followed by the addition of 2.5 ml of concentrated sulfuric acid. The solution was mixed and allowed to cool at room temperature for 1 h. The absorbance was measured at OD485 using a spectrophotometer.

The total amount of Extracted EPS was quantified using following formula.

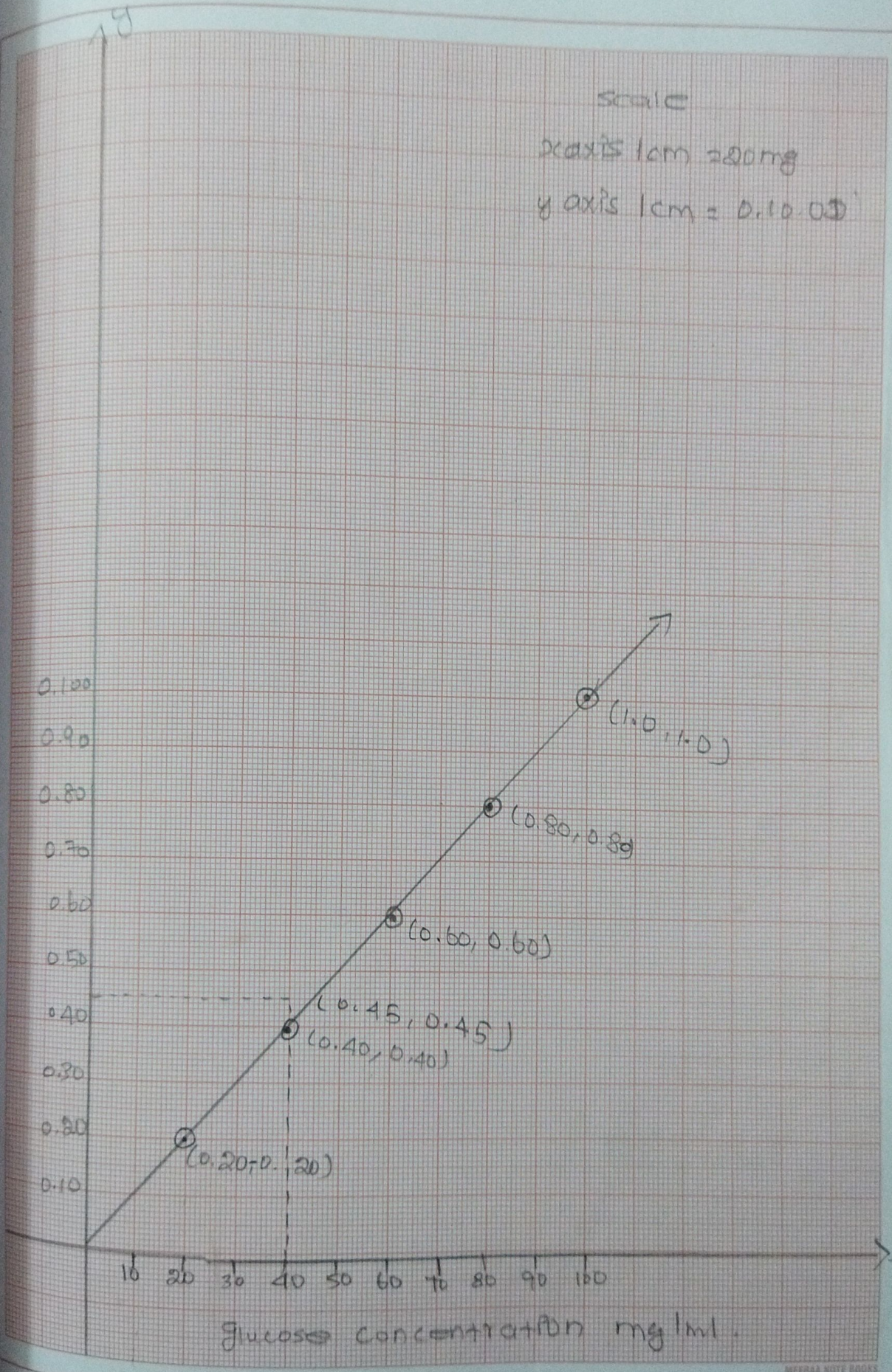
Calculation:

Absorbance corresponds to 0.1mL of the test = 'x' mg of glucose

100mL of the sample solution contains = ('x' ÷ 0.1) × 100mg of glucose = % of total carbohydrate present

Characterisation of EPS by FTIR:

FTIR is widely used for the partial characterization of polysaccharides. The molecular structure and important functional group of EPS from the above isolated bacterium was analysed using FT-IR system and the characteristic absorption bands were assigned. The EPS was dispersed on the universal attenuated total reflectance (UATR). The IR spectra (50 scans) were recorded at room temperature (referenced against air) with the wave number range of 450–4000 cm^{-1}. Spectra were analysed with spectrum ES software



Glucose concentration.

RESULT

RESULT

Isolation by using serial dilution technique:

The isolation of Exopolysaccharide producing bacteria from sea water sample was performed with serial dilution technique and the number of colonies for each dilution was calculated and then selective organisms were maintained for further identification and the results were noted. (Fig 3 and Table 1)

Identification of Exopolysaccharide producing bacteria:

The isolated colonies were identified with microscopic observation, cultural characteristics and Biochemical tests, the suspected colonies were streaked on the respective selective media and the results are tabulated. (Fig 6 and table 2 &3)

Growth on selective media:

After biochemical confirmation by standard biochemical tests, the confirmed Halophile sps was developed on Bacillus Zobell Marine medium. (Fig 7).

Confirmation of EPS producing marine Bacteria:

a) Congo red plate assay

In order to confirm the production of EPs from the selected isolate Congo red agar plate assay was done. Isolates were cultivated on YMG at 30°c for 24 hours having Congo red (with 250µg /L dye concentration).Mucoidness of colonies was observed and the result was noted (Fig : 8)

b) String test

Mucoid colonies have a glistening and slimy appearance on agar plates was the primary criteria for selection of bacteria, the colonies when extended with wire loop formed a long filament that was confirmed visually and the result was noted (Fig 9)

Precipitation of EPS

In order to obtain the exopolysaccharide released in the medium, the bacterial isolate was kept in YMG medium in the shaker environment for 3 days at 25°c, and then the crude broth culture was centrifuged and the supernatant was subjected for deproteination using sevag method and the result was noted

Quantification of EPS by Phenol - sulphuric acid method:

The total amount of Exopolysaccharide from the isolated *Halophile* sps was quantified by phenol – sulphuric acid method using glucose as standard and the results was noted. (Fig 12)

FTIR - Composition of Exopolysaccharide:

Fourier transform infrared was obtained by grinding a mixture of EPS sample with dry KBr and pressing in a mold. An IR spectrum was recorded on a Fourier transform infrared spectrophotometer Brucker Scientific 500- IR (Ray 2006) and the result was noted. (Fig 11)

RESULT TABLES

RESULT TABLES

Table 1: SERIAL DILUTION METHOD:

S.NO	DILUTION FACTOR	COLONIES
1.	10^{-1}	260
2.	10^{-2}	200
3.	10^{-3}	180
4.	10^{-4}	129
5.	10^{-5}	97
6.	10^{-6}	64

CALCULATION:

For Dilution 10^{-4}:

No. of. Cells present = $\frac{\text{No. of. Colonies}}{\text{Volume of sample}}$ x DF in sample

$$= \frac{129}{1} \times 10^{-4} = \frac{129}{1000}$$

$$= 1.29 \times 10^{-4} \text{ CFU/ML}$$

For Dilution 10^{-5}

No. of. Cells present = No. of. Colonies x DF in sample

Volume of sample

$= \frac{97}{1} \times 10^{-5} = \frac{97}{10000}$

$= 9.7 \times 10^{-5}$ CFU/ML

Table 2: MICROSCOPIC EXAMINATION

STAINING	RESULT
Simple staining	Rod
Gram Staining	Positive
Motilily	Motile

Table 3 : BIOCHEMICAL TEST

TEST NAME	RESULT
Indole	Positive
Methyl Red	Positive
Voges Proskaeur	Negative
Citrate	Positive
Starch Hydrolysis	Positive
Gelatin Hydrolysis	Positive
Urease	Positive
Catalase	Positive

Table 4: Glucose estimation (Phenol – Sulphuric acid method)

S.No	Working standard concentration in mg / ml	Sample concentration in mg / ml
1.	0.20	0.45
2	0.40	
3	0.60	
4	0.80	
5	1.00	

IMAGES

IMAGES

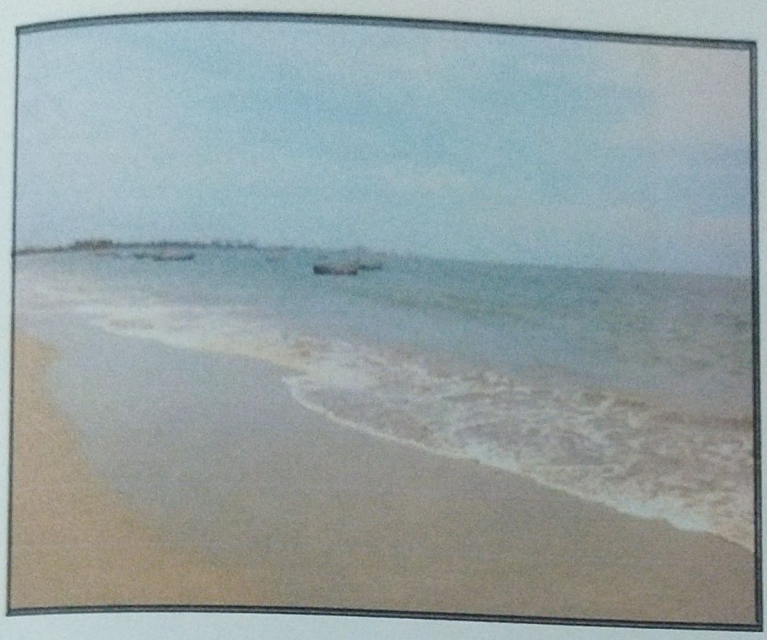


Fig 1: Sea water sample collection site

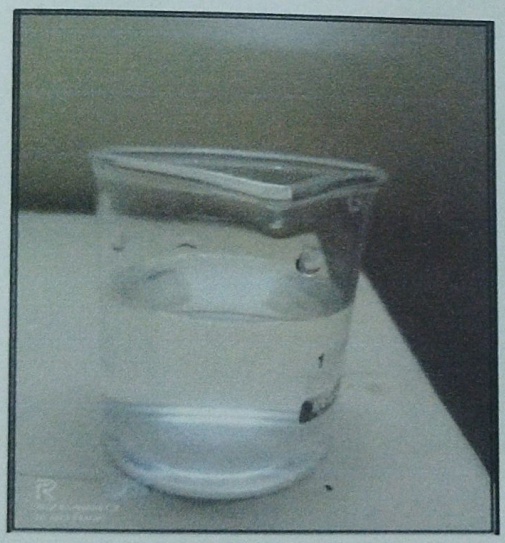


Fig 2: Marine water sample

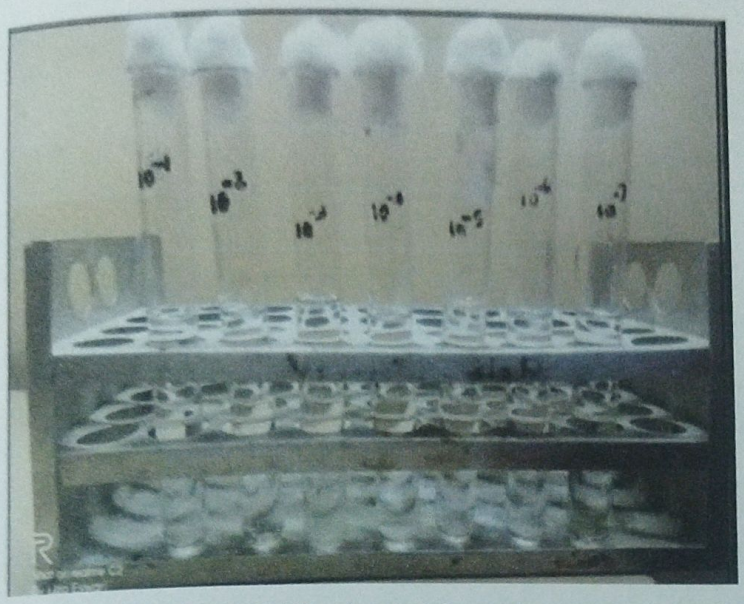
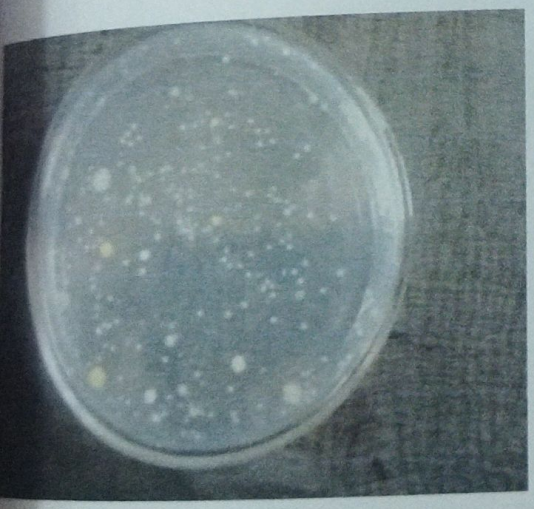


Fig 3 : Serial dilution with maine water sample.



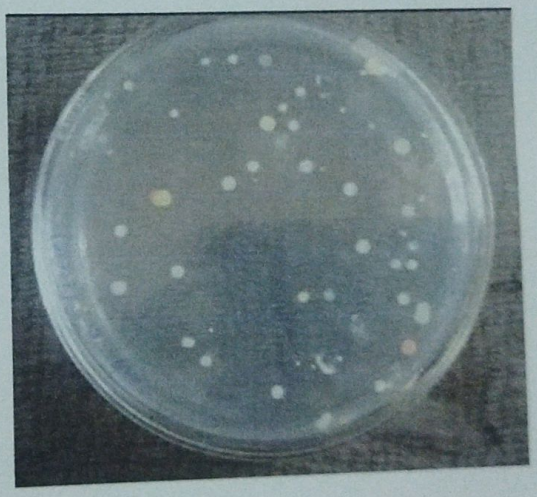
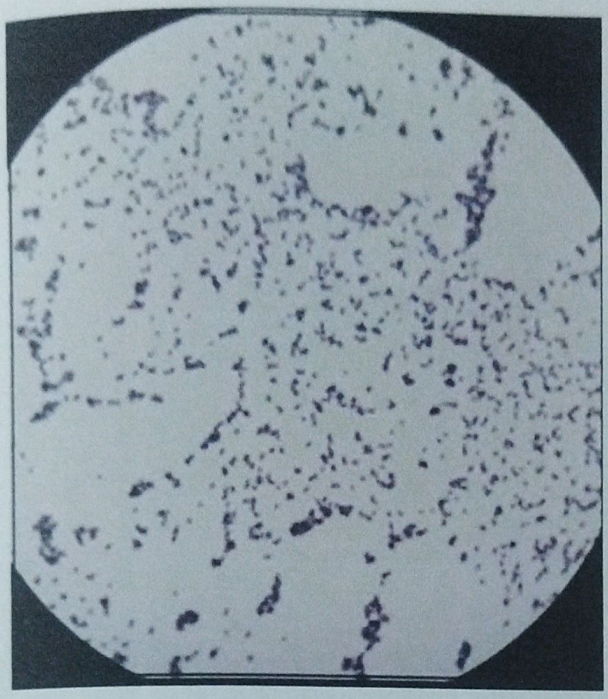
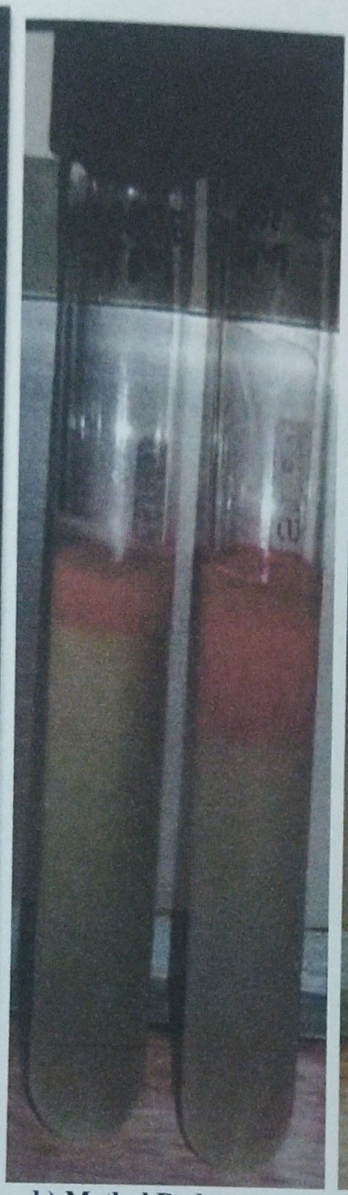


Fig 4:Serial dilution plates on marine agar media (10^{-4} & 10^{-5})

Fig 5: Microscopic examination- Simple and Gram staining



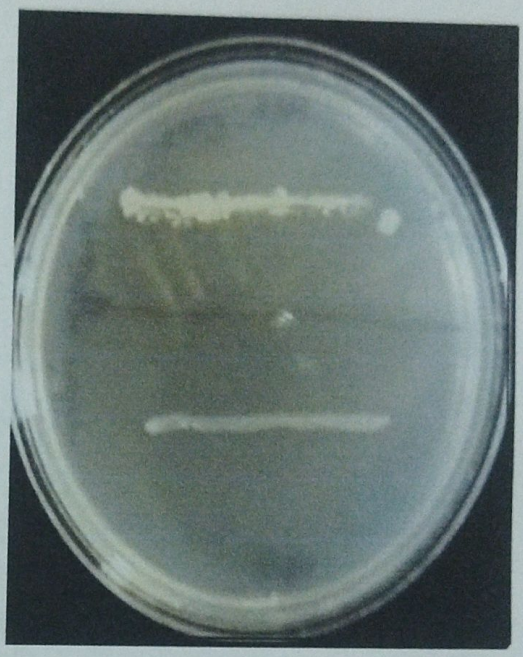
a) Indole test



b) Methyl Red test

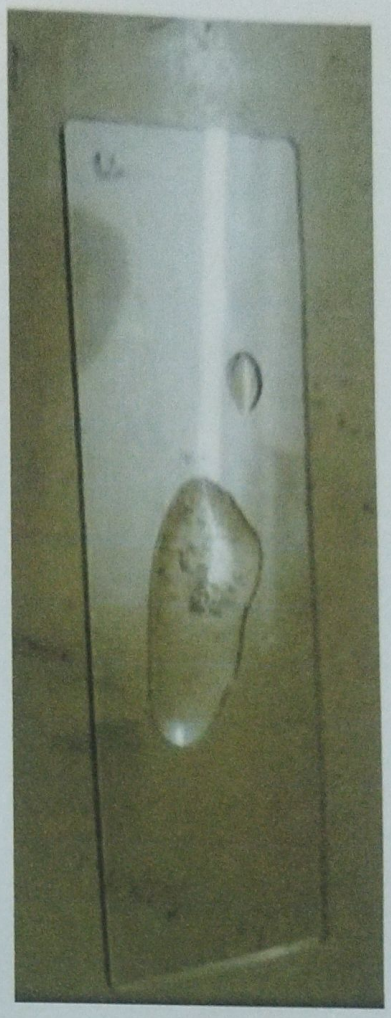
c) VP - test

d) Citrate test



e) Starch hydrolysis

F) Gelatin Hydrolysis



G) Catalase test

Fig 6: Biochemical test

Fig 7 : Growth of Halophilic bacteria on Zobell Marine Agar medium

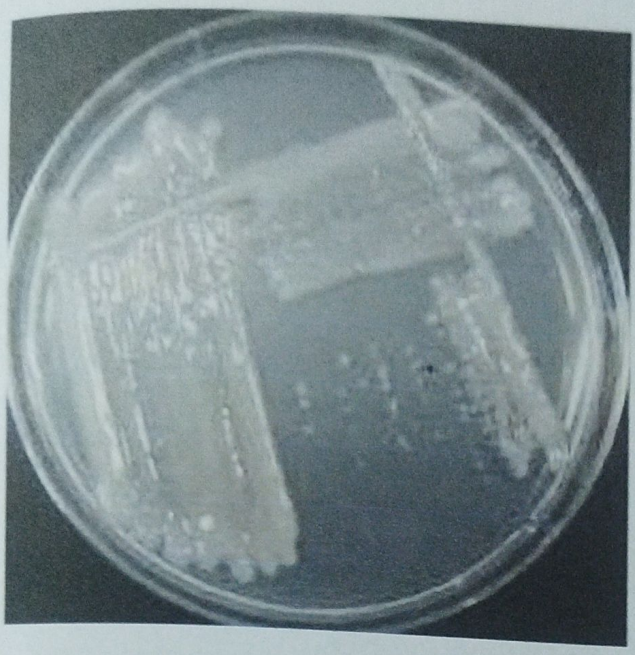


Fig 8: string test

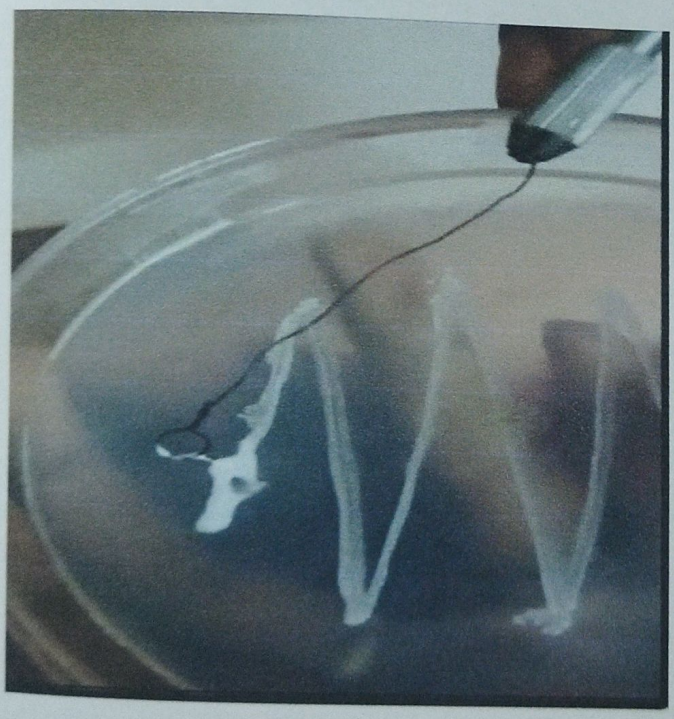


Fig 9 : Congo red

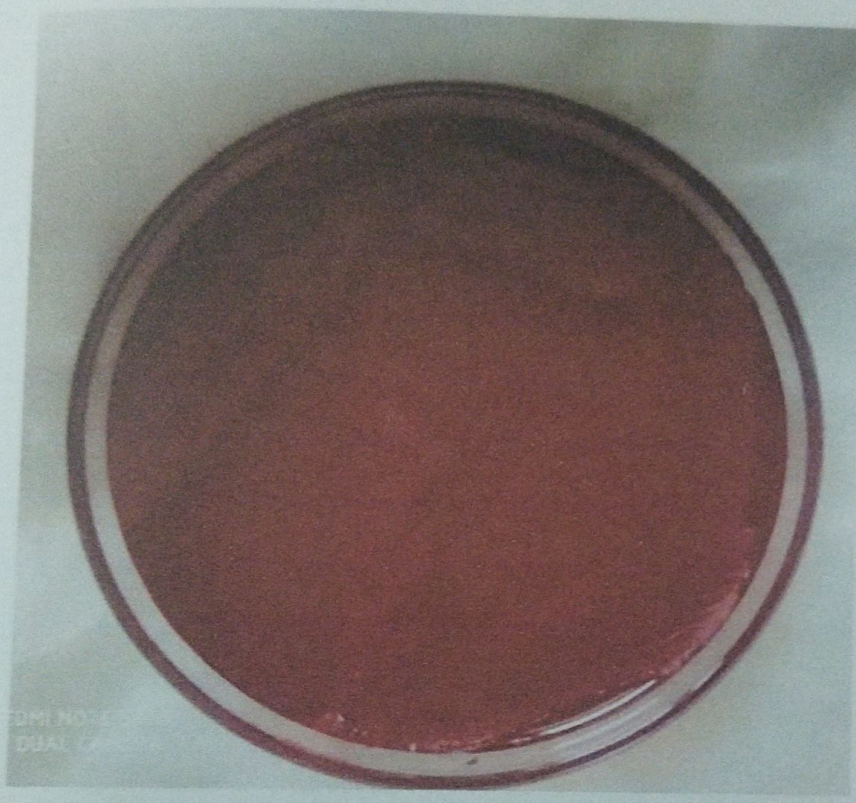


Fig 10:Harvesting EPS producing culture in the shaker in YMG medium

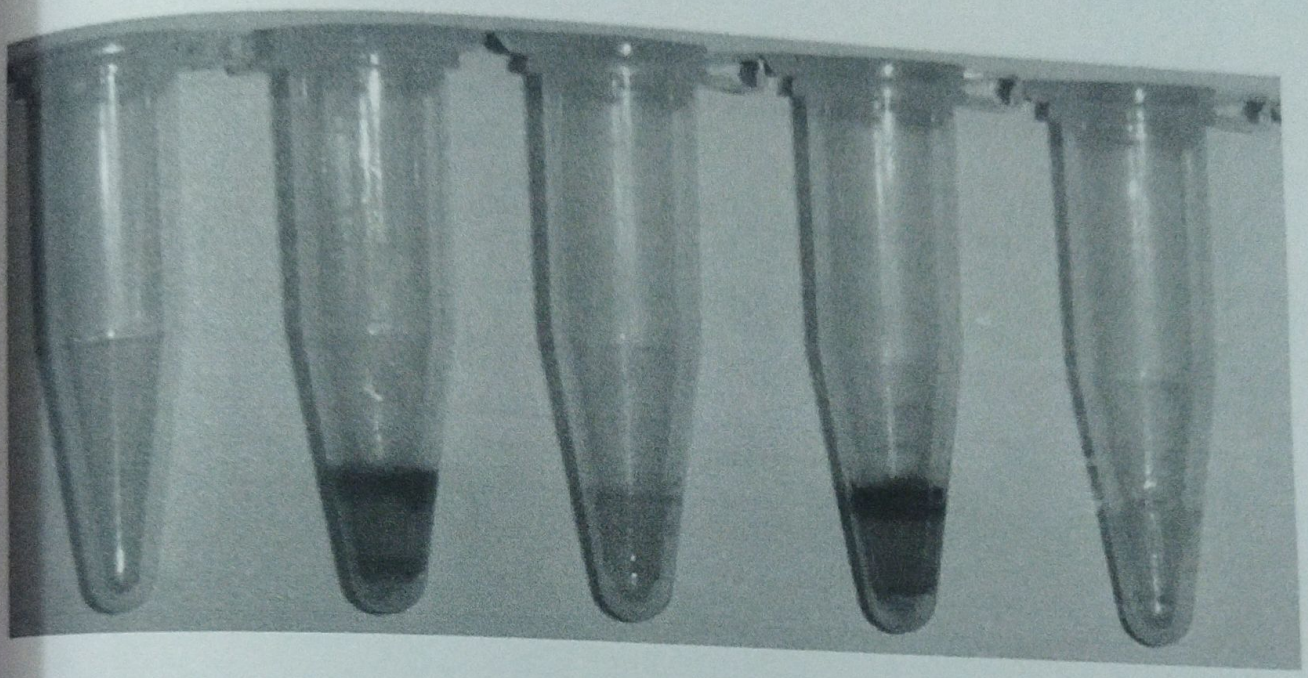


Fig 11:Recovery of EPS after treating with sevag reagent

Glucose standard & Sample

Fig 12: Quantification of Glucose (Exopolysaccharide) by Phenol sulphuric acid method

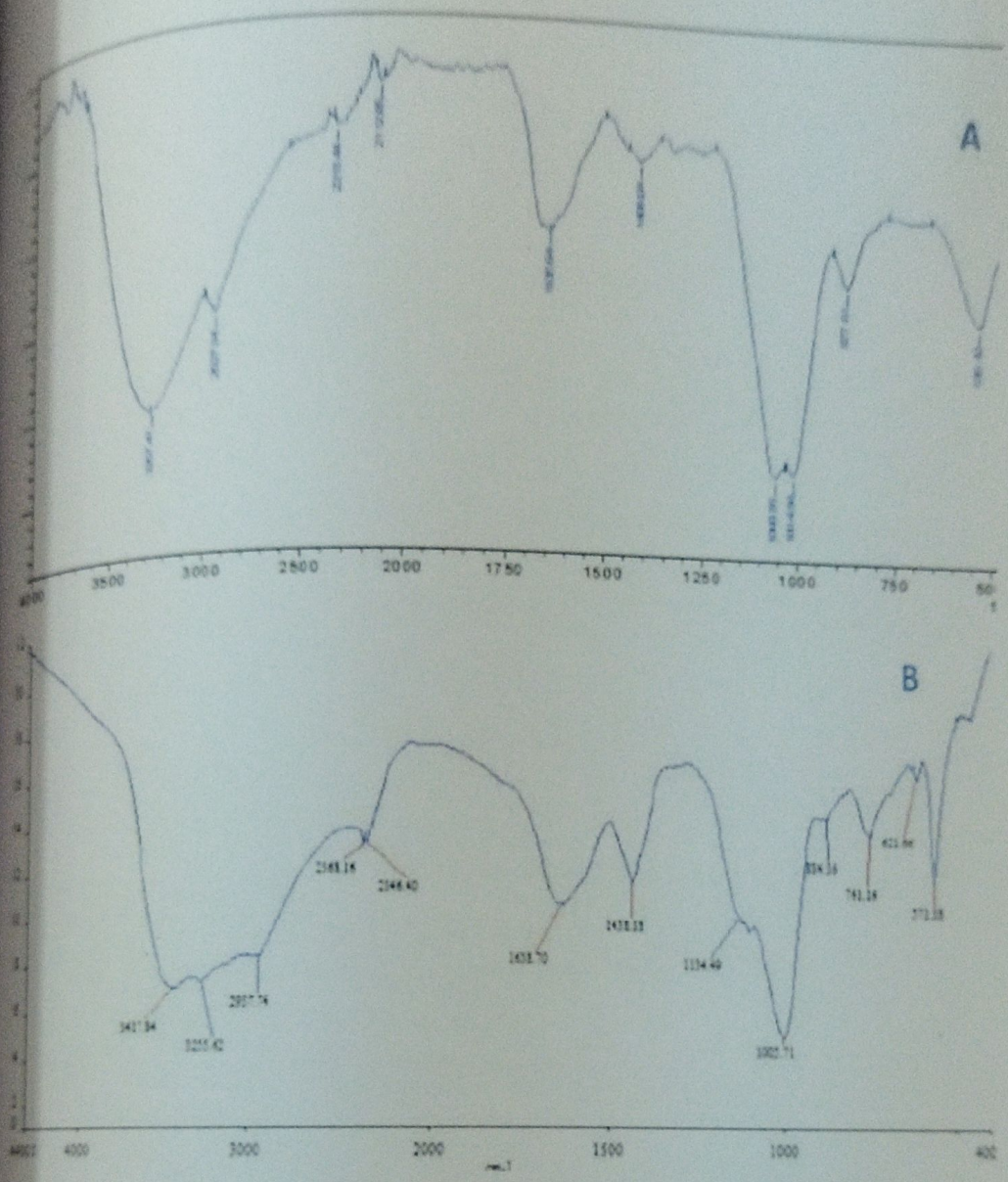


Fig 13:FTIR Analysis of Exopolysaccharide from halophilic bacteria

DISCUSSION

DISCUSSION

The results of experiments conducted in connection with isolation and characterization of exopolysaccharide from marine are discussed as follows.

The total number of colonies isolated from the serial dilution method are given in (Table 1 and Fig 3 and 4) and it reveals that the number of colonies in each plate decreases as the dilution factor increases and (10^{-4} and 10^{-5}) dilution plates were selected for further study. Then microbial identification of colonies were performed based on Bergeys's manual, and from the results of microscopic examination, Biochemical characterization and cultural characteristics on growth medium, it is confirmed that the isolated colony is identified as *Halophilic sps* (Fig 5,6 and table 2 &3) Exopolysaccharides can be also identified by bacteria phenotypes, both in liquid and solid media. Usually, a "mucoid" strain indicates that the microorganism is able to produce exopolymeric substances but only the determination of carbohydrates content can ensure the presence of exopolysaccharides (Arundhati Paul et.al 2013) The strain CC30 grown in 11% skimmed milk produced a viscous and ropy curd. EPS was extracted from the ropy curd and dialyzed.(K.Jayanth et.al.,2001)

Some previous studies have purified the EPSs from extreme halophilic archaea belonging to *Haloferax, Haloarcula,Halococcus, Natronococcus, Halobacterium*, and *Haloterrigena* genera (Antón et al. 1988; Paramonov et al.1998; Nicolaus et al. 1999; Squillaci et al. 2016; Lu et al.,2017), and no haloarcheon related to *Halorubrum* genus has been reported as an EPS producer.

Mucoid colonies have a glistening and slimy appearance on agar plates formed long filament when extended with wire loop >2mm which confirmed the exopolysaccharide production .Mucoid colonies have a glistening and slimy appearance on agar plates were the primary criteria for selection of bacteria (Fusconi and Godinho, 2002). The colonies when extended with wire loop form a long filament (Vescovo *et al.*, 1989). Exopolysaccharide production was confirmed visually or through the string test (Fang *et al.*, 2004) by formation of a string (>5 mm) upon lifting of the loop indicates positive result.

The isolated culture formed black, smooth, humid with mucoid type colony onto Zobell marine agar medium supplement with 0.08% Congo red which confirms the production of exopolysaccharide. The isolates were streaked onto Zobell marine agar medium supplement with 0.08% Congo red. The positive result determined by black, smooth, humid with mucoid type colony (Freeman *et al.*, 1989).

Most thermophilic bacteria often produce massive quantities of EPS like *Bacillus licheniformis, Geobacillus thermodenitrificans,and* and *Bacillus thermantarcticus*, isolated from shallow warm sea winds and Thermotoga maritima's co-cultures and Methanococcus jannaschii H2 consuming methanogen, found to produce large biofilms. At last, Geobacillus tepidamans V264 isolated from the earth's hot spring, which can create an unusually heat-resistant EPS that begins to degrade at around 280°C (P.Ramya et .al 2020) A total of 14 morphologically different marine bacterial cultures were isolated and screening was done for the efficiency to produce extracellular polysaccharides. Out of 14, only four bacterial isolates were produced EPS. Further the four bacterial isolates were analyzed to check the polysaccharide content. The marine isolate PMSS12 exhibited maximum yield of exopolysaccharides (4.3 g/l). Selim *et al.*, 2018 reported that 18 isolates were isolated from the Ageeba beach sediment (MarsaMatrooh Governorate) and 9 isolates only produced EPS.

In this study, quantification of total carbohydrate content present in precipitated EPS by phenol sulphuric acid assay was employed for EPS producer from sea water and it was found to be 0.45mg / ml.(Fig 12) The five isolates designated as WAS1, WAS11, SC6, SOS7 and SOS10 produced copious amount of EPS of above 1.0 g/l and confirmed them to be the most potent producers of EPSs. (Ifnia et al.2019) The purified fractions of the EPS after ethanol precipitation produced 1.95 g/L of EPS and showed the negligible amount of protein and nucleic acid. Purified EPS was then subjected to morphological, structural, and physicochemical characterization to explore its potential applications. (Sri Lakshmi Ramya Krishna et al 2013)

Fourier-transform-infrared spectroscopy (FT-IR):

The spectrum of the purified EPSs produced by halophilic organism *was* determined (Figure 11). The IR-spectra showed absorption bands and peaks characteristic of carbohydrate, carboxylate, and hydroxyl groups as well as urinate, amine and C-O-C ester linkages. The spectrum of extracted EPSs revealed that a dominant absorption that is often attributed to O-H stretching vibration at 3848.26 cm-1 of O-H in carboxylic acid which is accompanied with the bands at 2932.23 cm-1, known to be typical carbohydrates which corresponds to C-H asymmetric stretching vibration in carboxylic group. The band at 2342.34 cm-1 approves the bending vibration of P-H phosphine group. An asymmetrical stretching peak observed at 1641.13 cm-1 is characteristic of C=C medium stretching vibration in conjugated alkene. The band observed at 1418 cm-1, indicated the presence of urinate. The spectrum also displayed

a minor peak at 1379.82 cm-1, indicating the presence of carboxylate in the polymer. The peak at 1238.82 cm-1 identifies the medium vibration stretching of amine C-N in amine group. In addition, the absorption band at 1062.59 cm-1 indicated asymmetrical stretching vibration of a C-O-C ester linkage. The small absorption band at 877.45 cm-1 could be associated with glycosidic linkages between the sugar monomers. The absorption peaks around 1000-1100 cm-1 are known to be characteristic for all sugar derivatives.

A broad stretching at 3448 cm−1 represents the stretching vibration of hydroxyl groups and is characteristic of a carbohydrate ring. The main absorption band at 927 cm−1 indicates the vibrations of the glycoside link C-O-C .In the fingerprint region (the region below 1500 cm−1), small peaks indicate the presence of sulfated groups and/or that the substance is polysaccharide The band at 836 cm−1 is characteristic of α-D glucan. The absence of characteristic absorption peak around the region of 1700–1770 cm−1 suggests that neither glucuronic acid nor diacetyl ester is present in the EPS The presence of carboxyl groups in the FTIR spectra of the polymer indicates that they may serve as the binding site for divalent cations.(Masouid Hamidi et.al 2019).

Based on the above result, it is clearly understood that the halophilic bacteria isolated from the sea water has the ability to produce exopolysaccharides under normal cultural conditions. Further the extracted EPS could be subjected to various structural and molecular analysis with advanced techniques. As the EPS has more applications in pharmaceutical, Nutraceutical, Textile and medicine industries. Hence, it is recommended to screen more EPS producer from various extremophilic region to understand its diversity and utilize as a substitute for human high value product.

SUMMARY

SUMMARY

In the present research an attempt was made to isolate the Exopolysaccharide producing marine bacteria from the Thoothukudi ocean water sample collected 7 – 8 Km distance from the sea shore. In order to isolate the EPS producing bacterium, the serial dilution technique was done and the plate was prepared on Zobell Marine agar medium and essential number of colonies were seen on the respective dilutions. (Fig 3,4 and Table 1). Then microbial identification of colonies were performed based on Bergeys's manual, and from the results of microscopic examination, Biochemical characterization and cultural characteristics on growth medium, it is confirmed that the isolated colony is identified as *Halophilic sps* (Fig 5,6 and table 2 &3).Then the production of EPS producing organism was confirmed through string test and Congo red plate assay. Then the isolated organism was subjected to release exopolymers in the medium by keeping it in the shaker for 3 days . Next the extracted EPS was quantified with Phenol – Sulphuric acid assay and the amount was calculated as 0.45mg / ml and the graphic representation also made. Finally, the extracted EPS was characterized by FTIR – analysis and found to have various peaks at different ranges confirming the presence of different groups.(Fig 13) Hence it is finally concluded that *Halophilic* sps isolated from the sea water sample could be further analyzed till molecular level and more details of its chemical structure such as the glycosyl linkages, the main repetitive units, and the putative branching sugars as well as its other biological activities are warranted. Thus, these EPSs could apply a gainful activity in the nourishment industries, cosmetic, agricultural and medicine field.

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ISOLATION OF HYDROCARBON DEGRADING MICROORGANISMS AND ASSAY OF ITS BIODEGRADING ABILITY

A PROJECT SUBMITTED BY

ST.MARY'S COLLEGE (AUTONOMOUS), THOOTHUKUDI

Affiliated by Manonmaniam Sundaranar University,

In partial fulfillment of the requirements for the award of the degree of

BACHELOR OF SCIENCE IN MICROBIOLOGY

SUBMITTED BY

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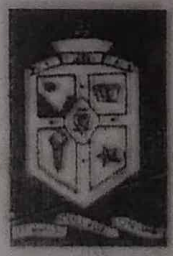

DEPARTMENT OF MICROBIOLOGY

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CERTIFICATE

This is to certify that the project work entitled **"Isolation of hydrocarbon degrading microorganisms and assay of its biodegrading ability"** submitted to **St Mary's College (Autonomous), Thoothukudi** affiliated to **Manonmaniam Sundaranar University, Tirunelveli** for the partial fulfillment for the award of Bachelor of Science in Microbiology is a bonafide research carried out by **E. Muthu Priya (19SUMB19), S.Nandhini (19SUMB20), R. Nidharshana (19SUMB21), S.Niranjani (19SUMB22), R.Nivetha (19SUMB23), A. Pavithra (19SUMB24), A.P. Pavya Askila (19SUMB25)** under the guidance and supervision of **Ms. A. Maria Heartina Adlin Vaz,** Assistant Professor, Department of Microbiology, St Mary's College (Autonomous). Thoothukudi, for academic year 2021 - 2022.

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SIGNATURE OF THE EXAMINER

PLACE: THOOTHUKUDI

DATE: 25.05.2022

DECLARATION

I hereby declare that the project work entitled **"Isolation of hydrocarbon degrading microorganisms and assay of its biodegrading ability"** is a bonafide record of the work completed by me during the academic year 2019-2022 in St. Mary's College (Autonomous), Thoothukudi and submitted as a partial fulfilment of requirements for the award of the Degree of Bachelor of Science in Microbiology prescribed by the Manonmaniam Sundaranar University. We also affirm that this is a original work done by me under the supervision of **Ms. A. Maria Heartina Adlin Vaz,** Assistant Professor of Department of Microbiology, St. Mary's College (Autonomous), Thoothukudi.

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A. P. Pavya Askila

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PLACE : THOOTHUKUDI

DATE : 25.05.2022

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ABBREVIATIONS

%	Percentage
µg	Microgram
µl	Microliter
cm	Centimeter
g	gram
Hr	Hours
Kg	Kilogram
L	litre
m	meter
mcg	microgram
Min	Minutes
ml	Milliliter
mm	Millimeter
˚C	Degree Celsius
PDA	Potato Dextrose Agar
sp	Species
Vol.	Volume

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INTRODUCTION:

POLLUTION:

The world in which we live as it is today, is the world in which everything we do as regards human growth, biological, physical, economic, industrial and infrastructural growth, science and technological growth etc. revolves around energy. Apart from the traditional firewood, wind, and hydropower, petroleum hydrocarbon continues to be used as the most principal and versatile source of energy. Environmental pollution with petroleum and petroleum products has been recognized as one of the most serious current problems especially when associated with accidental spills on large scale (Butier and mason 1997). Petroleum oil is complex mixture of many thousands of compounds. Petroleum hydrocarbons are the major constituents of crude oil (50-98%) and alkenes represent 20-50% of oil depending on the source of the oil. Crude-oils are mainly short-chain hydrocarbons, it is composed of complex mixtures of paraffinic, alicyclic and aromatic hydrocarbons and a smaller proportion of nonhydrocarbon compounds such as naphthenic acids, phenols, Thiol, heterocyclic nitrogen, Sulphur compounds as well as Metallo-prophyrins and asphaltenes. Crude oil as a complex Mixture is produced by incomplete decomposition of plant and animal biomass over a long time. The carbon content normally is in the range 83-87%, and the hydrogen content varies between 10 and 14%. In addition, varying small amounts of nitrogen, oxygen, sulfur and metals (Ni and V) are found in Crude oils. Petroleum based products are the major source of energy for industry and daily life. Leaks and accidental spills occur regularly during the exploration, production, refining, transport, and storage of petroleum and petroleum products. The amount of crude oil seepage was estimated to be 600,000 metric tons per year with a range of uncertainty of 200,000 metric tons per year. Polycyclic Aromatic Hydrocarbons are important pollutants which are introduced into the environment through different ways such as anthropogenic activities, combustion, undesirable discharging of oil tankers, spills around the petroleum refineries and gas plant facilities. Hydrocarbons are considered to be of biological origin since short and long chains hydrocarbons (alkanes; C10 – C20; C20 – C40) appear to be exclusively the origin of biological processes. These compounds have toxic, carcinogenic, and mutagenic properties and considered as a serious hazard to human health and environment. there has been extensive evidence on microbial degradation of petroleum hydrocarbons. since the biodegradation of petroleum hydrocarbons is a natural process controlled by temperature, pH,

and scarcity of nutrients such as N and P, bioremediation is a viable and promising method for clean-up and remedy of hydrocarbon polluted environment.

The harm that oil pollution causes to the ecological environment is well known (Sikkema et al., 1995) for example the deep water horizon oil spill accident in the Gulf of Mexico produced a profound impact on the economy and environmental safety, which is still the focus of people's attention. (Xue et al., 2015). Although people are becoming increasingly aware and concerned about the toxic effects of soil pollution on humans and animals affected areas, (Diez et al., 2007; Mason et al., 2012) the strong toxic impacts of petroleum hydrocarbons on affected microbial communities are often overlooked. In diesel exposure experiments the researchers found that the primary effects of diesel fuel toxicity were reductions in species richness, evenness and phylogenetic diversity, with resulting community heavily dominated by a few species, principally *Pseudomonas*. Several surveys have also reported that metabolic intermediates with relatively high solubility produced from the degradation of petroleum hydrocarbons by bacteria may have higher cytotoxicity than the parent molecules and therefore damage the bacteria (Houet al., 2018). However, indigenous bacteria form very large aggregates, and each species has its own function. Accordingly, while some bacteria that are sensitive to petroleum hydrocarbons are greatly inhibited upon exposure to petroleum hydrocarbons, others that can efficiently degrade petroleum hydrocarbons, as well as bacteria that can take advantage of cytotoxic intermediate metabolites, will flourish. However, clean-up of petroleum oil pollutants by relying on the strength of these indigenous microorganisms alone will take a long time; therefore, it is necessary to develop intervention measures to speed the process up.

REMEDY:

In terms of managing soil pollution there are two types of strategy. the first, which at the beginning of the 21st century is the preferred option for many regulatory agencies, has the remediation goal expressed as a maximum allowable total concentration of pollutant in the soil. To comply with this approach the total concentrations of pollutant in soil must be lowered and this can be achieved using the group of technologies termed as 'clean up' technologies. The second strategy does not require a reduction in the total concentrations of a pollutant in soil, but aims to manage exposure to the pollutant, and hence environmental and health risks through technologies that lower pollutant mobility and bioavailability. these are termed as 'Contaminant' technologies

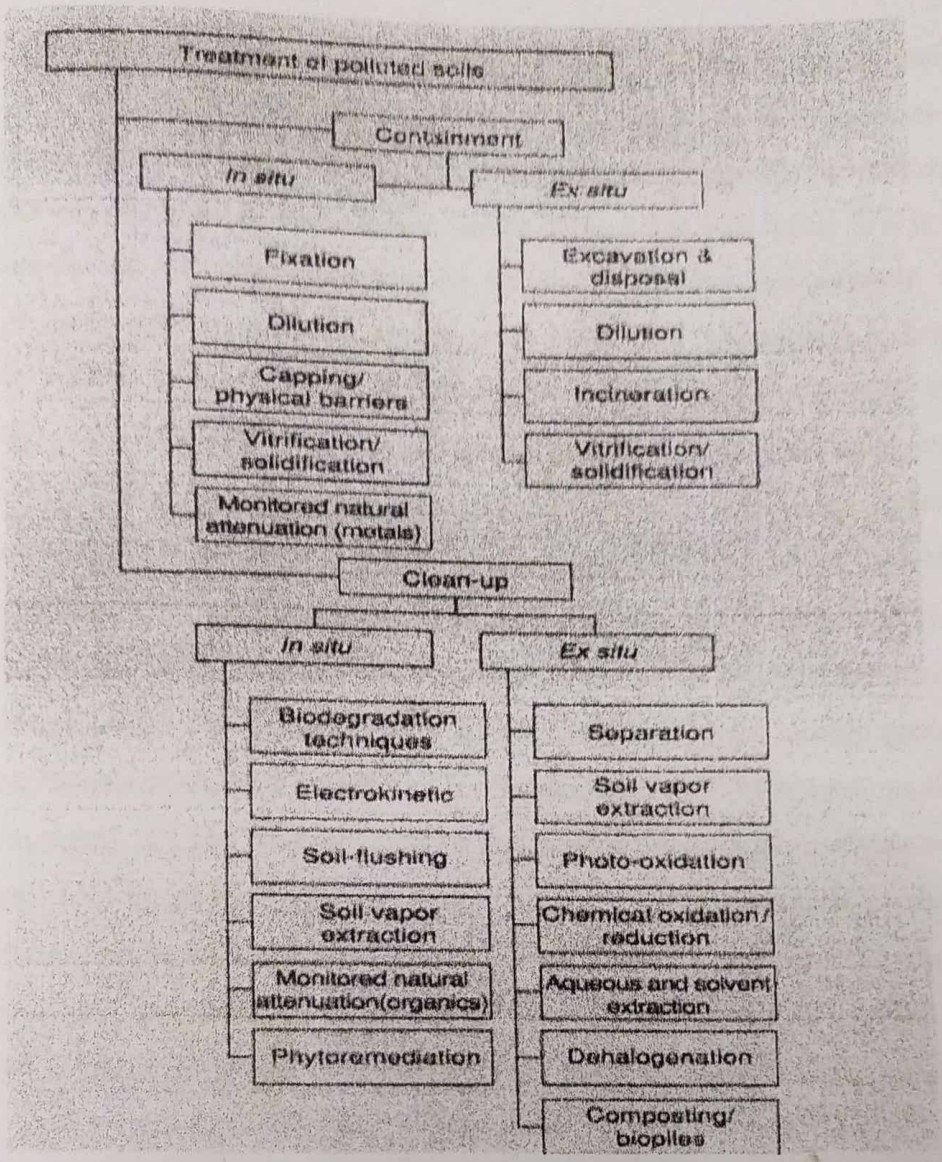


Fig 1.1 : REMEDY OF POLLUTED SOILS

Physical and chemical methods for treatment of these pollutants are not only expensive because of high costs of chemicals and equipment, but also generate excessive amounts of sludge which further pollutes the environment. As a better alternative, biological methods are preferred for their simple, cheap and environmentally friendly operations. In biological means, Microorganisms play a vital role over other bioremediation agents because of advantages such as rapid growth with minimum growth requirements.

BIOREMEDIATION:

Bioremediation is a complex process with biological degradation taking place in the cells of microorganisms which absorb pollutants, where if they have specific enzymes, the degradation of pollutants and their corresponding metabolites will take place. Bioremediation is the best technique to completely remove PAH's Polycyclic aromatic hydrocarbons from

the environment or convert them to less harmful compounds. This process uses living organisms to degrade or detoxify hazardous wastes into harmless substances such as carbon di oxide, water and cell biomass. It relatively uses low cost, low technology techniques which have a high public acceptance. Bioremediation can be divided into two types: first is Natural attenuation, which can be applied when the natural conditions are suitable for the performance of bioremediation without human intervention and, the second is engineered bioremediation which is used when is necessary to add substances that stimulate microorganisms. The first one is more attractive because of its low cost, minimum maintenance and minimal environmental impact. Engineered bioremediation is faster than natural attenuation because it includes microbial degradation stimulation by controlling the concentrations of pollution, oxygen, moisture, pH, temperature, etc. Fritsche and Hofrichter (Fritsche & Hofrichter, 2000) reported that aerobic degradation of hydrocarbon involves the following major principle: (1) the accessibility of the chemicals by the microorganisms. For instance, biosurfactants production is required for the degradation of hydrocarbons since they are insoluble in water. (2) The first intracellular attack of the pollutants in which oxygenase and peroxidases activate and incorporate oxygen which is the key enzymatic reaction. (3) Conversion of the organic pollutant stage by stage into peripheral degradation pathways into central intermediary metabolism intermediates like Krebs cycle. (4) Cell biomass biosynthesis from the central precursor metabolites such as acetyl- CoA, pyruvate, and succinate.

MECHANISM OF BIODEGRADATION:

Mechanism of oil degradation is done by the production of surface-active agents(surfactants). Such surface-active agents are believed to bring the fine oil droplets in close contact with the aqueous media there by emulsifying the oil in water. This facilitates transport of contaminants to the intracellular enzyme to microbes. A hydrocarbon utilizing bacteria producing a potent emulsifier is that of *Pseudomonas sp* producing the emulsifier Rhamnolipid. Many types of bio surface active agents are synthesized by wide variety of microorganisms.

MICROORGANISMS INVOLVED IN BIODEGRADATION:

Microorganisms have evolved their capacity to degrade hydrocarbon compounds over millions of years. These compounds are a rich source of the carbon and energy. The application of bacterial isolates in degrading crude oil involves the manipulation

of environmental parameters to allow microbial growth and degradation to proceed at a faster rate (Vidali 2001). Microbes which are dependant on nutrients will be used to synthesize the enzymes for the breakdown of hydrocarbons.

Various groups of indigenous bacteria, fungi, cyanobacteria, and algae are involved in petroleum hydrocarbon utilization/degradation. Among all these groups bacteria are reported as the most active bioremediation agents.

BACTERIA:

Bacteria are the most active agents in petroleum biodegradation and there is evidence of their fundamental role as primary degraders of spilled oil. Effect of various nutrients on the degradation of crude oil by different bacteria was investigated by several scientists. Several factors, both physio-chemical and biological, affect the rate of microbial degradation of hydrocarbon in soil. However, application of statistical approaches for bioremediation of crude oil was reported. Nearly hundred species of bacteria, representing thirty microbial genera had hydrocarbon oxidizing properties. Many species and genera had been found to have this ability. The heterotrophic microorganisms found in soil include naturally occurring populations that have the ability to degrade petroleum products of *Pseudomonas, Arthrobacter , Alcaligenes , Corynebacterium , Flavobacterium , Achromabacter , Micrococcus , Nocardia and Mycobacterium* appear to be the most consistently isolated hydrocarbon degrading bacteria from soil.

A huge number of hydrocarbons degrading bacteria have previously been isolated all over the world. Some of them are:

SUBSTRATE	BACTERIA
Crude oil	*Pseudomonas aeruginosa , Bacillus cereus , Dyadobacter koreensis , Campylobacter hominis , Micrococcus luteus*
Spent engine oil	*Bacillus* spp., *Pseudomonas* spp., *Staphylococcus* spp., *and Streptococcus* spp.
Diesel, kerosene and petrol	*Enterobacter aerogenes , Pseudomonas aeruginosa , Aerococcus viridian , Clostridium sporogenes , Staphylococcus*

	aureus , Lactobacillus acidophilus , Micrococcus luteus , Streptococcus faecalis and Bacillus sp.
Crude oil	*Bacillus , Acinetobacter , Micrococcus and Pseudomonas .*

Recently growing interest in the use of Pseudomonas sp. During degradation of crude oil is reported.

Pseudomonas sp. is Gram negative rod measuring 0.5 to 0.8µm by 1.5 to 3.0µm. Almost all strains are motile by means of single polar flagellum , and some strains have two or three flagella . the flagella yield heat liable antigens (H antigen). The significance of antibody directed against three antigens , aside from its value in serological classification is unknown. Clinical isolates usually have oil, which may be antiphagocytic and probably aids in bacterial attachment, thereby promoting colonization.

Domain: Bacteria

Phylum : Pseudomonadota

Class : Gammaproteobacteria

Order : Pseudomonadales

Genus : *Pseudomonas*

Most petroleum hydrocarbons encountered in the environment are ultimately degraded or metabolized by indigenous bacteria because of their energetic and carbon needs for growth and reproduction as well as to relieve physiological stress caused by the presence of petroleum hydrocarbons in the microbial bulk environment. Many normal and extreme bacterial species have been isolated and utilized as biodegrades for dealing with petroleum hydrocarbons. the degradation pathway of a variety of petroleum hydrocarbons (e.g., Aliphatic and polyaromatics) have been shown to employ oxidizing reactions, however these pathways differ greatly because of the specific oxygenases found in different bacterial species. For instance, some bacteria can metabolize specific alkanes while others break down aromatic or resin fractions of hydrocarbons. This phenomenon is related to the chemical

structure of petroleum hydrocarbon components. Recent studies have identified bacteria from more than 79 genera that are capable of degrading petroleum hydrocarbons; several of these bacteria include *Achromobacter* , *Arthrobacter*, *Kocuria* , *Pandoraea* , *Staphylococcus* , *Pseudomonas* , *Rhodococcus*, etc.

The fuel eating bacteria known as *Pseudomonas* sp. have evolved a taste for hydrocarbons, a major component of the fossil fuel. Degradation of oils by *Pseudomonas sp.* is the best carrier-based inoculums. These bacteria are found in different environments such as soil, water, plant and animal tissues. They are ubiquitous in soil and water and are considered as scientific and technologically important. They comprise a taxon of metabolically versatile organisms which are capable of utilizing a wide range of simple and complex organic compounds. They are known to be involved in biodegradation of natural or manmade toxic chemicals. This species is an excellent microorganism for use in bioremediation because of its flexibility and plasticity of its metabolic pathways. Nevertheless, this expanded and variable metabolome is also responsible for the larger genome sequence of *Pseudomonas sp.* cand can also be considered to be a genetic and metabolic "burden". Identifying the pathways and the corresponding genetic framework responsible for the biotransformation and / or the biodegradation of specific compounds of interest is of extreme value in order to 1) create simpler strains of *Pseudomonas sp.* by knocking out unnecessary genes and pathways. 2)import pathways of interest in model organisms such as *E. coli*.

FUNGI:

Fungi also play an important role with their ability in removing hazardous compounds from the water. Sediment particles contaminated with crude oil from oil spills is one of the desired ecological niches to fungi which inhabit such substrates and use them as carbon source. Fungi have been found to be better degraders of petroleum than traditional bioremediation techniques including bacteria. Although, hydrocarbon degraders may be expected to be readily isolated from a petroleum oil associated environment, the same degree of expectation may be anticipated for microorganisms isolated from a totally unrelated environment.

Penicillium is a genus of ubiquitous saprobic fungi with >300 known species, but few are recognized as pathogens of dogs or people

Kingdom: Fungi

Division: Ascomycota

Class : Eurotiomycetes

Order : Eurotiales

Family : Trichocomaceae

Genus : *Penicillium*

Penicillium is one of the most abundant fungal floras with the intention that there are 106 –108 spores in one gram of normal soil and 104 spores in one millilitre of unpolluted groundwater (Gallegos Martinez et al. 2000). Recently, a considerable attention has been focused on using physical, chemical, and biological methods to remove or modify the environmental contamination created by petroleum products

The ability of most fungi to produce extracellular enzymes for the assimilation of complex carbohydrates makes possible the degradation of a wide range of pollutants. They even have advantage of being relatively easy to grow in fermenters, thus being suited for large scale production. Another advantage is the easy separation of fungal biomass by filtration due to its filamentous structure. In comparison to yeasts, filamentous fungi are less sensitive to variations in nutrients, aeration, pH, temperature and have a lower nucleic content within the biomass. In addition, several Penicillium strains are able to live in saline environments, an advantage of these microorganisms over the others in the bioremediation field.

The study was therefore designed to monitor rate of biodegradation petroleum by microorganisms isolated from oil contaminated sites. Petroleum degrading microorganisms were isolated from oil contaminated soil sample. The isolated strains were identified by morphological and biochemical characterization. The isolates were screened to determine the degradation of petrol.

AIM AND OBJECTIVES:

- To collect the sample from motor oil stained patch in a workshop
- To enumerate the microorganisms from the soil sample
- To isolate the hydrocarbon utilizing microorganisms from oil contaminated soil.
- To identify the selected isolates by morphological characterization.
- To identify the selected isolates by biochemical characterization
- To determine the petroleum utilization by the selected strains
- To analyze the parameters for the biodegradation test.

Atlas and Bartha (1972) reported that bacterial cultures are mixed to use them to degrade crude oil in soil because bacterial strains when mixed are more effective in Biodegradation of crude oil.

Dibble and Bartha (1979) reported that the rate of biodegradation of petroleum in increased the amended soil. Since petroleum degrading bacteria grow and utilize hydrocarbons better at slightly alkaline pH.

Rittman and Johnson (1989) isolated bacterial hydrocarbon degraders such as *Pseudomonas sp* from oil contaminated soil.

David .M. Palatko (1991) stated that the production of biosurfactants were during the microbial degradation of organisms. These organisms activity helped to bring out the nature of their synthetic surfactants

Amund et al., (1993) stated that oil degrading bacteria using motor oil as a carbon source it could be enumerated on minimal salt medium.

Lyle .G. Whyte et al.,(1997) described that psychrotrophic microorganisms possessed alk and nap pathways , which were responsible for biodegradation . Hence these strains potential for bioremediation of low temperature contaminated site

Salmon et al., (1998) reported that neutral pH of 7.0 is found to be optimal for biodegradation of petroleum hydrocarbon.

W.Meredith, S.-J. Kelland, D.M.Jones (2000) stated that the biodegradation of oil produced high concentration of carboxylic acid , Showed high total acid number value.

W.Meredith et al., (2000) showed that carboxylic acids are a major group of compounds responsible for the high TAN values of the oils and studied that biodegradation is the dominant process that produces high concentration of carboxylic acid in these oils

C Raghukumar, V Vipparty, J David, D Chandra mohan (2001) reported that the marine cyanobacteria *Oscillatoria Salina, Aphanocapsa sp, Plectonema terebrans* degraded the crude oil and they were measured by gravimetric and gas chromatotrophic method.

K.S.M. Rahman , J. Thahira Rahman , P. Lakshmanaperumalsamy , L.M. Banat (2002) studied optimal conditions for biodegradation of crude oil. Bacterial culture isolated from oil contaminated soil samples. *Pseudomonas sp, Bacillus sps, Micrococcus sp* were selected for study based on efficiency of crude oil utilization. Temperature of 300C and PH 7.5 were found to be optimum for maximum biodegradation.

T. Mandri and J Lin (2004) isolated microorganisms pseudomonas degraded 71% flavobacterium cannot maintain at 0 degree for more than three days. *A. Coacetium* degraded the oil 84%.

E. Lombi and R.E. Hamo (2005) described that there are different technologies for remediation of organic and inorganic contaminant in soil bioremediation and phytoremedates would be highly used in future technology.

G Emtiazi and H. Shakarami I nahvi, SH Mida madian (2005) isolated from the petroleum Contaminated Soil Instable. They were more stable when they were immobilized on the perlite. Isolate did not produce chemotaxin to octadecane, dedecane, but they utilized octadecane and dedecane but not utilized Kerosene. The E.coli petrol degradation and octadecane transformed by lysozyme treatment.

Antony .I Okeh (2006) stated that remediation of petroleum contaminated system could be achieved by either physiochemical or biological method. However the attendant negative

consequence of the physiochemical approach are currently directing greater attention to the exploitation of the biological alternatives.

Kishore das et al., (2006) stated that the experimental analysis of *Pseudo bacillus subtilis* isolated from petroleum contaminated soil showed good reduction. In total petroleum hydrocarbon reduction is effectively done by Pseudomonas.

Ashis k- Mukherjee (2006) reported that bioremediation of petroleum contaminated soil was degraded by the species of *P. aeruginosa* and *B. Subtilis*. They degraded petroleum due to their thermophilic nature.

Das and Mukherjee (2007) reported that *Pseudomonas* is a common bacteria capable of degrading hydrocarbons.

S.Y.Adeline , HC Tan carol (2009) studied potential use of the isolate Pseudomonas as a hydrocarbon degrader. Biodegradation was first detected on Bushnell-haes agar plate and again broth cultures. In both tests, the use of potential isolate for biodegradation of hydrocarbon contaminated environment.

Aouad linda , Ab bouni , Bouziane (2012) stated that organisms were isolated from soil and water samples for the industrial refinery the bacterial strain were selected their capacity of growing in presence of hydrocarbon, optimized conditions to improve biodegrading activity of isolated strains were studied using concentration of petroleum oil and surfactant.

Ainon Hamzah, Mazni Abu Zarin, Aidil Abdul Hamid, Othman omar, Sahidan Senafi (2012) stated that the sixth day trichoderma was meant for degradation of heavy crude oil followed by degradation of petrol in ninth day.

R.C. John and G.C. Okpokwasili (2012) stated that *Nitrobacter Sp.* , *Nitrosomonas Sp.* were isolated and they degraded the Crude oil for the source of carbon and energy.

Bhaben tanti , Alak Burergohain (2013) stated that process of crude oil is one of the earths major pollutants . potentials of certain soil bacteria in the biodegradation of petroleum to develop an active indigenous bacterial.

Shirendra sharma (2014) mentioned that bioremediation is the removal of pollutants from natural environment by conversion of them to less harmful one , could highly achieved by microbiological community of Pseudomonas .

A.Esmacili and E. Sadeghi (2014) stated that the oil degraded by the water derived fungus family of penicillium The Strain of fungus degraded hydrocarbon 95.4% by bio removal activity.

Shivendra Sharma and Hardik Pathak (2014) reported that large number of hazardous compounds are released into the environment. The most common chemical involved in environmental contamination are petroleum hydrocarbon. Less harmful microorganism contaminated to the environment is bioremediation. Pseudomonas is the most frequently found bacteria in nature which is used in biodegradation process.

Vijay Kumar et al., (2014) isolated strain Pseudomonas sp has potential in diesel degradation and can be recommended for bioremediation of sites that are contaminated with diesel. Bacterial culture studied based on optimum condition, PH, nitrogen source, carbon source in addition to increased growth by the isolate indicating maximum utilization of diesel.

Jesubunmi and Christianah Olubunmi (2014) isolated five bacteria they were Pseudomonas, Micrococcus, Proteus, Bacillus, Klebsiella. Four fungus they were penicillium, Streptomyces, aspergillus, Cheatomium. The density heterotrophic increases the density of utilization of hydrocarbon.

Oluwafeni obayori and Lateaf B Salam and Oluwa Toba s ogunwumi (2014) stated that Pseudomonas had the ability to degraded pyrene the growth profile of Pseudomonas aeruginosa isolated had been studied.

K.V. Darsa et al., (2014) mentioned that the isolate had the ability to tolerate petrol concentration and grow on them. HPLC analysis for degradation showed new peak appearance confirming Pseudomonas aeruginosa could be used as bioremediation

Raed S. Al-Wasify and shimaa R. Hamed (2014) stated the effect of those microorganisms were tested by capillary gas Chromatography Method

Udgire M et al., (2015) evaluated the capability of native bacterial strain to utilize the petroleum oils as the sole carbon source under invitro conditions

Mohammad saud safdari et al., (2016) studied indigenous microbial isolates for degradation of diesel fuel . *Pseudomonas aeruginosa* showed higher biodegradation efficiency in shaking flask containing diesel contaminated water. It isolates showed higher capability in biodegradation of diesel contamination of the refinery

Sunita and Varjan et al., (2017) concluded that *Pseudomonas aeruginosa* isolated from petroleum oil well ecosystem of south Gujarat supported bioremediation process but if the Halophilic or Halotolerant nature was increased in them could be applied and managed in bioremediation of marine oil spill.

A.Benchouk and A . Chibani (2017) presented indigenous microorganisms in water and soil sample are capable of degrading hydrocarbon contaminants . *Pseudomonas aeruginosa* and *P.fluorescence* isolated strain from a contaminated soil of a refinery. Capability of isolated strain to degrade petroleum was performed by measure optical density , colony forming unit, and concentration of total petroleum hydrocarbons.

ANM Fakhruddin (2018) reported that the worst pollutants petroleum hydrocarbons were degraded by phytoremediation, bio augumentation. Bio stimulation method were proceeded.

M.J. Jenisha et al., (2021) concluded that the hydrocarbon classic butera found in workshop soil had the ability of hydrocarbon degradation. *Proteus sp* undergone oil spreading test which confirmed that they produced / secreted biosurfactant which could be used for remediating oil spill.

Haijun Liu, Grus yang, Hui jia, Bingjle Sun (2022) reported that *Pseudomonas aeruginosa* degradation was analysed by gas- chromatography mass Spectrometry.

METHODOLOGY:

STUDY SITE:

The soil samples were collected from mechanic workshops around to Thoothukudi

SAMPLE COLLECTION:

Soil samples were collected in a sterile polyethylene bag using a sterile spatula from a oil stained patch in the workshops by scooping to about 5 centimeter. They were immediately transported to the laboratory for the analysis

ENUMERATION OF MICROORGANISMS:

10 grams of the oil contaminated soil was suspended in 90 ml of distilled water and ten-fold serial dilutions of the soil samples from 1:10 to 1:1000000 were carried out and 0.1 ml of the 10^{-5} and10^{-5} dilution for each soil samples were plated in triplicate on nutrient Agar and potato dextrose Agar using poor plate method. The nutrient Agar plates were incubated at 35°C for 48 hours while the potato dextrose Agar plates were incubated at 28°C for 120 hours. The number of viable microorganisms in the sample was calculated from the number of colonies formed and the volume of inoculum and the dilution factor expressed in colony forming unit.

ENRICHMENT AND ISOLATION OF HYDROCARBON UTILIZING MICROORGANISMS:

One gram of oil stained soil from motor mechanic workshop was inoculated in Bushnell Haas broth medium in test tubes. The test tubes were incubated at room temperature for five to seven days. After incubation the content of each test tube was seriously diluted using distilled water. 1ml aliquot of 10-5 dilution was plated in Bushnell Haas medium for hydrocarbon utilizing fungi and bacteria.The plates were incubated at 37°C for 120 hours.

GRAM STAINING :

place a small drop of bacterial sample on a slide. Heat fixed the bacteria to the slide by passing it through the flame of a bunsen burner three times. Applying too much heat or for too long can melt the bacteria cell wall, distorting their shape and leading to an inaccurate result. If too little heat is applied the bacteria will wash off the slide during staining. Then Use a dropper to apply the primary stain (crystal violet) To the slide and allow it to sit for one minute. Gently rinse the slide with water not longer than five seconds to remove excess stain. Rinsing for a long time can remove too much color while not rinsing long enough may allow too much stain to remain on the gram negative cells. Use a dropper to apply grams iodine to the slide to fix the crystal Violet to the cell wall. Let it sit for one minute. Then Rinse the slide with alcohol or acetone for about 3 seconds, followed immediately with a gentle rinse you using water. The gram negative cells will lose color while the gram positive will remain Violet or blue. However if the decolorizer is left on for too long all the cells will lose color. Apply the secondary stain safranin and allow it to sit for one minute. Gently rinse with water no longer than 5 seconds, the gram negative cells should be stained red or pink while the gram positive cells will still appear purple or blue. View the slide using a compound microscope. A magnification of 500X to 1000X may be needed to distinguish cell shape and arrangement

LACTOPHENOL COTTON BLUE STAINING:

Lactophenol Cotton Blue is used as staining solution for fungi. Place a drop of Lactophenol Cotton Blue reagent on a clean and dry slide. The stain imparts a blue coloration on hyphae. By using a nichrome inoculating wire, carefully tease the fungal culture, into a thin preparation

BIOCHEMICAL TESTS:

INDOLE TEST:

Take a sterilized test tube containing 4ml of SIM medium. Inoculate the tube aseptically by taking the growth from 18 to 24 hours culture. Incubate the tube at 37 degrees Celsius for 24 - 28 hours. Add 0.5 ml of Kovac's reagent to the broth culture. Observe for the presence or absence of ring

METHYL RED TEST:

Using sterile experimental organisms where inoculated into approximately labeled tubes containing M R broth by means of loop inoculation. Uninoculated tube was kept as control. Both tubes were incubated at 37°C for 24-48 hours. After proper incubation 5 drops of MR indicator was added to both tubes including control. It was mixed well and color change were observed.

VOGES PROSKAUER TEST:

Using sterile technique, the experimental organism was inoculated into VP broth by means of loop inoculation. One tube is kept uninoculated as control. The tube will be incubated at 37°C for 24-48 hours. After proper incubation, about 3ml of Barret's reagent A and 1ml of Barret's reagent B was added into both tubes including control. The tubes were shaken gently for 30 seconds with the caps off to expose the media to oxygen. The reaction was allowed to complete in 15-30 minutes and the tubes were observed.

CITRATE TEST:

Using a sterile technique, Simmons Citrate agar slant was inoculated with the test organism by means of a stab and streak inoculation. An uninoculated tube was kept as control. Both the tubes were inoculated at 37°C for 24-48 hours and we're observed.

CATALASE TEST:

Transfer a small amount of bacterial colony to a surface of clean dry glass slide using a loop or sterile wooden stick. Place a drop of 3% H2O2 on the slide and mix. A positive result is the rapid evaluation of oxygen as evidenced by bubbling. A negative result is shown by no bubbles or only a few scattered bubbles. Dispose of your slide in the glass disposal container

BIODEGRADATION TEST:

For testing the biodegradation efficiency of the isolated bacterial strain, 100 ml of minimal broth (dextrose 1g ammonium sulphate 1g, dipotassium 0.7g, monopotassium phosphate 2g, sodium citrate 0.5g and magnesium sulphate)

Containing 2.5%, 5%, 7.5% and 10% petrol concentrations separately in 250ml Erlenmeyer flasks were prepared. To each flask one ml inoculum of pure culture of the isolated strain during the logarithmic phase was added. the culture flasks were incubated in a shaker at 30°C at 100rpm

PARAMETER ANALYSIS:

After 4, 8, 12 and 16 days of treatment pH, optical density, and CO_2 were determined for each petrol concentrations.

pH ESTIMATION:

pH of the culture broth was determined after 0,4,8,12 and 16 days of treatment using a pH paper. Dip the end of the pH strip into the culture broth. After a couple of seconds remove the paper and compare the color of the Ph strip to the color chart provided with the pH paper kit. Do not reuse a pH paper to retest or test another chemical

OPTICAL DENSITY:

The optical density of the culture broth was determined after 0,4,8,12 and 16 days of treatment. Select a blank cuvette and place it in the spectrophotometer. Close the lid. Click on 0 ABS 100% T button, the instrument now reads 0.00000A. Choose a solution with known concentrations and measure the absorbance between the wavelength 350nm to 700nm. Record the wavelength at the maximum absorbance value

CO_2 ESTIMATION:

1ml of the culture broth was taken after 4,8,12 and 16 days of treatment and titrated against 0.05N NAOH solution. Phenolphthalein was used as an indicator and appearance of stable pink color was considered as the end point. the amount of CO_2 was calculated using the following equation

$$CO2\ (mg/L) = \frac{\text{titre value} \times \text{Normality of NAOH} \times 1000 \times 44}{\text{Volume of the sample}}$$

RESULTS AND DISCUSSION:

ENUMERATION OF MICROORGANISMS :

In this study microorganisms were isolated from oil contaminated soil collected from two workshops in Thoothukudi. 2 samples were named as Sample I and Sample II respectively Samples were serially diluted and plated on Nutrient agar plates and Potato Dextrose agar plates and incubated at 37°C for 24 hours and 37 hours.

TABLE 5. 1 : ENUMERATION OF MICROORGANISMS

MEDIUM	SOIL SAMPLE	NO. OF COLONIES
NUTIRENT AGAR	I	107 COLONIES
	II	126 COLONIES
POTATO DEXTROSE AGAR	I	6 COLONIES
	II	26 COLONIES

Fig 5.5- 5.9 Divulges the development of the colonies in the nutrient agar from the soil sample I and II plates. Fig 5.10-5.11 ensure the development of colonies in the PDA agar media plate from the soil sample I and II . Fig 5.14 shows the gram staining process. After staining of microscopic observations of isolate 1 confirmed the presence of rod shaped bacteria. It is identified as *Pseudomonas sp* .

Fig 5.1 : STUDY SITE 1

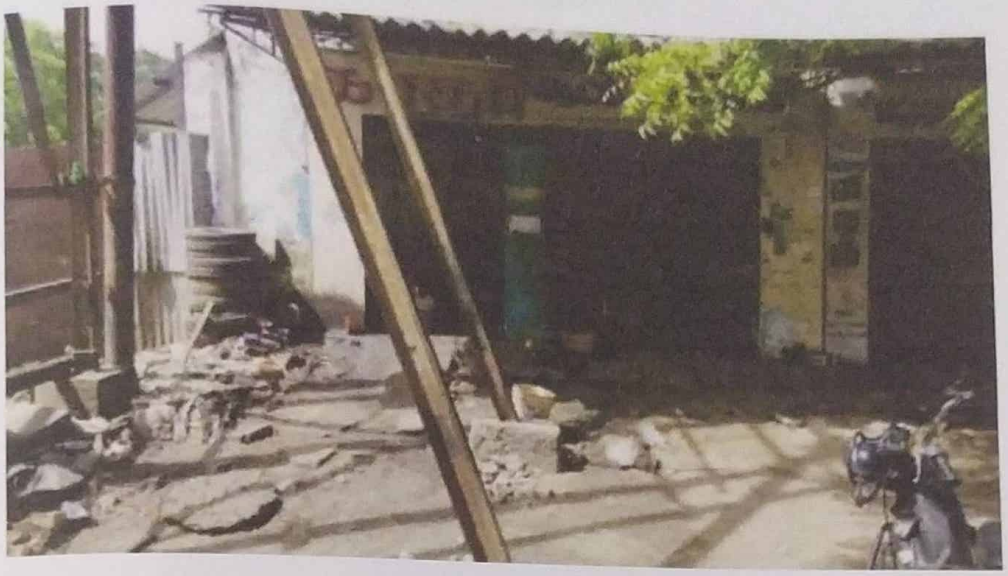


Fig 5.2 : STUDY SITE 2

SAMPLE COLLECTION

Fig 5.3: SAMPLE 1

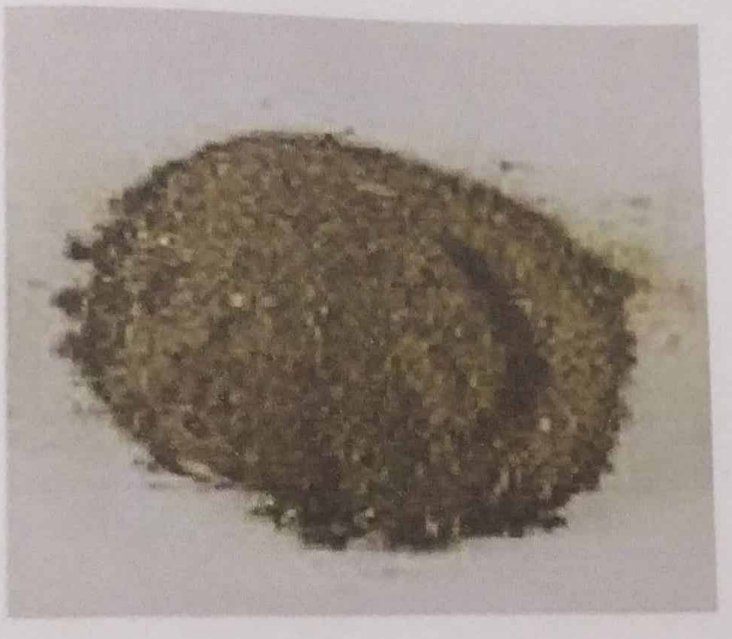


Fig 5.4 : SAMPLE II

Fig 5.5 –5.9 :ENUMERATION OF MICROORGANISMS -NUTRIENT AGAR

Fig 5.5 : NUTIRENT AGAR CONTROL

Fig 5.6 : SAMPLE 1 – 10^{-5} dilution

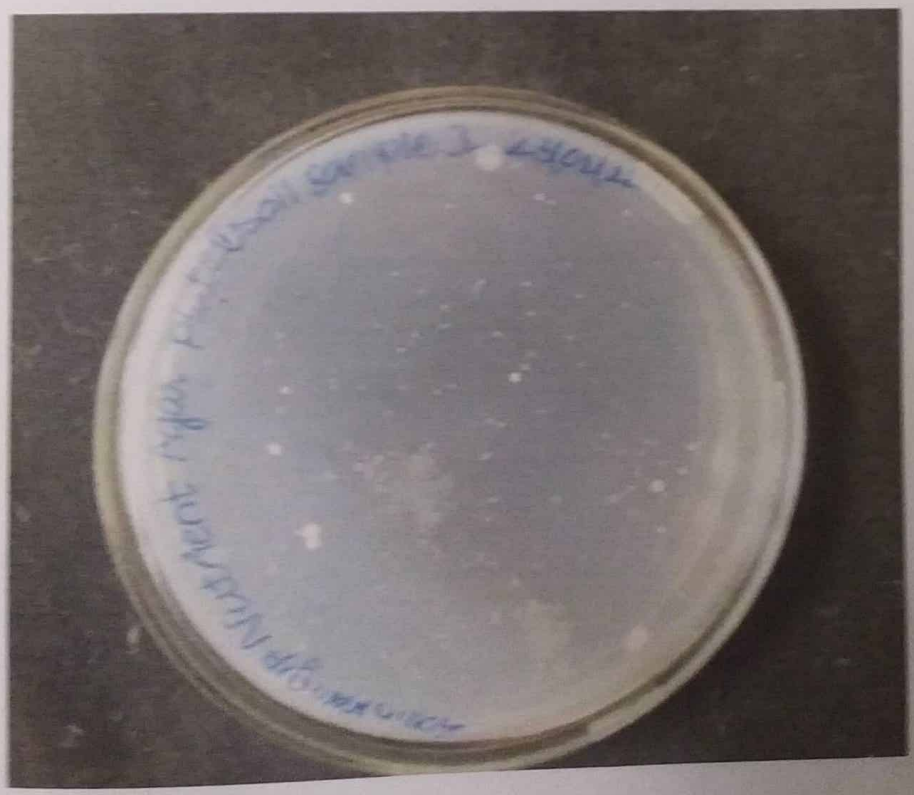


Fig 5.7 : SAMPLE 1 – 10^{-6} dilution

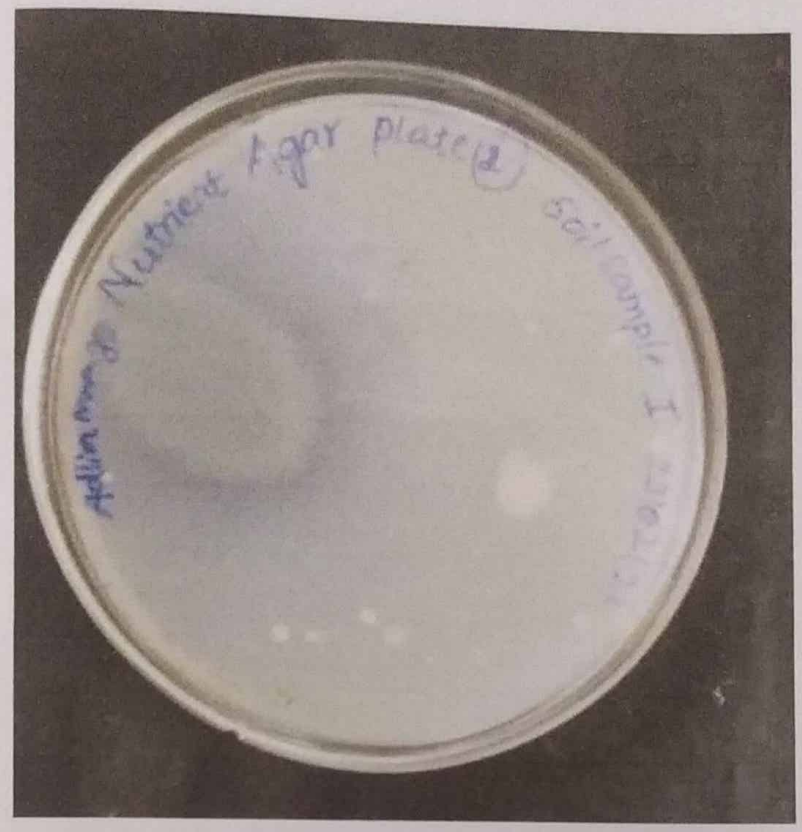


Fig 5.8 : SAMPLE II – 10^{-5} dilution

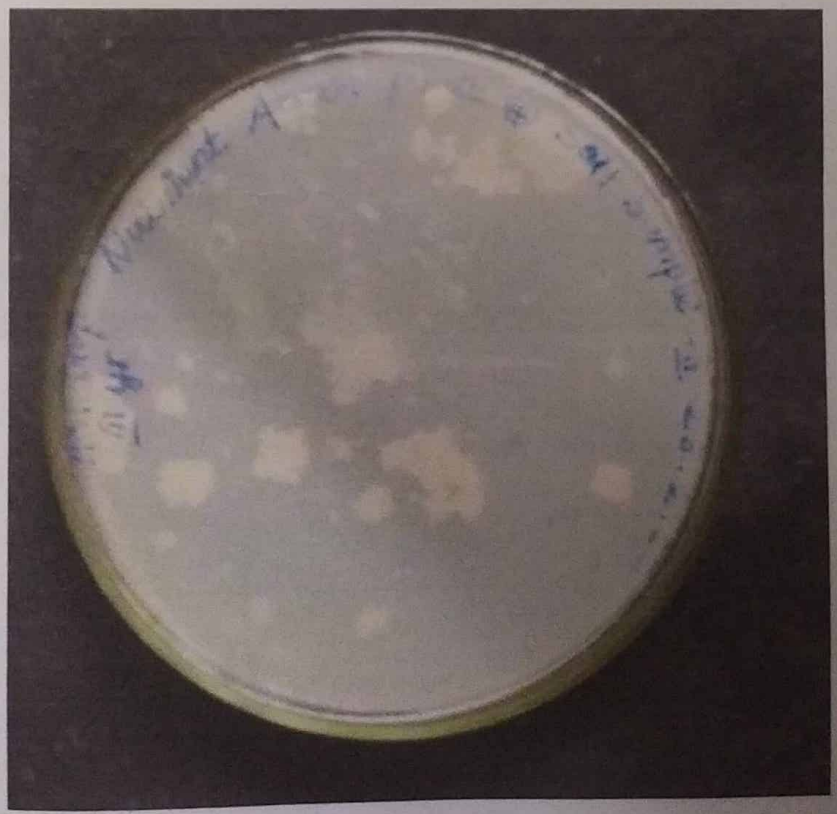


Fig 5.9 : SAMPLE II – 10^{-6} dilution

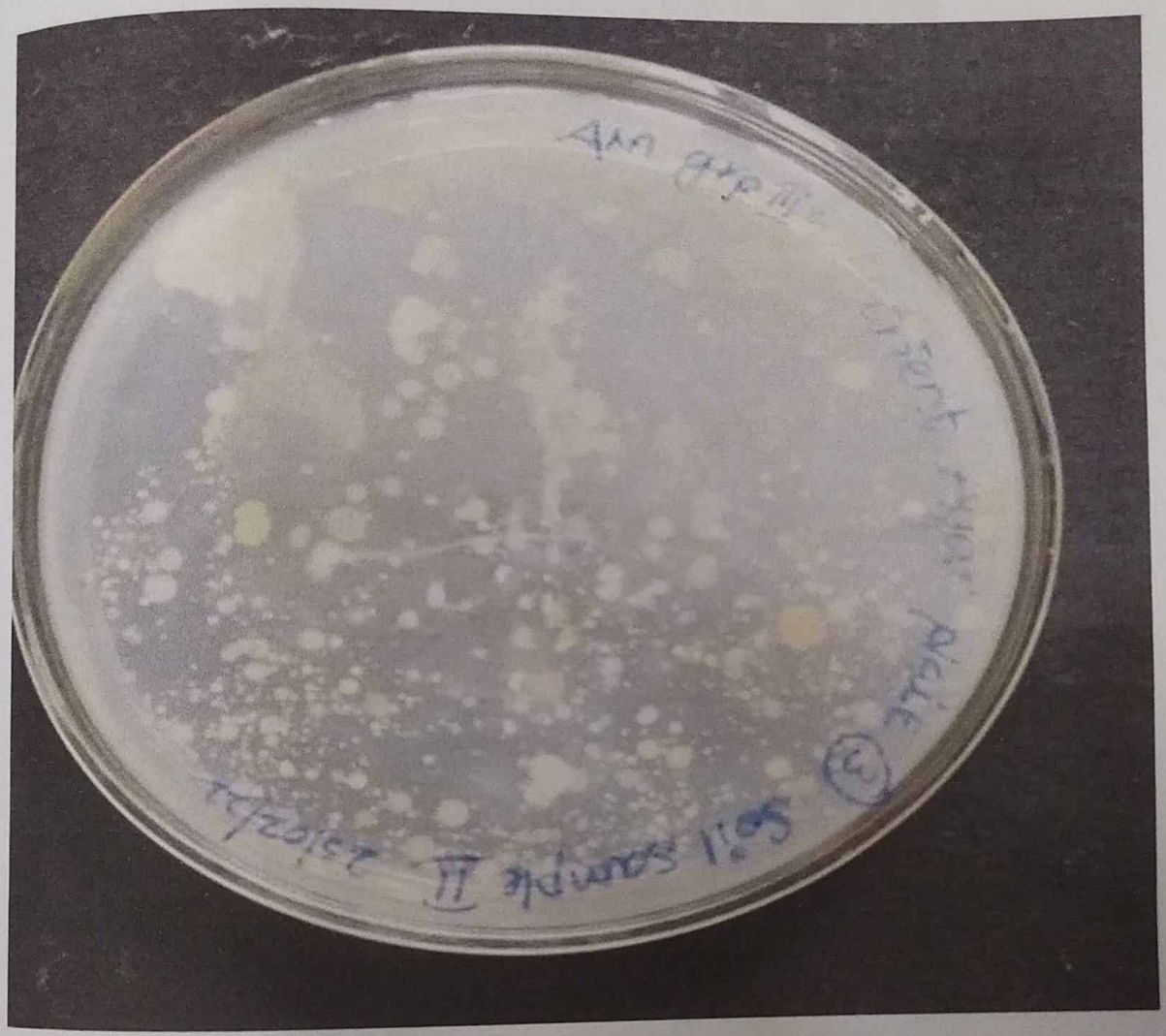


Fig 5.10 -5.11 : ENUMERATION OF MICROORAGANISMS -POTATO DEXTROSE AGAR

Fig 5.10 : SAMPLE I – 10^{-5} dilution

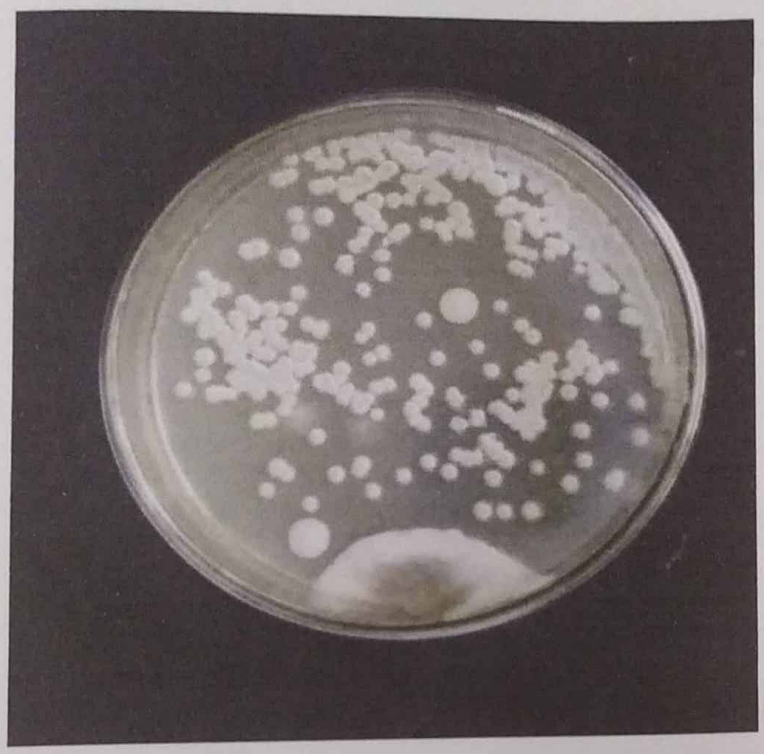
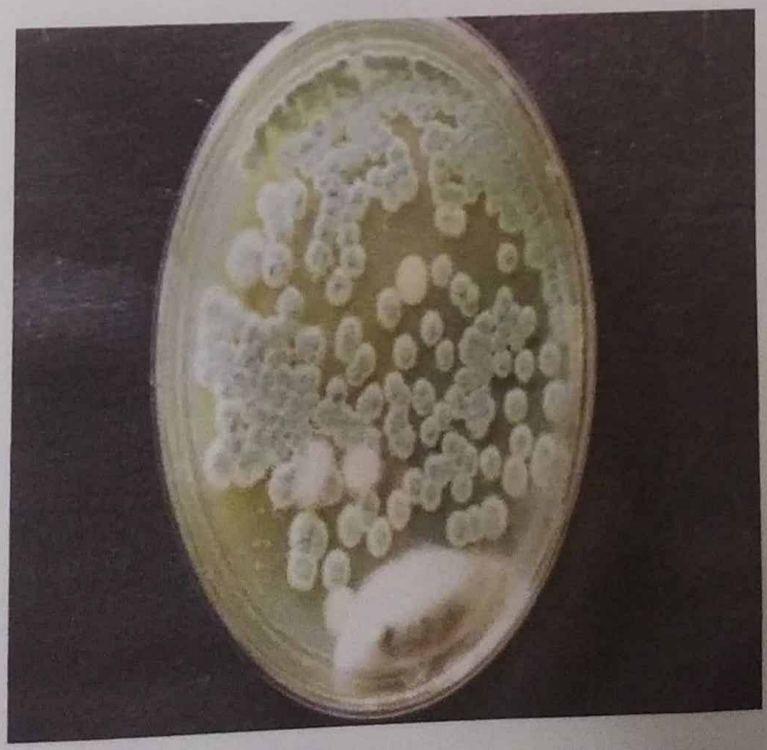


Fig 5.11: SAMPLE II – 10^{-6} dilution



ISOLATION OF HYDROCARBON UTILIZING MICROORGANISMS (TABLE 2):

Sample I and II was enriched in Bushnell Haas Broth at 37°C for 7 days .After enrichment the organisms were inoculated in Bushnell Haas Medium for 7 days. A number of colonies only 2 of them were isolated.

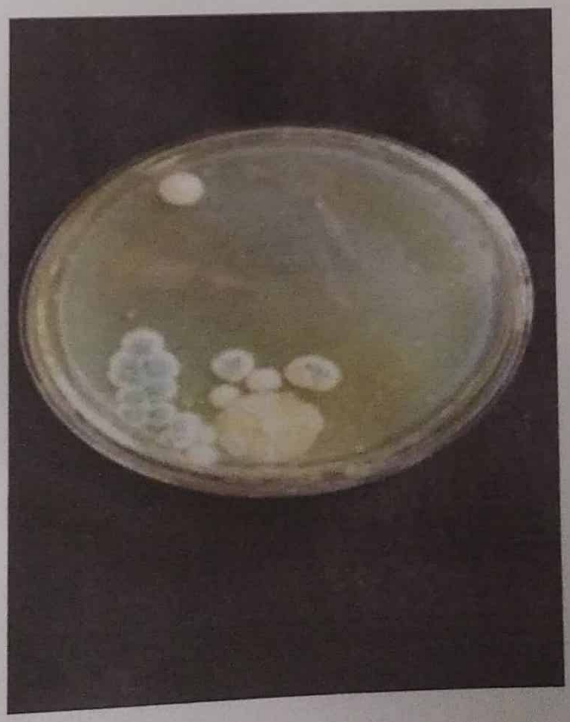
TABLE 5.2: ISOLATION OF HYDROCARBON UTILIZING MICROORGANISMS

S.NO	SAMPLE DESCRIPTION	NO. OF ORGANISMS ISOLATED
1	Sample I – Bushnell Haas Medium	1
2	Sample II – Bushnell Hass Medium	1

Fig 5.12 & Fig 5.13 : ISOLATION OF HYDROGEN UTILIZING MICROORGANISMS

Fig 5.12 : ISOLATE 1

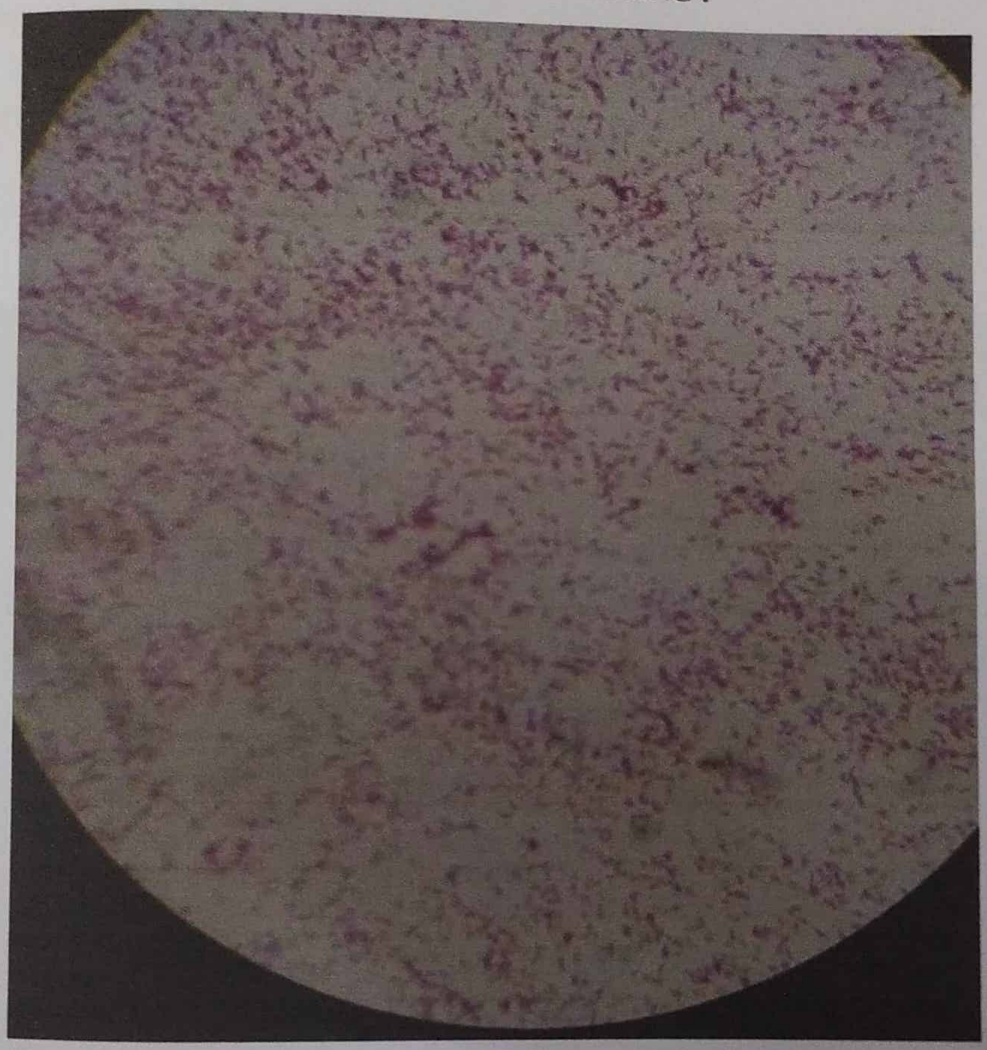
Fig 5.13 : ISOLATE 2



GRAM'S STAINING :

The basic principle of gram staining involves the ability of the bacterial cell wall to retain the crystal violet dye during solvent treatment . gram positive microorganisms have higher peptidoglycan content whereas gram negative organisms have higher lipid content.Based on this principle , after staining , Microscopic observation of isolate 1, confirmed the presence of Rod shaped bacteria .

Fig 5.14 : GRAM'S STAINING :



LACTOPHENOL COTTON BLUE STAINING :

Fungi are eukaryotic organisms with both Macroscopic and Microscopic characteristics. The fungal spores cell wall is made up of chitin , of which the components of the Lactophenol cotton blue solution stains for identification. Different fungi under Lactophenol Cotton Blue Wet mount will show morphological structures including hyphae and spores

After staining, during microscopic observation of thus slide it reveals a blue colour stained fungal spore , hyphae and fruiting structure against the pale blue background.

After this procedure, the isolate 2 was identified as *Penicillium sp.*

Fig 5.15 : LACTOPHENOL COTTON BLUE STAINING:

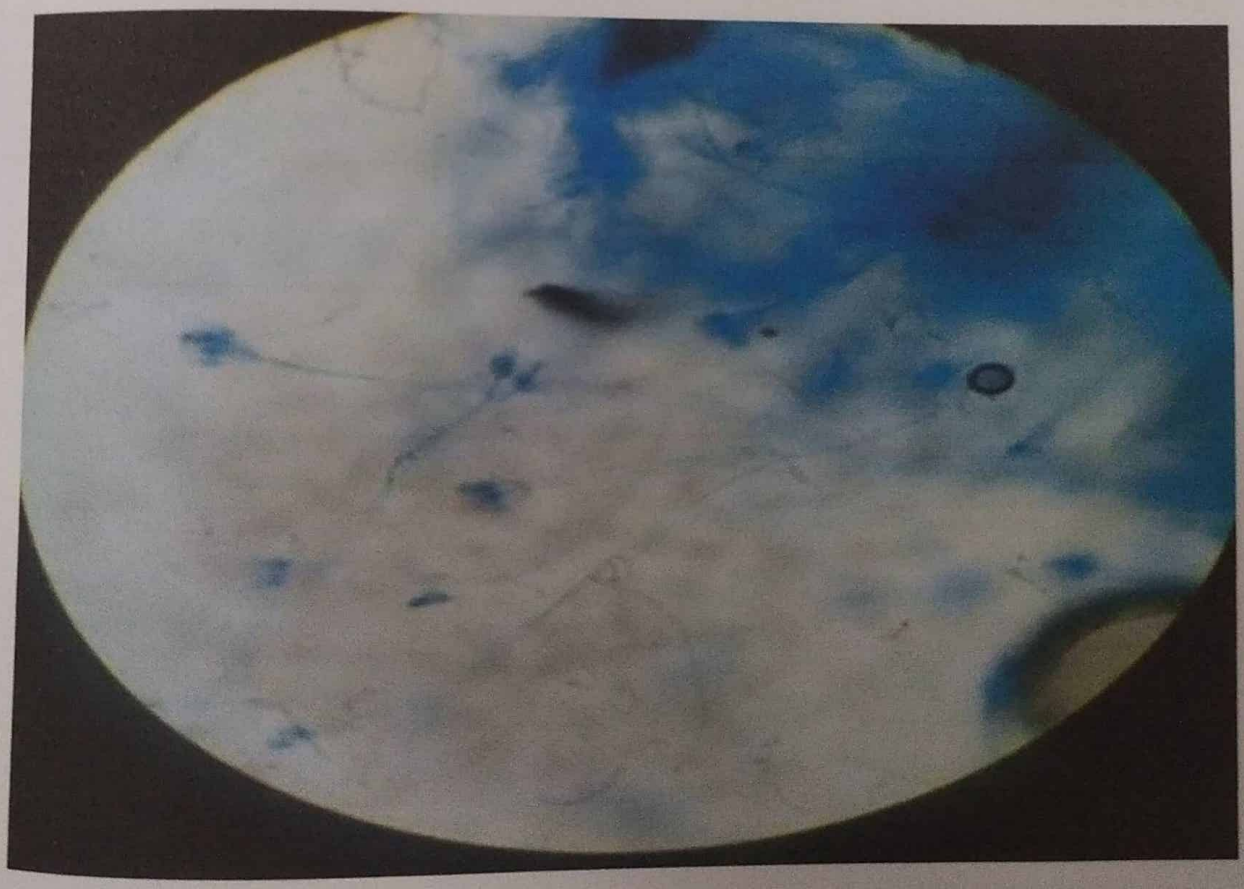


Fig 5.15 Lactophenol cotton blue staining in microscopic observation of isolate II were confirmed the presence of filamentous fungi and it is identified as *Penicilium sp* . Parameter analysis of degradation of petroleum broth .After 4,8,12 and 16 days of treatment of PH, Optical Density and CO_2 were determined for each petrol concentration.

BIOCHEMICAL TESTS OF THE BACTERIAL ISOLATE:

From the microscopic examination colony morphology and Biochemical characteristics, the selected bacterial isolate was identified as *Pseudomonas sp*. The biochemical characteristics exhibited by *Pseudomonas sp* is presented in the table

TABLE 5.3 : BIOCHEMICAL TEST

S.NO	CHARACTERISTICS	RESULTS
1	INDOLE	NEGATIVE
2	METHYL RED	NEGATIVE
3	VOGES PROSKAUER	NEGATIVE
4	CITRATE UTILIZATION	POSITIVE
5	CATALASE	POSITIVE

BIOCHEMICAL CHARATERISTICS OF ISOLATE 1

Fig 5.16 : INDOLE TEST – NEGATIVE

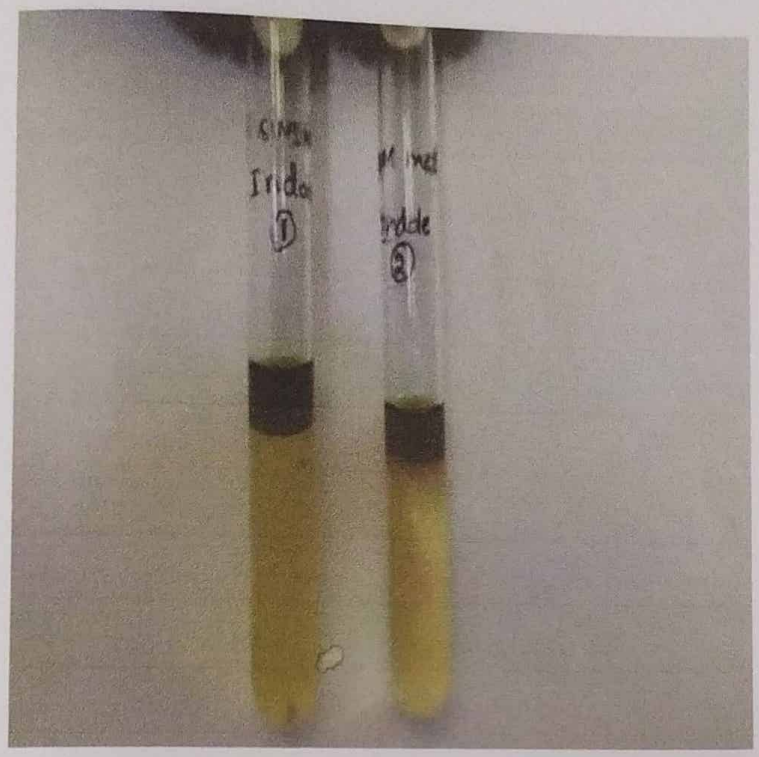


Fig 5.17 : METHLY RED TEST – NEGATIVE

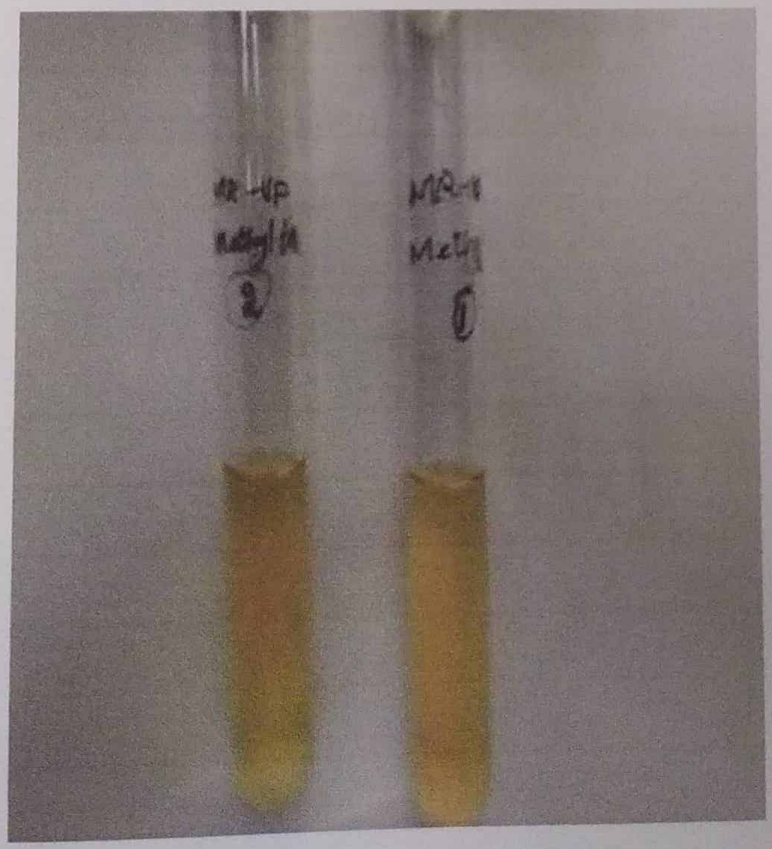


Fig 5.18 : VOGES PROSKAUER TEST – NEGATIVE

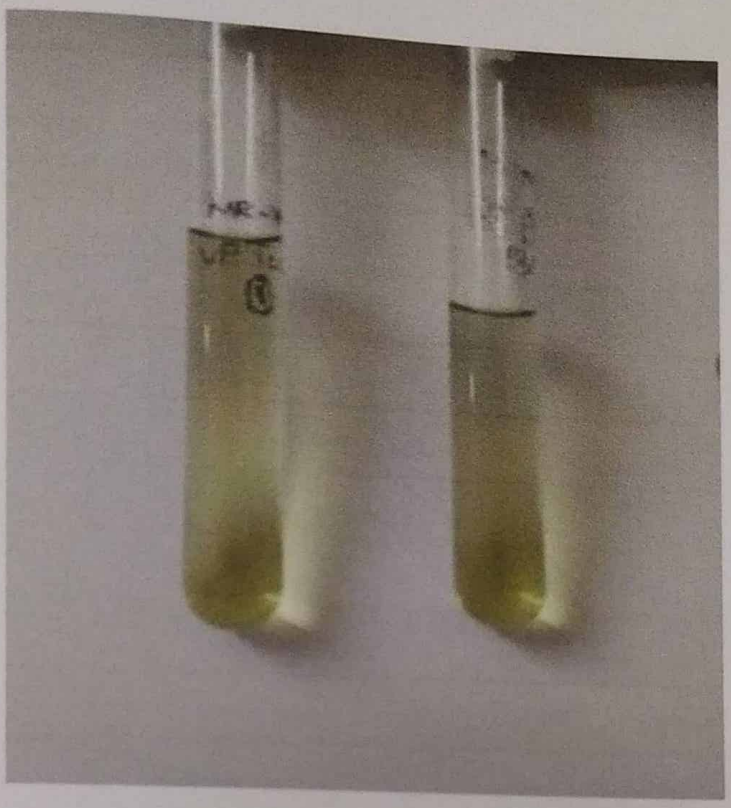


Fig 5.19 : CITRATE TEST - POSITIVE

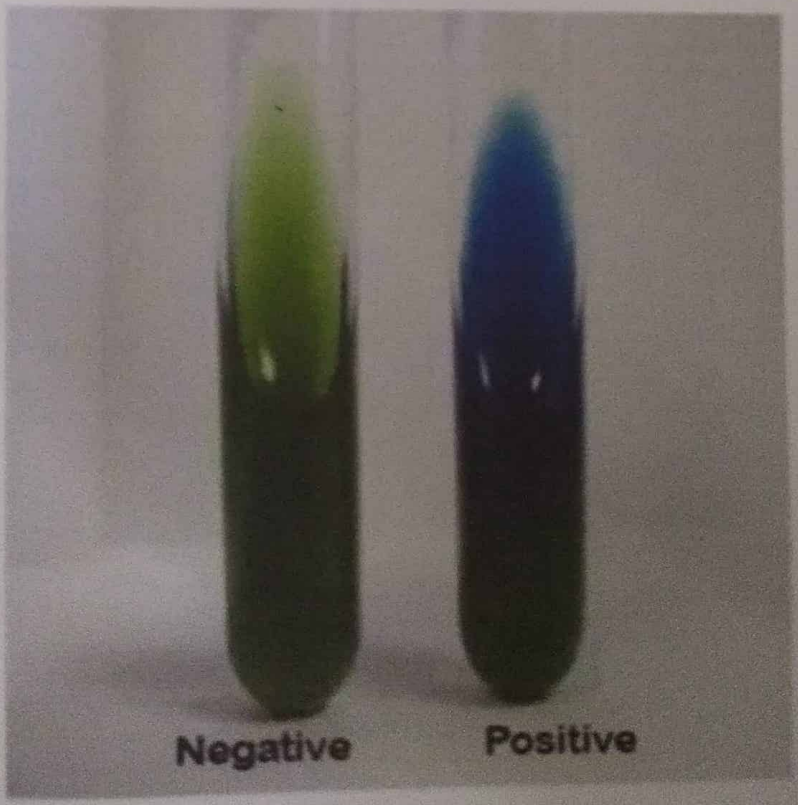
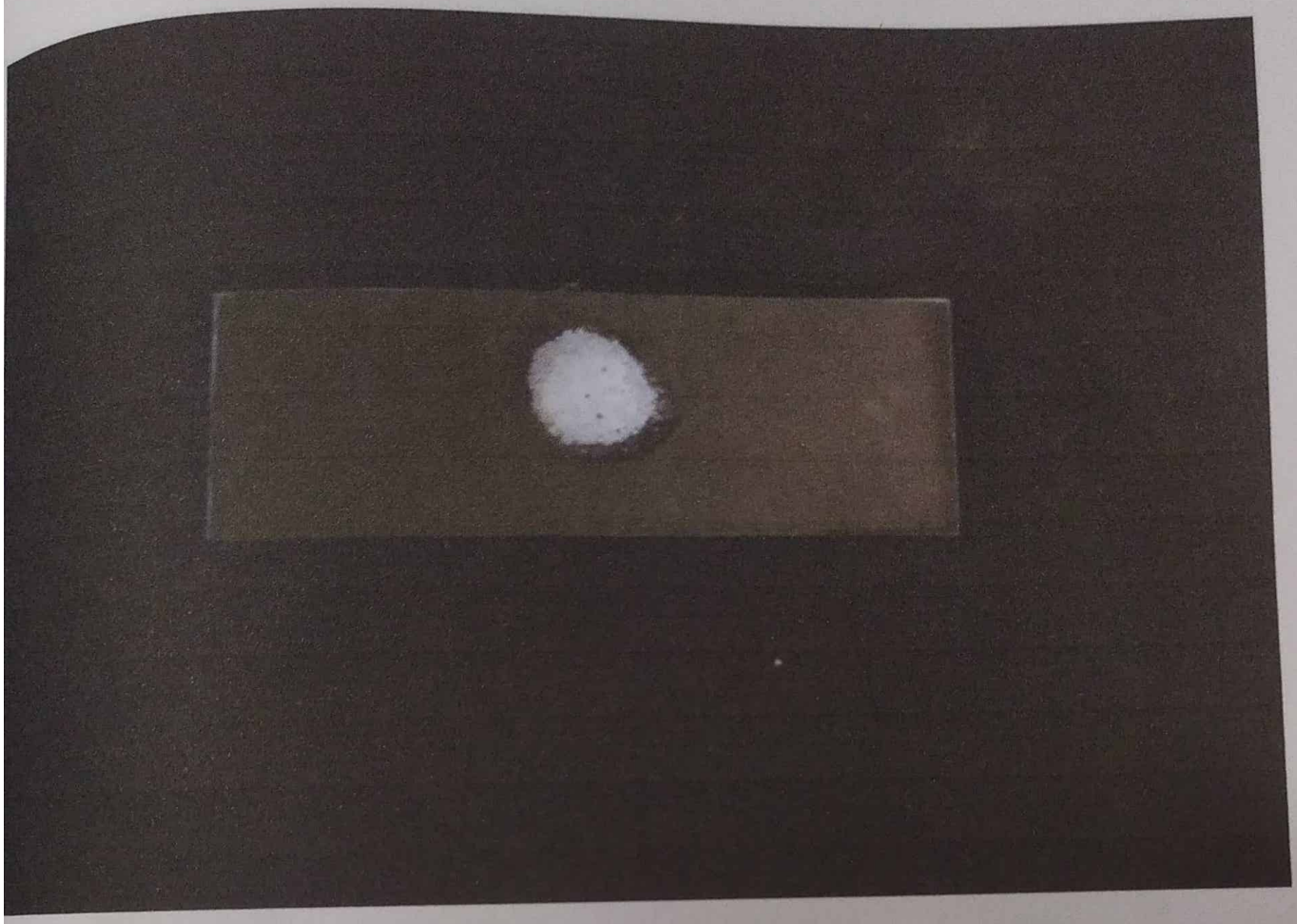


Fig 5.20 : CATALASE TEST – POSITIVE



Biodegradation Test:

For testing the biodegradation efficiency of the isolated bacterial strain, 100 ml of minimal broth (dextrose 1g ammonium sulphate 1g, dipotassium 0.7g, monopotassium phosphate 2g, sodium citrate 0.5g and magnesium sulphate)

Containing 2.5%, 5%, 7.5% and 10% petrol concentrations separately in 250ml Erlenmeyer flasks were prepared. To each flask one ml inoculum of pure culture of the isolated strain during the logarithmic phase was added. the culture flasks were incubated in a shaker at 30oC at 100rpm

The biodegradation treatment was analysed on 4^{th} , 8^{th} , 12^{th} , and 16^{th} Day.

Fig 5.22 : The changes in PH record after 4,8,12 and 16 days of treatment with Pesudomonas sp up to 8 days of treatment decline in PH was observed in all the petrol concentration expect 7.2 % indicating the formation of organic acids after petrol degradation .After 8 days of treatment PH level was increased in all the petrol concentration .

Fig 5.21: BIODEGRADATION OF PETROLEUM ON 4^{th} DAY

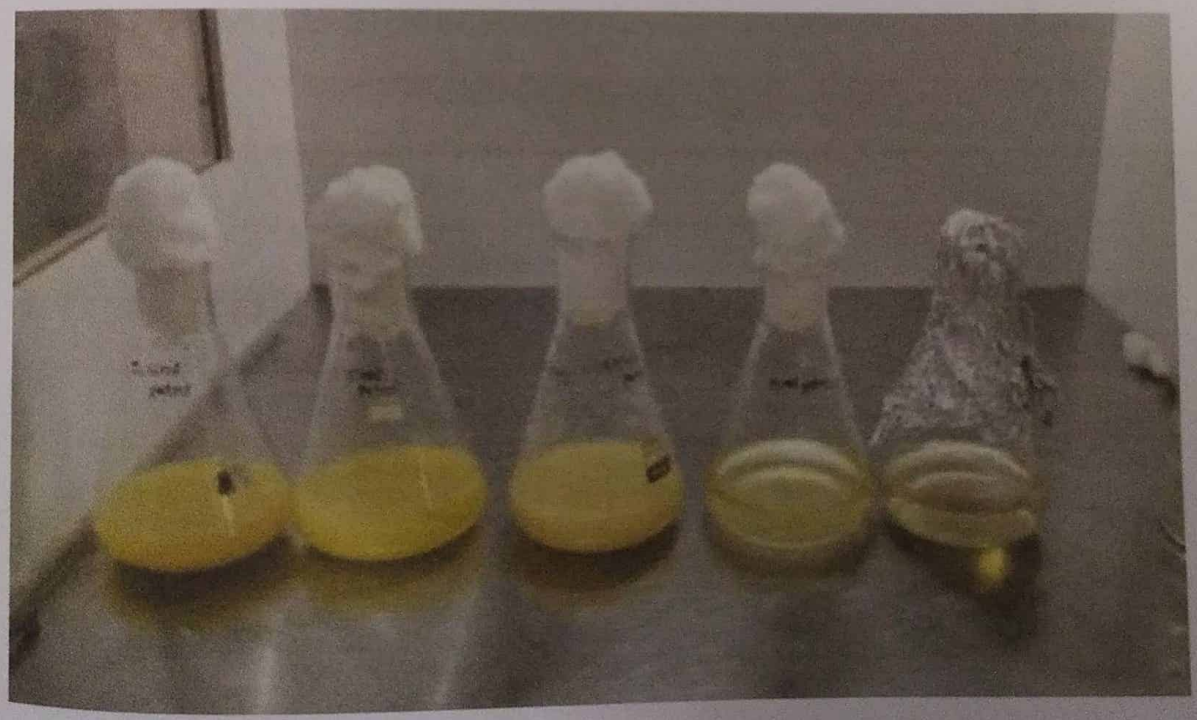
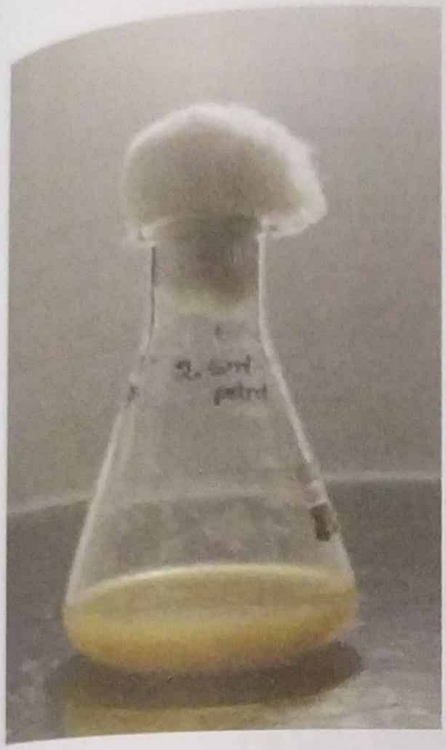


Fig 5.22 & 5.23: BIODEGRADATION OF PETROLEUM ON 12th DAY



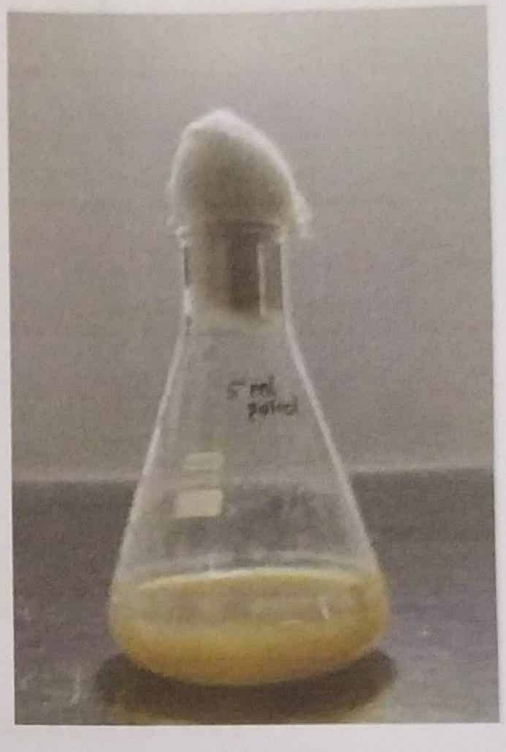
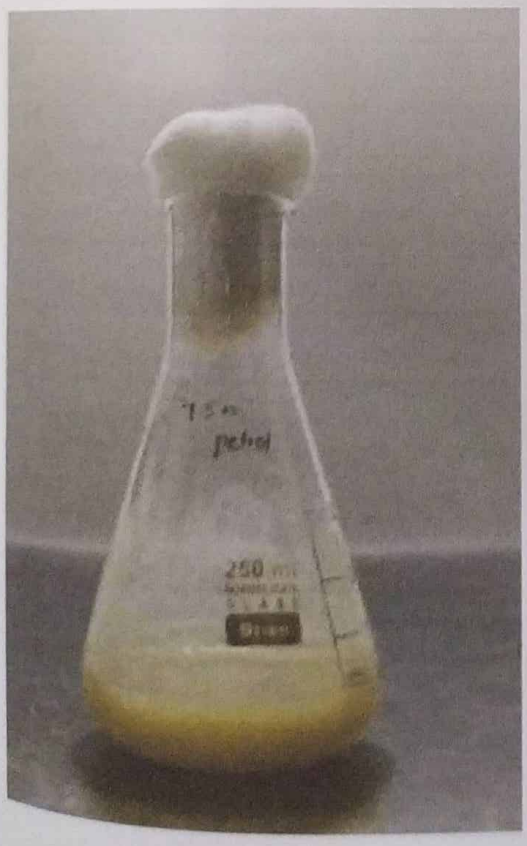


Fig 5.24 & 5.25: BIODEGRADATION OF PETROLEUM ON 12th DAY



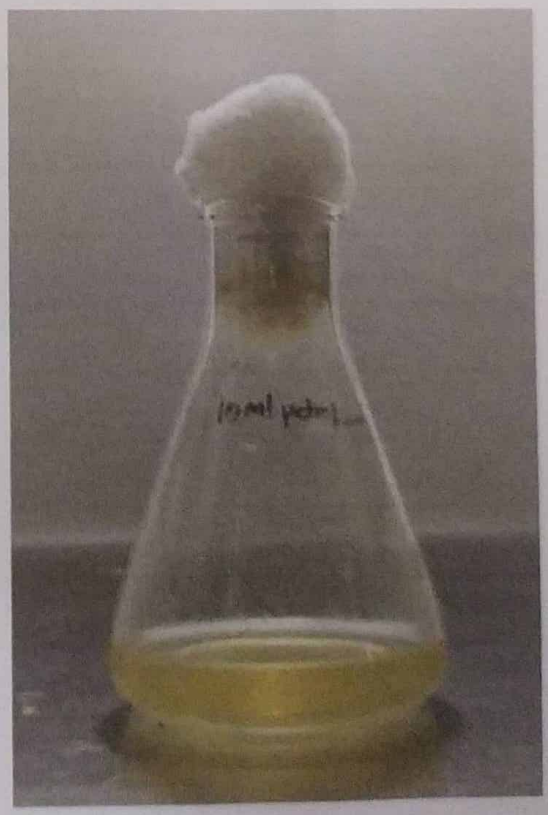


Fig 5.26 : BIODEGRADATION OF PETROLEUM – CONTROL

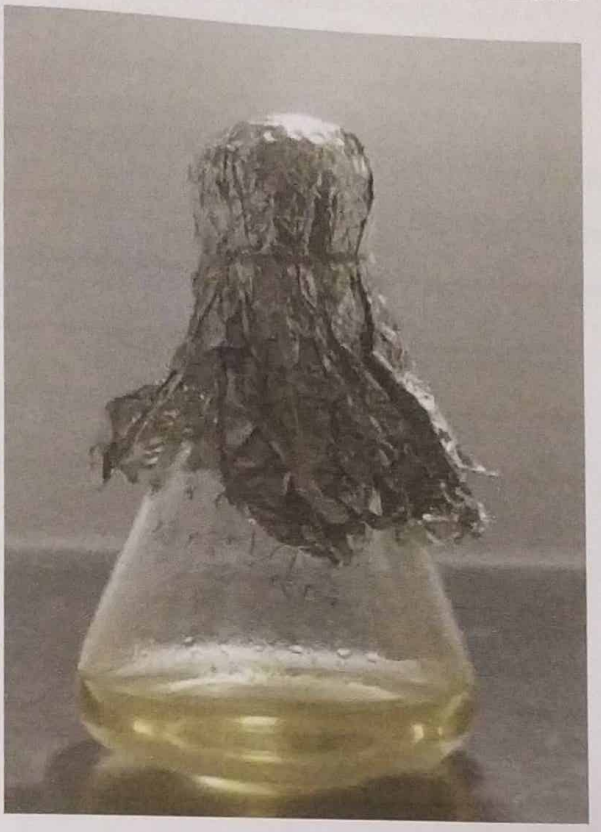
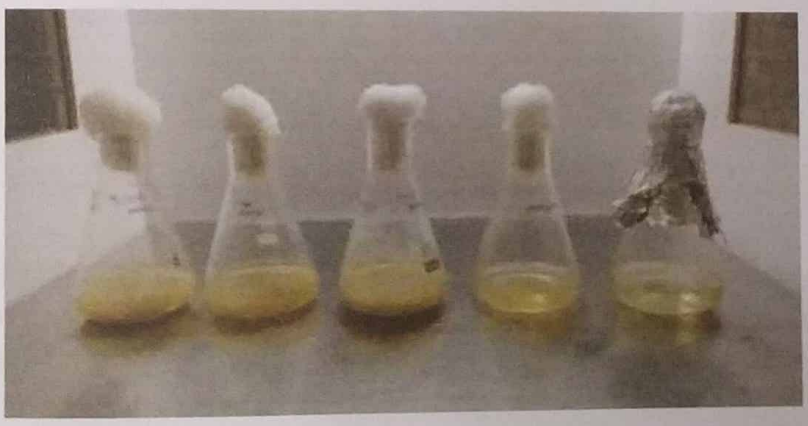


Fig 5.27 : BIODEGRADATION OF PETROLEUM 16th DAY



PARAMETER ANALYSIS:

After 4, 8, 12 and 16 days of treatment pH, optical density, and CO2 were determined for each petrol concentrations.

pH ESTIMATION:

Changes in pH were recorded at 4, 8, 12, 16 days of treatment with Pseudomonas sp. At various concentrations of petrol. The variation in pH of the medium during the treatment period was found to be increasing from 6 – 7.5 gradually. The results are tabulated

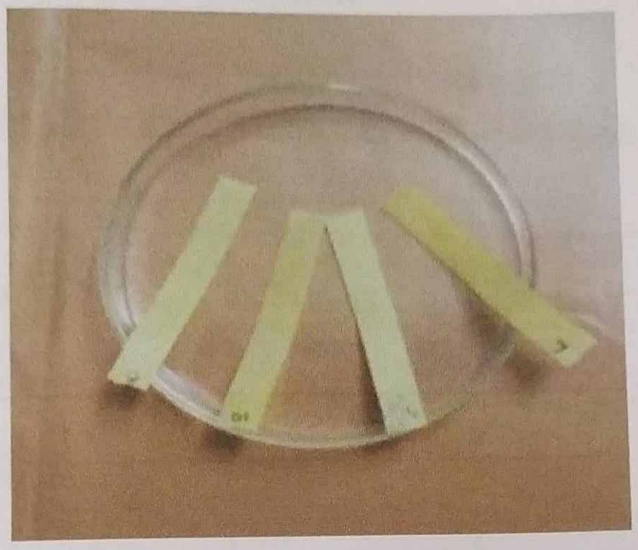
TABLE 5.4 : PARAMETER ANALYSIS – pH ESTIMATION

CONCENTRATION OF PETROL	4th DAY	8th DAY	12th DAY	16th DAY
2.5%	6.8	6.4	7	7.2
5%	6.4	6.8	6.8	7.2
7.5%	6.4	6.8	7	7.4
10%	6.8	6.8	7.2	7.4

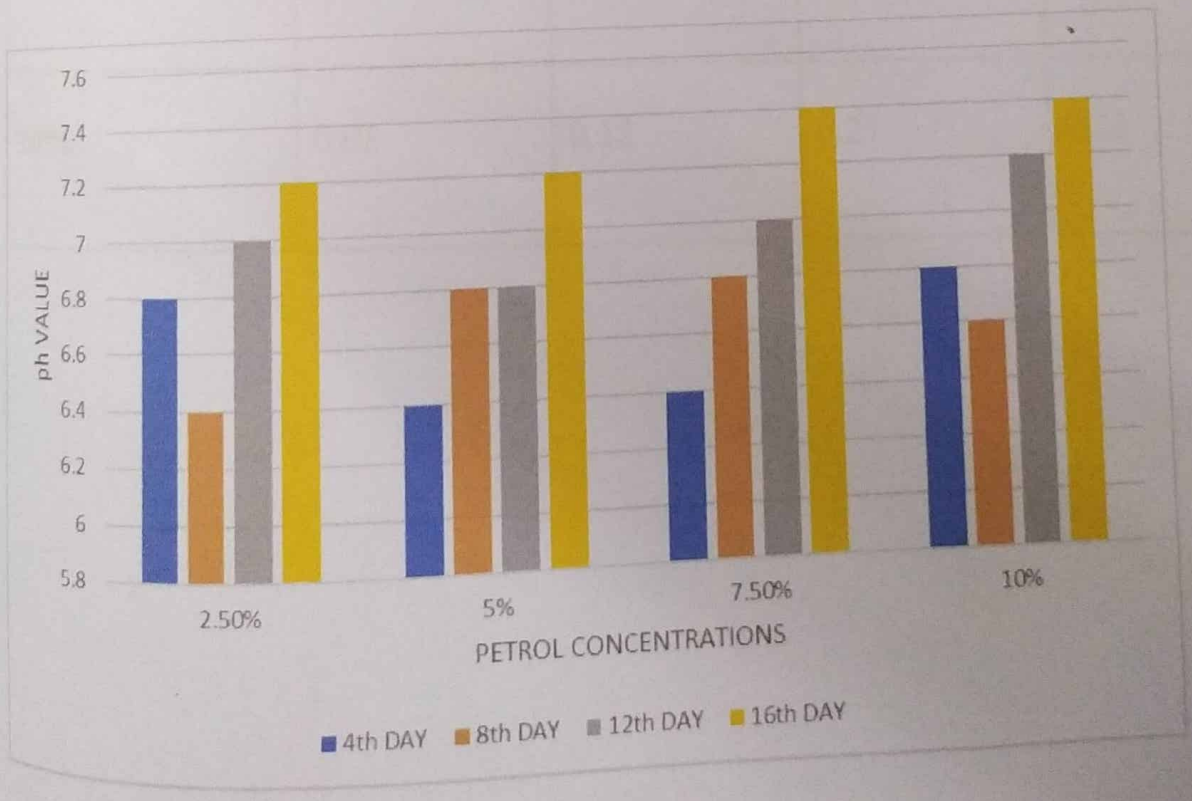
Tables 5.1- 5.6 Divulges the two way analysis of variance for the parameters, Ph, optical density and co2 with the variables treatment period and petrol concentration. Variations due to petrol concentration were statistically significant for optical density and co2 while they were not statistically significant due to treatment period

Graph 5.2 Increase in optical density values during the initial period of treatment was noticed .But afterwards there was a decline and the maximum optical density was observed at 7.5% petrol concentration after eight days of treatment

Fig 5.28 : PARAMETER ANALYSIS – pH ESTIMATION



GRAPH 5.1: CHANGES IN pH DURING DEGARADTION OF PETROL BY *Pseudomonas sp*



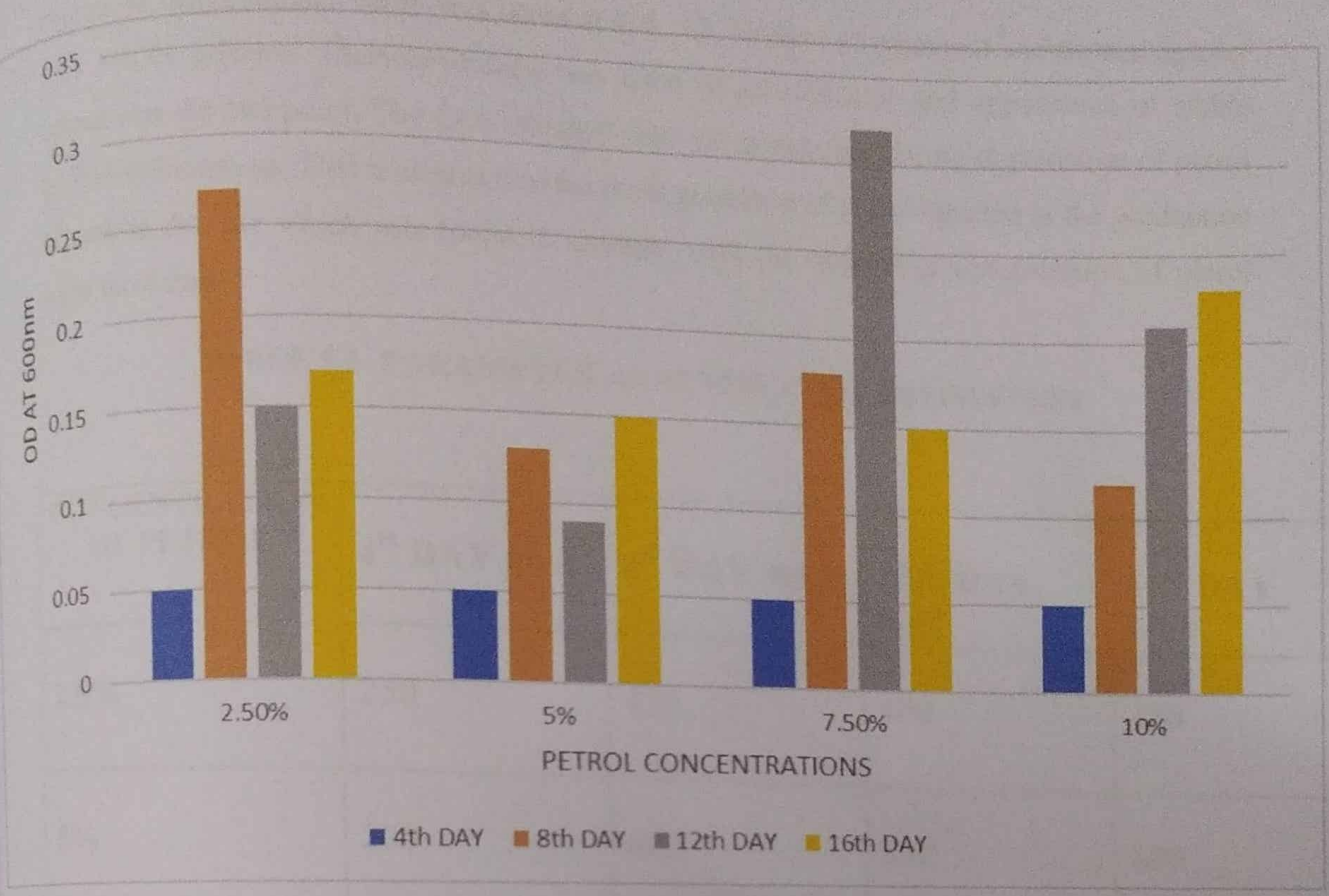
OPTICAL DENSITY:

Changes in optical density at 600nm were recorded at 4, 8, 12, 16 days of treatment with Pseudomonas sp. for the various concentration of petrol. still an increase in growth rate was observed with increase in the days of treatment while there was a decrease in optical density with increasing concentration of petrol were tabulated .

TABLE 5.5 : PARAMETER ANALYSIS – OPTICAL DENSITY

CONCENTRATION OF PETROL	4th DAY	8th DAY	12th DAY	16th DAY
2.5%	0.05	0.27	0.15	0.17
5%	0.05	0.13	0.09	0.15
7.5%	0.05	0.18	0.32	0.15
10%	0.05	0.12	0.21	0.23

GRAPH 5.2: OPTICAL DENSITY OF VARIOUS CONCENTRATIONS OF PETROL DURING DEGRADATION BY *Pseudomonas sp*



CO_2 ESTIMATION:

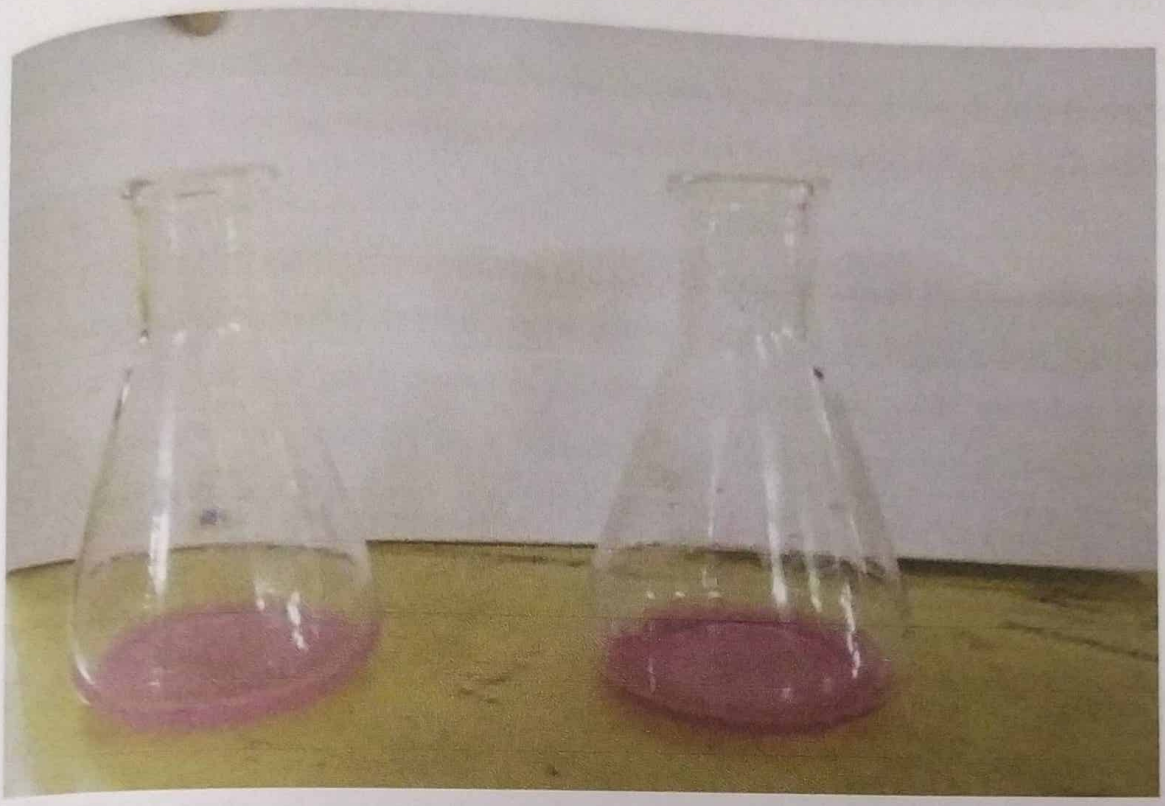
One ml of the fermented broth was taken at 4, 8, 12, 16 days of treatment and titrated against 0.05 NaOH solution. Phenolphthalein was used as an indicator and appearance of stable colour was the end point. The CO_2 released showed an increase during degradation of petrol by Pseudomonas sp. This indicates that the biodegradation of petrol resulted in the production of carbon dioxide which was found to increase with the increasing concentration of petrol were tabulated.

TABLE 5.6: PARAMETER ANALYSIS – CO_2 ESTIMATION

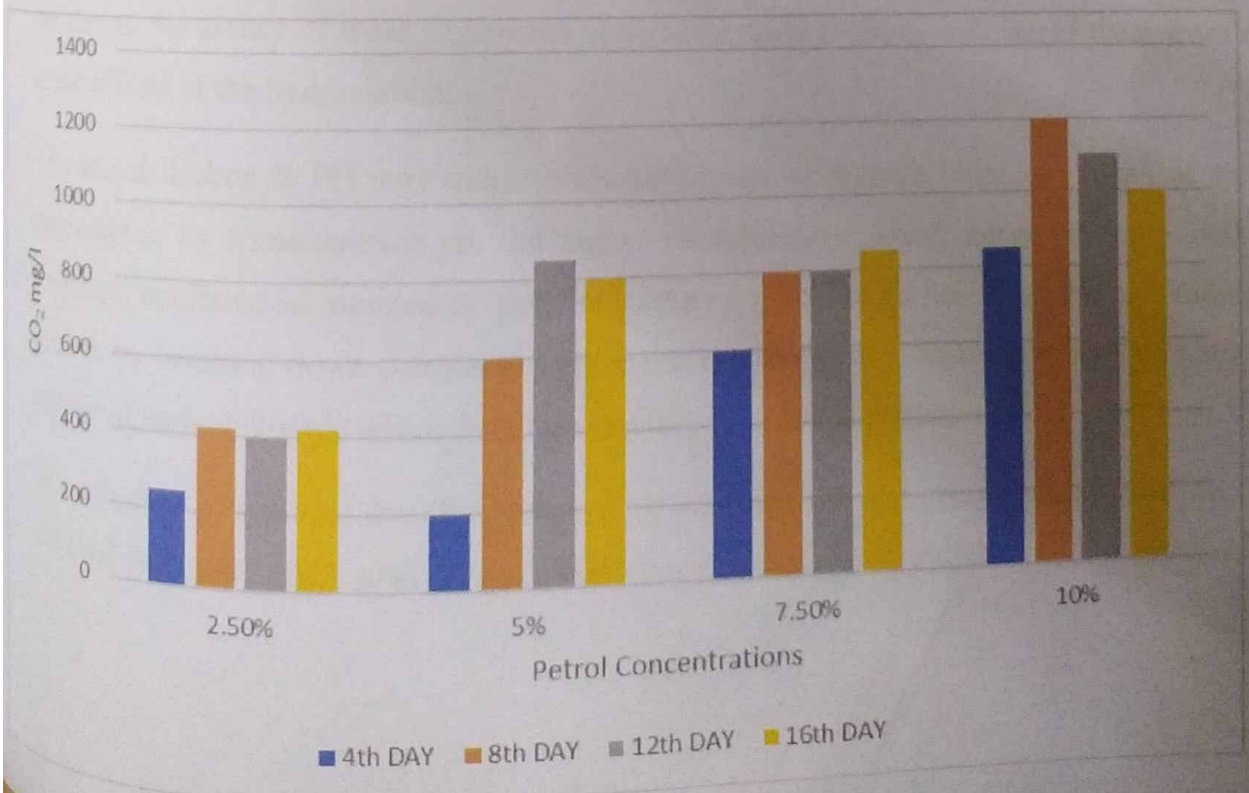
CONCENTRATION OF PETROL	4th DAY mg/l	8th DAY mg/l	12th DAY mg/l	16th DAY mg/l
2.5%	250	420	400	420
5%	200	600	850	800
7.5%	600	800	800	850
10%	850	1200	1100	1000

Graph 5.3 shows the changes in the CO_2 level of the culture medium during the treatment of petrol by Pseudomonas aeruginosa. Petrol degradation resulted in the degradation of CO_2 was observed for 10% petrol after eight days of treatment CO_2 release showed on increase during initial petrol and later remained in the asymptote level except 5% petrol concentration.

Fig 5.29: PARAMETER ANALYSIS – CO_2 ESTIMATION



GRAPH 5.3 : CARBON DI OXIDE RELEASED DURING DEGRADATION OF PETROL BY *Pseudomonas sp*



K.V.Darsa *et al.*, reported that the most rapid and complete degradation of the majority of organic pollutants is brought about under aerobic conditions. The initial intracellular attack of organic pollutants is an oxidative process and the activation as well as incorporation of oxygen is the enzymatic key reaction catalyzed by oxygenases and peroxidases. Peripheral degradation pathways convert organic pollutants step by step intermediates of the central intermediary metabolism, for example the tricarboxylic acid cycle. Biosynthesis of cell biomass occurs from the central precursor metabolism for example acetyl coA, succinate and pyruvate.

Sugars required for various biosynthesis and growth are synthesized by gluconeogenesis. The microorganisms implicated in oil degradation are widely distributed in nature and have been isolated from soil and water with this oil degrading potentials . The microbes capable of utilizing oil and oil products as a sole source of carbon and energy occur practically everywhere in air,water and soil. It is estimated that in one gram of soil polluted by oil, their number increases to 1×10^{6} to 5×10^{7} cells especially if pollution occurred repeatedly and for a long time. Taxonomic characteristics of these isolates identified them as *Bacillus sp*, *Staphylococcus sp*, *Micrococcus* , *Pseudomonas putida*, *Staphylococcus* and *Alcaligens* were also reported to degrade diesel oil.

The rate of crude oil biodegradation in the soil was rapid and it might be due to the Microorganisms in the soil having efficiency in utilizing the residual crude oil as a source of carbon and energy. Crude oil contain hydrocarbon and does not resist attack by Microorganisms. The hydrocarbon utilizing microorganisms isolated from the soil were species of *Bacillus*, *Lactobacter*, *Arthrobacter*, *Pseudomonas*, *Micrococcus*, *Zoopage* and *Articulosporium*. *Bacillus sp* predominated especially in the crude oil polluted soil. This may be due to the ability of these organisms to produce spores which may shield them from the toxic effects of the hydrocarbon.

The initial decline in PH may indicate the formations of organic acids as a result of petrol degradation by *Pseudomonas sp*. the higher concentration petrol, during initial period of treatment exhibited an increase in biomass confirming the isolate being capable of exhibiting growth by breaking down petroleum hydrocarbons. This is also confirmed by the increased release of carbon dioxide which may be a product of petrol degradation.

Wongsa, P.,*et.al* reported that the pattern of degradation showed that the microorganism first attacked the lower and higher hydrocarbon chains and those of middle length were attacked

later in the course of incubation. Considerable information on the microbial degradation is available in the literature, but less is known on the biodegradability of some petroleum commercial products. The dominant mechanism that breaks down these petroleum products is biodegradation , which is carried out by natural microbial population. In the present study *Pseudomonas sp.* is able to degrade petrol which is used as carbon and energy source.

SUMMARY

The present study was conducted to gather and reveal information about the biodegradation of petroleum by microorganisms isolated from oil contaminated soils. Using serial dilution technique the microorganisms enumerated using crowded plate method. One bacterial isolate and one fungal isolate from oil contaminated soil sample were isolated.

The morphological and biochemical characterization was performed for the selected isolates. The bacterial isolate showed positive result for Citrate test and Catalase test; and negative result for Indole test, Methyl Red test, Voges Proskauer test and was identified as *Pseudomonas* sp .

The fungal isolate was identified as *Penicillium* sp after performing Lactophenol cotton blue staining technique and studying its morphological characters.

Hydrocarbon biodegradation by Pseudomonas sp was studied using minimal broth and adding petrol in different measurements such as 2.5ml, 5ml, 7.5ml,10ml respectively then by inoculating 1 ml the bacterial isolate of *Pseudomonas sp* and incubated for sixteen days. During sixteen days of incubation the parameters such as pH, optical density, CO_2 concentration was performed at 4th , 8th , 12th, 16th days of biodegradation analysis.

The rate of biodegradation of petroleum by *Pseudomonas sp* increases with increase in incubation period. It showed an increase in petroleum degradation upto sixteenth day of incubation after that it decreased.

From the present day, it can be concluded that *Pseudomonas sp* was effective in petroleum degradation. It can be used for bioremediation which keeps our planet clean and healthy.

CONCLUSION:

The world in which we live as it is today, is the world in which everything we do as regards human growth, biological, physical, economic, industrial and infrastructural growth, science and technological growth etc. revolves around energy. Apart from the traditional firewood, wind, and hydropower, petroleum hydrocarbon continues to be used as the most principal and versatile source of energy. Pollution is the introduction of harmful materials into the environment these harmful materials are called pollutants. Environmental pollution with petroleum and petroleum products has been recognized as one of the most serious current problems. The harm that oil pollution causes to the ecological environment is well known. It alters the soil biodiversity , reduces organic matter and soil's capacity to act as a filter. It also contaminates the water stored in the soil and groundwater , and causes an imbalance of soil nutrients. Bioremediation is a complex process with biological degradation taking place in the cells of microorganisms which absorb pollutants, where if they have specific enzymes, the degradation of pollutants and their corresponding metabolites will take place. Most petroleum hydrocarbons encountered in the environment are ultimately degraded or metabolized by bacteria like *Pseudomonas sp* because of their energetic and carbon needs for growth and reproduction as well as to relieve physiological stress caused by the presence of petroleum hydrocarbons in the microbial bulk environment. The fuel eating bacteria known as Pseudomonas sp. have evolved a taste for hydrocarbons, a major component of the fossil fuel. This study was therefore designed to monitor rate of biodegradation petroleum by *Pseudomonas sp* isolated from oil contaminated sites. So we conclude that *Pseudomonas sp* can be effective for biodegradation of petroleum.

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PREPARATION OF BIOPLASTIC USING BANANA PEEL (*Musa acuminata*) AND DETERMINATION OF ITS ANTIMICROBIAL ACTIVITY.

A PROJECT SUBMITTED TO

ST.MARY'S COLLEGE (AUTONOMOUS), THOOTHUKUDI

Affiliated by Manonmaniam Sundaranar University

In partial fulfillment of the requirements for the award of the degree of

BACHELOR OF SCIENCE IN MICROBIOLOGY

SUBMITTED BY

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M. PRIYA (19SUMB27)

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DEPARTMENT OF MICROBIOLOGY

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May – 2022

CERTIFICATE

This is to certify that the project work entitled **"Preparation of bioplastic using banana peel (*Musa acuminata*) and determination of its antimicrobial activity."** submitted to **St.Mary's College (Autonomous), Thoothukudi** affiliated to **Manonmaniam Sundaranar University, Tirunelveli** for the partial fulfillment for the award of Master of Science in Microbiology is a bonafide research carried out by **K.Pradeepa, M.Priya, H.Sabira banu, M.S.Sameen Afroze, M. Santhiya, M. Saranya, S. Sheba Christina** under the guidance and supervision of **Dr.C.Siluvai Kirubagari Aneeshia, M.Sc., Ph.D.** Assistant Professor of Microbiology St. Mary's College (Autonomous), Thoothukudi, for academic year 2021-2022.

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DATE : 25.05.2022

DECLARATION

I hereby declare that the project work entitled **" Preparation of bioplastic using banana peel (*Musa acuminata*) and determination of its antimicrobial activity"** is a bonafide record of the work completed by me during the academic year 2019-2022 in St. Mary's College (Autonomous), Thoothukudi and submitted as a partial fulfillment of requirements for the award of the Degree of Master of Science in Microbiology prescribed by the Manonmaniam Sundaranar University. I also affirm that this is a original work done by me under the supervision of **Dr.C.Siluvai Kirubagari Aneeshia, M.Sc., Ph.D.** Assistant Professor of Department of Microbiology, St. Mary's College (Autonomous), Thoothukudi.

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ABBREVIATIONS

ml	-	Millimetre
kg	-	Kilogram
DMSO	-	Dimethyl Sulfoxide
EMB	-	Eosin Methylene Blue
TCBS	-	Thiosulfate citrate Bile salts Sucrose agar
PHA	-	Poly hydroxy alkenoate
P4HB	-	Poly (4 – hydroxy Butyrate)
PHB	-	Poly hydroxy butyrate
PHBH	-	P – Hydroxy – Benzoate Hydroxylase
MHA	-	Muller Hinton Agar
%	-	Percentage
°C	-	degree Celsius
PLA	-	Poly Lactic Acid
GHG	-	Green House Gases
PHH	-	Poly hydroxy Valerate
CH_4	-	Methane
Co_2	-	Corbon dioxide
USS	-	United States Standard
g	-	gram
kcl	-	Kilo calories
mg	-	Milligram
mm	-	Millimetre
Vit	-	Vitamins
+ve	-	Positive
-ve	-	Negative

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INTRODUCTION

INTRODUCTION

PLASTIC:

Plastic is a polymer material consisting of a wide range of synthetic or semi synthetic compounds. "The word plastic comes from the Greek word 'plastikos' which means to be molded into different shapes" (Joel 1995). Plastics are low budget, light weight, strong, durable, corrosion-resistant materials, with high thermal and electrical insulation properties (Andrade *et al.*, 2016).

Plastic waste is derived from hydrocarbon - based material, which can be used for incineration or boiler. Despite burning of plastics at lower temperature it may liberate deadly and poisonous chemical gases into the air, including dioxins which is corrupting to the human being on the other hand, if plastic is made from 100% hydrocarbon-intermediates, it is very serviceable but it leads to slow degradation. According to the plastic pollution organisation, plastic materials that are used in our daily consumption has become attractive that initiates an indisputable behavioral need which led to over consuming but at the same time it also pollutes the environment. Plastics, yet being something that's destroying the biomes, it is something that can be of precious use in the field of construction if the waste processed, used and recycled discreetly as per needs.

To reduce the problem of plastic waste that has continuously suffocated the planet and leading to contamination of the environment, there is another way of choice to give a solution to this issue, form where bio plastic emerges. Hence, there is need to produce plastics from materials that can be readily eliminated from our biosphere in an "eco-friendly" fashion.

Fig:1 Hazardous Plastic

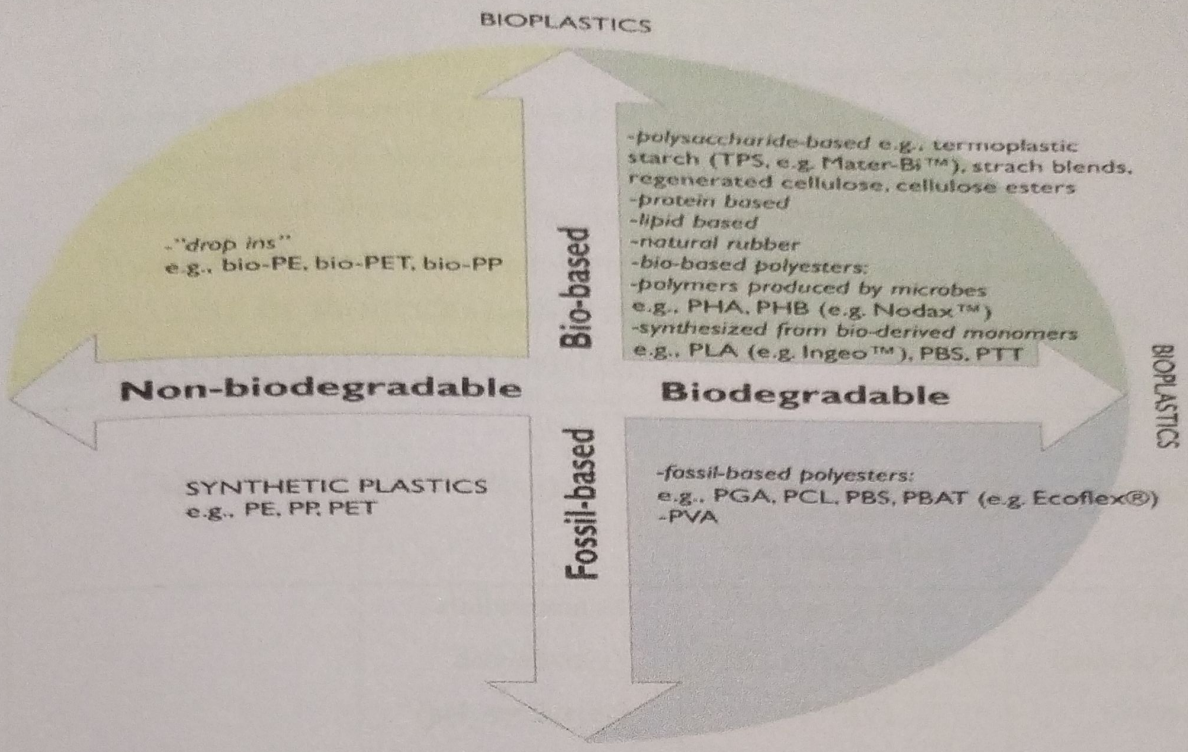


Fig: 2 Biodegradable and Non-Bio degradable

BIO PLASTICS:

The bio plastics are biodegradable plastics derived from plant and/or microorganisms, instead of fossil fuels. As such as conventional plastics, the bio plastics can be used in several ways under ordinary condition The biodegradable plastics are defined as materials whose physical / chemical properties undergo deterioration and completely degrade when they are exposed to micro organisms into carbon dioxide as in aerobic processes, methane as in anaerobic process with in a specific time limit. The time required to decompose depends on the material, environmental conditions such as temperature and moisture and the location where decomposition takes place. Since bio plastics are 100% degradable, the large emissions of carbon dioxide that occur during plastic production are reduced with the production of bio plastics between 0.8 and 3.2 tons (BIO PLASTIC BASED ON BANANA PEEL Ruth Castilo, Eliasury Escobar ***et al.***, 2015)

TYPES OF BIO PLASTIC:

Bio plastics are a large group of polymers; their list is always increasing due to new innovation and search for improvement of qualities. Their types include:

Starch -based Simple bio plastics produced from corn starch

Cellulose -based - Produced with cellulose esters and cellulose derivatives

Protein -based - Formed from proteins such as wheat gluten, casein, and milk

TABLE 1 : LIST OF BIODEGRADABLE PLATE DERIVED FROM PLANT, BIO-BASED MICROORGANISMS AND ANIMALS:

Types	Plant	Bio-Based Microorganism	Animal
BIODEGRADABLE (Bio-based plastic)	Cellulose and its derivatives (polysaccharide) Lignin Starch and its derivates (monosaccharide) Alignate (polysaccharide) Lipids (triglycerides) Wheat, corn, pea, potato, soy, potato (protein) Gums (e.g., cis-1,4-polyisoprene) Carrageenan PLA (from starch or sugarcane)	PHAs (e.g., P4HB, PHB, PHBH, PHBHx, PHBV) PHF Bacterial cellulose Hyluronan (polysaccharide) Xanthan (polysaccharide) Curdlan (polysaccharide) Pullulan (polysaccharide) Silk (protein) gellan	Chitin (polysaccharide) Chitosan (polysaccharide) Hyaluronan (polysaccharide) Caesin (protein) Whey (protein) Collagen (protein) Albumin (protein) Keratin, PFF (protein) Leather (protein)

Aliphatic polyesters - They are bio-based polyesters such as poly lactic acid (PLA), polyhydroxy valerate (PHV), polyhydroxyhexanoete (PHH) etc.

Organic polyethylene -Formed from fermentation of raw crops such as sugar cane, and corn.

TABLE 2 : COMPARISON BETWEEN CONVENTIONAL PLASTIC AND BIO PLASTIC:

Conventional Plastic	Bio plastic
• Eco-toxic	Non-toxic
• Unsustainable	More sustainable
• Reduces soil fertility	Increases soil fertility
• Increases global warming	Eco-friendly
• Leads to a biotic depletion	No harm to a biotic factor
• More energy usage during production	Less usage of energy

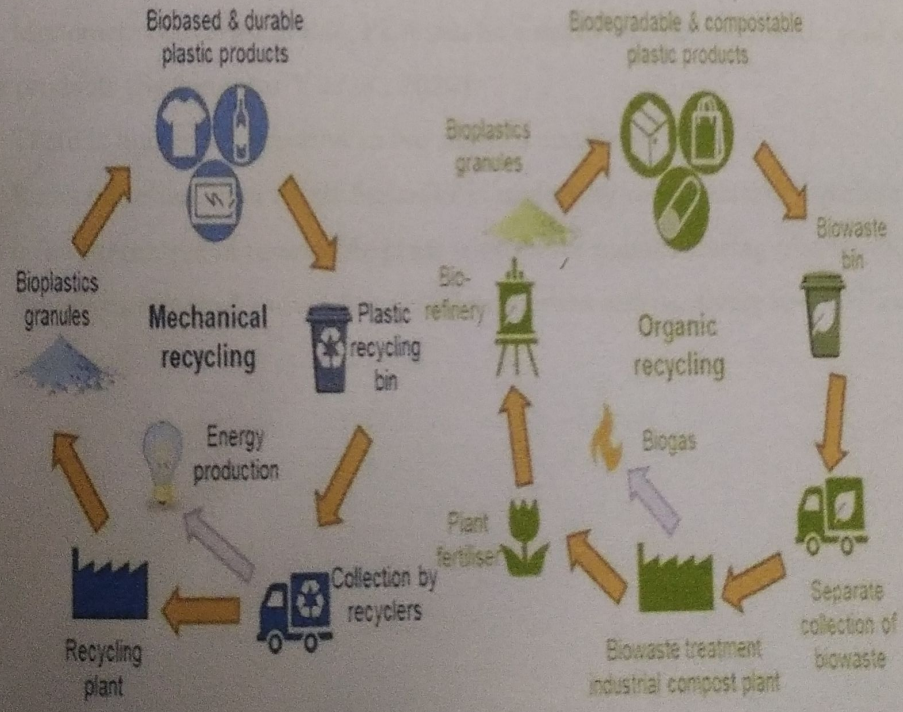


Fig: 3 Mechanical Recycling – Organic Recycling

ADVANTAGES:

Eco-friendly-Conventional plastics are formed from oil-based sources. Exploration and utilization of oil-based materials to form plastics is harmful to the environment. Whereas, bio plastics are normally obtained from renewable sources, then reducing pollution of the Environment. (Pathak S *et al.*, 2014).

Biodegradable -Bio plastics are easily degraded by microbes into end products such as water, carbon dioxide, methane, biomass, and inorganic compounds in few months thereby saving the environment from the pollution elicited by conventional plastics. Some of the plastics are durable, requiring few years to be completely degraded. (Chen YJ ***et al.***, 2014). This property is not harmful to the environment because the end-products are naturally occurring in the ecosystem (Folino A ***et al.***, 2020).

Compostable -Bio plastic can be broken down by microbes in controlled conditions in the laboratory, industry, or home composting facility. Parable, PLA is an example of compostable bio plastic used in single-use packaging.

Recyclability -Bio plastics can be mechanically recycled.

Incineration-Bio plastic are subjected to incineration in a situation of slow biodegradation. This presents less effects to the environment compared to incineration of conventional plastics.

Monomer recovery-Parable, PLA can be used to recover 99% lactic acid at end-life of the PLA products (Sarkingobir Y *et al.*, 2020)

There is higher job creation in bio plastics economy.

Reduced reliance on fossil fuels-Oil is underway to extinction, therefore there is need to search for alternative in renewable plant sources for manufacturing plastics (Kate *et al.*, 2011).

Bio plastics include novel functional properties and relatively low GHG emissions during manufacture.

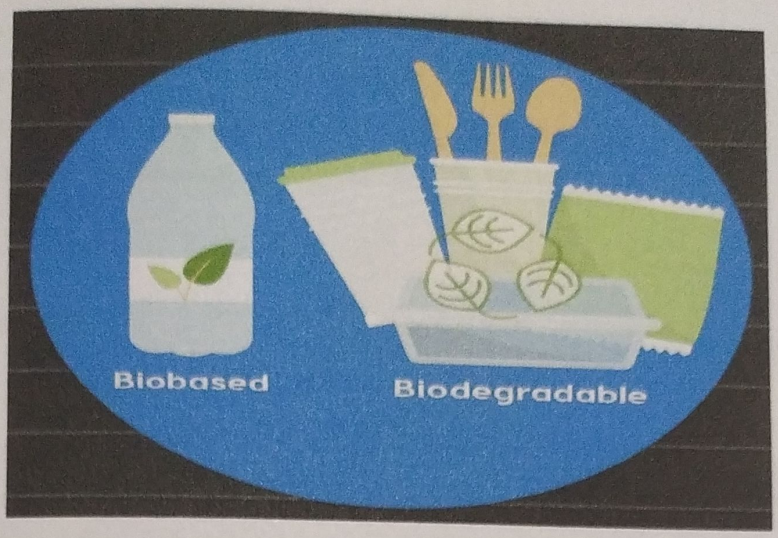


Fig : 4 Bio-degradable Products

TABLE 3 : COMPARISON BETWEEN BIO-PLASTIC AND PETRO PLASTIC.

Characteristics	Bio plastics	Petro plastics
Renewable	Yes, or partially	No
Sustainable	Yes	No
Break down in the Environment	Biodegradable and/ or compostable	Some degradable by polymer oxidation
Fossil fuel usage	Usually, low	Relatively high
Arable land use	Currently low	None
GHG emissions	Usually, low	Relatively high
Polymer range	Limited but growing	Extensive

Most bio plastics can be broken down in the environment by micro-organisms in a process called biodegradation. This process produces CO_2 and water under aerobic conditions or CH4 under anaerobic conditions (in the absence of air) such as in landfill. Mixed bio plastics are usually biodegradable, but some are not and can be either recycled or processed for energy recovery.

APPLICATIONS:

- **MEDICAL DEVICES:**

Biodegradable dental implants, made of porous polymer particles, are being used to quickly fill the hole after a tooth has been extracted. Biodegradable plastic pins, tacks and screws which are used to hold shattered bones together while they heal, to reattach ligaments and for delicate reconstructive surgery for ankles, knees and hands.

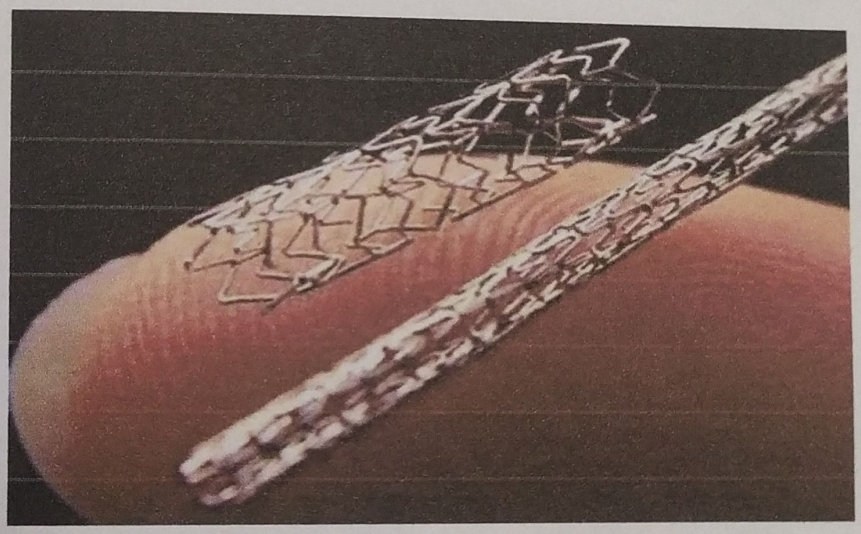


Fig: 5 Bio Medical devices

- **AGRICULTURE AND HORTICULTURE:**

Foils, yarns, and nets made out of bio plastics help to protect freshly created slopes and mounds and secure them from erosion until the roots of the plants have developed as required. Compostable seed belts and active component capsulations made out of bio plastics have also proven to be beneficial. Horticulture includes films for banana bushes and grapevine bushes which have to be protected from dust and environmental influences.

Fig: 6 Bio Agricultural Products

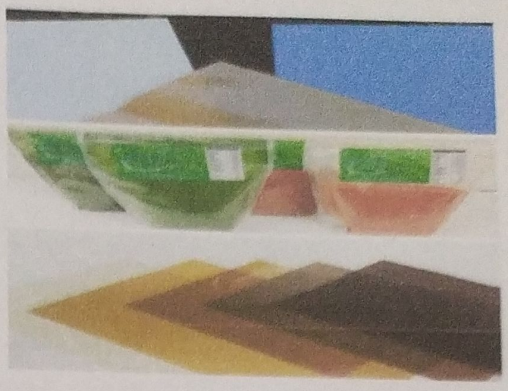


Fig: 7 Soloplast Compostable films

- **FOOD PACKAGING:**

Bio plastics for packaging is applicable for both short shelf life as well as long which shelf-life products which do not require that much very good oxygen/water barrier properties entails the commercial exploitation of these bio packaging materials. It is clear that bio-based packaging materials offer a versatile potential in packaging industry.

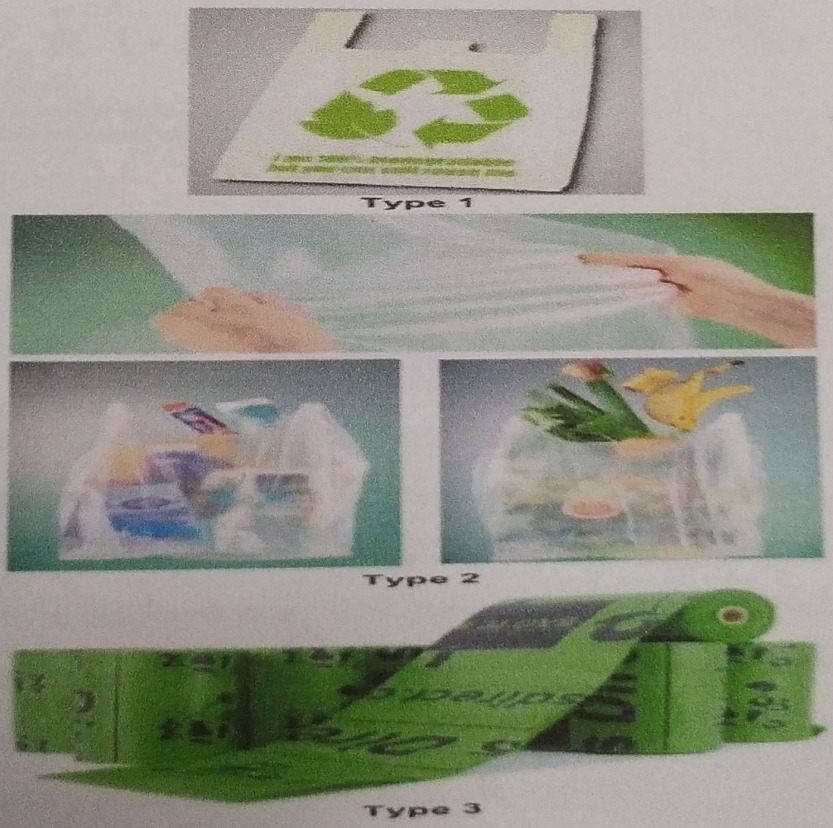


Fig: 8 Bio Bag Types

GLOBAL BIO PLASTIC MARKET:

The global bio plastic market is predicted to be growing about 20-25% per year. Roughly about 10%-15% bio plastics of the total plastics market will increase its market share to 25%-30% by 2020. The bio plastic market reached over 1 billion USS in 2007 and it will be over 10 billion by 2020. More and more companies are entering and investing in this market.

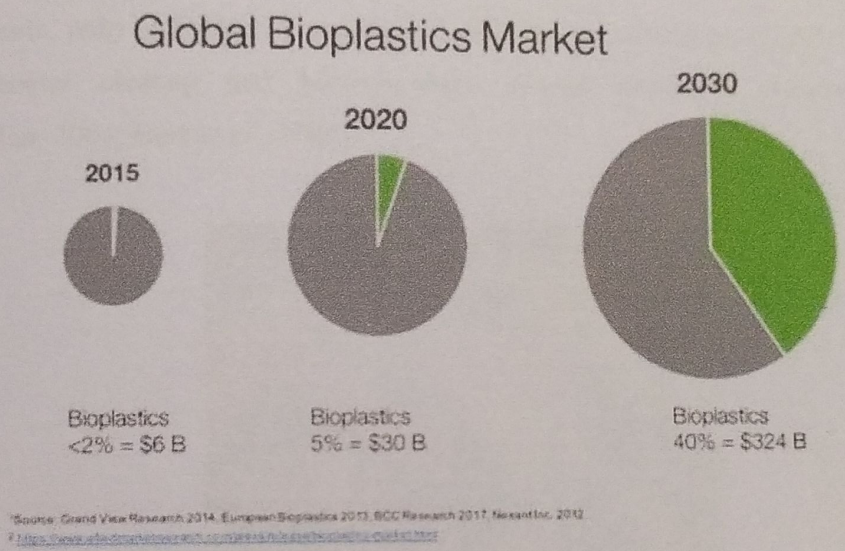


Fig: 9 : Global Bio-plastic Market

EVOLUTION OF BIO PLASTIC:

Food waste is a challenge for sustainable development, meanwhile plastic waste is a big problem in environmental pollution. This made scientist to explore the potential of food waste as bio plastic material as an environmentally friendly alternate packaging. Food wastes have the potential to be developed as bio plastics if they contain biopolymers such as starch, cellulose, or another biopolymer. The development of bio plastics from food waste has a double benefit that can be solve two problems indirectly, namely reducing plastic waste and food waste at the same time, thereby promoting environmentally sustainability.

USAGE OF BANANA FOR BIO PLASTIC:

Banana is a tropical fruit grown worldwide of about 122 countries. World leading banana and plantain producers are India, China, Uganda, Ecuador, Philippines,

and Nigeria. The production of Banana in India was 26509096 Metric Tonnes (25.58%) in 2015. Main banana producing states in India are Tamilnadu, Maharastra, Karnataka, Ejujarat, Andrapradesh, Assam, Bihar, Madhya Pradesh, Some varieties of Banana include Dwarf cavendish; Robusta; Rasthali ; poovan; Nendran; Red Banana; Karupaalli; Pachanadan; Virupakshi etc. Banana plantation occupies large part of the land, but it is acontamination source because after harvest, the tree is cut down and abandoned in the fields, which foments sigatoka (chillet *et al.*, 2009). Banana fruit peels make up a significant quantity of wastes produced from processing of banana. Banana peel has been utilized for various industrial applications including bio-fuel production, bio sorbents, pulp and paper, cosmetics, energy related activities, organic fertilizer, environmental cleanup and biotechnology related processes (Morian, 1987; Gunaseelan, 2004; Bori *et al.*, 2007)

Fig: 10 Banana Peel Bioplastic

Recovery of banana waste loss from production and processing

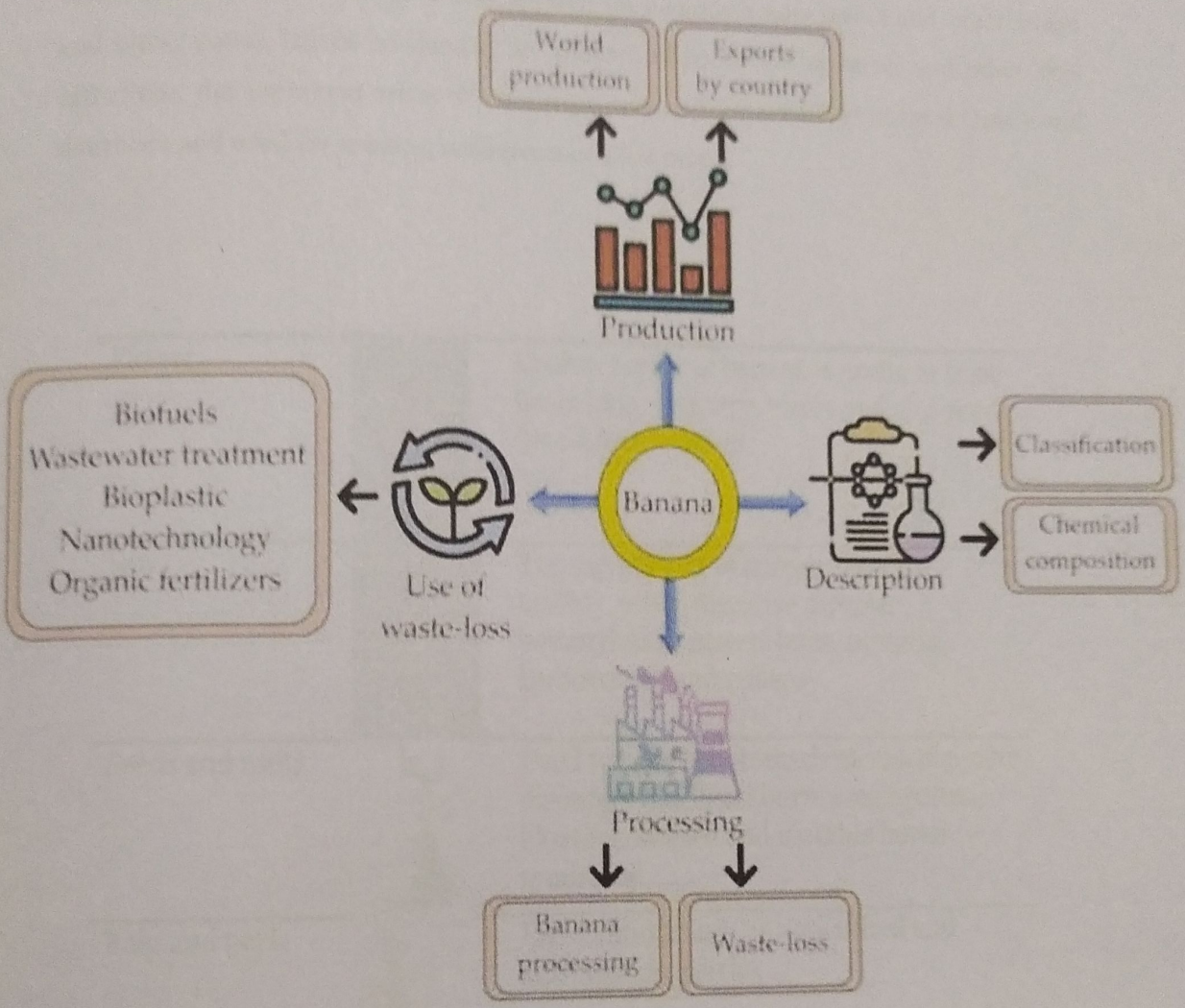


Fig: 11 Recovery of banana waste

Parts of banana plant and their health benefits:-

All parts of the Banana plant have medicinal properties, the flowers for bronchitts, dysentry, and fatal ulcers. Cooked flowers are given to treat diabetics; the astringent plant sap in the cases of hysteria, epilepsy, leprosy, fevers, haemorrhages, acute dysentry, diaarhoea, and also applied on haemorrhoids, insect and other stings and bites; young leaves of banana are placed as poultices on burns and other skin afflictions; the astringent ashes of the unripe peel and the are taken in for dysentry and diarrhoea and used for treating malignant or fatal ulcers.

Flower		Cooked flower of banana is useful to treat bronchitis, dysentry, ulcers and also good for diabetes patient.
Sap		The sap portion of banana treat the leprosy, fever, digestive disease, hemorrhoids, insects bites, hysteria, hemorrhage and epilepsy.
Seeds and roots		Used to treat the stomach ulcers, digestive disorders like heart burn, constipation, bloating, nausea and irritable bowel syndrome.
Pulp and peel		Have antioxidant, antimicrobial and antifugal properties

Fig: 12 Benefits of Banana Parts

Making bio plastic from banana peel instead of petroleum-based plastic is an effective solution that leads to a reduction in the use of non-renewable raw materials. Although the peel contains minerals, calcium, Magnesium, Potassium, Sodium, Zinc and Iron its main component starch (18.5%) (Riya Singh).The functional groups -OH. In a banana peel can be exploited to adsorb pollutants. The materials which are synthesized using banana peels have the properties of pliability, user friendliness, and

most importantly these materials are dehydration tractable. Nowadays, itcrucial to have a potential bio plastic material in alternative over conventional plastics. (Yarododdi J., Patil. V., Gjanchari. S *et al*., 2016)

SCIENTIFIC CLASSIFICATION OF BANANA:

Kingdom	:	Plantae
Class	:	Liliopsida
Order	:	Zingiberales
Family	:	Magnoliophyta
Genus	:	*Musa*
Sectio	:	*Tracheobionta*
Species	:	*Musa acuminata*

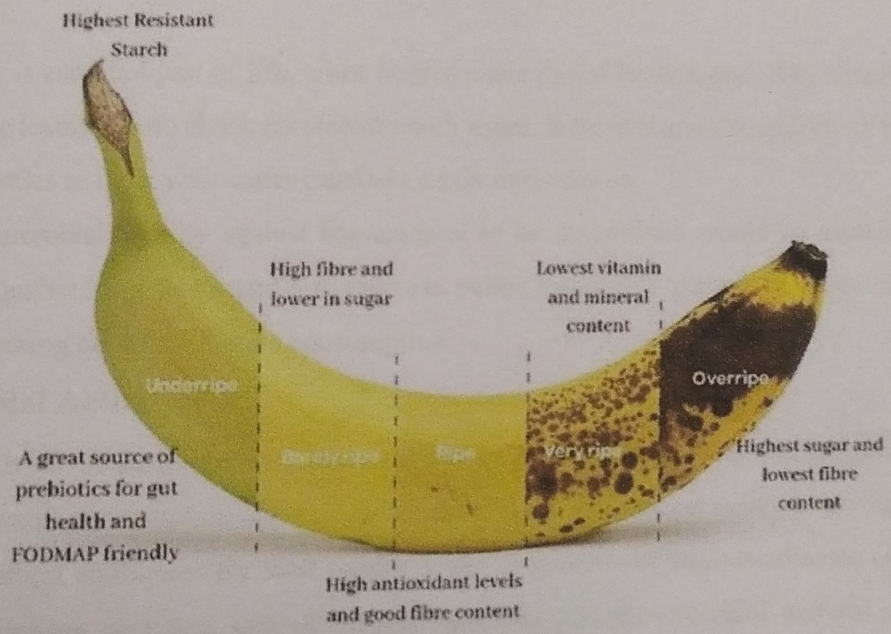


Fig: 13 Nutritional Properties of Banana

NEED FOR ANTIMICROBIAL ACTIVITY:

The Banana peel-based bio plastic had been obtained. When these bio plastics are applied over commercial purpose such as bags, toys, packaging materials and bottles, these application-oriented concept cannot be directly implemented. They should have higher safety for consumption. Thus, without practical examination we can't assure safety. Hence, the need

of antimicrobial had arised. When a packaging material being prepared out of banana. Peel should have the capacity to resist the microbial entry and should not cause spoilage to the product present inside. Hence the nature of banana peel to let the resistance of microorganisms can be determined by antimicrobial activity.

TOYS:

Children are very playful, when the toys made out from banana peel, is contact with child's metabolism after bitting them it should not cause any harmful pathogenic attack leading to microbial infection.

BAGS:

Poly bags are prepared from chemical-based polymers that are literally unsafe for consumption and difficult to decompose. Manufacturing bags from banana peel should give assurance that these bags can withstand against pathogenic microbes, entry, environment conditions and resistant nature of strains.

BOTTLES:

Water is essential part of life, when bottles made out of banana peel, they should non-toxic in nature leading to no chemical reaction with water, there is also no possibility of banana peel-based bottles to meet with water causing certain intoxication.

Hence, Antimicrobial activity against banana peel to be determined would be significantly impact on manufacturing sector and also increase public awareness towards its nature against all aspects assuring complete human consumption.

Antimicrobial Activity:

The advent of antibiotic resistance of certain food pathogens on the reluctance of consumers towards consumption of chemically treated good have encouraged the development of safe and natural antibiotics for food products and treatments of various ailments (Jadhav *et.al.*, 2013) Banana peel is a part of banana fruit that has antimicrobial activity against microorganisms. The peel was used for various purpose like acne, warts treatment and also as hair mask, face mask. The antimicrobial activity of *Musa acuminata* was determined by using ethanol as an organic solvent. The antimicrobial activity of banana peel extract was determined against standard strains. The bacterial strains showed sensitivity against alcoholic extract of banana peel. Well Diffusion assay has been carried out to detect the antibacterial activity of alcoholic extract of banana peel. Organic solvents dissolve more active compounds than water. Hence ethanol was used to dissolve more active compounds from banana peel. The bio active compounds contained in plants are responsible for the medicinal properties. (Singh *et.al.*, 2013) evaluated the antibacterial activity of three different colour banana such as red, green, yellow

against clinical pathogen. The red banana showed a maximum zone of inhibition of 27mm. The yellow banana peel exhibited 20mm of inhibition zone. Ighodaro demonstrated that the aqueous extract of *M.paradisiaca* gave an antibacterial effect against *Staphylococcus aureus*, *Escherichia coli* and *Proteus mirabilis* more than antifungal. Sumathy studied the antifungal and antimicrobial properties of yellow banana against different gram positive and negative bacteria. Therefore, banana peel wastes extracts could be potential antimicrobial alternatives and may be effective to utilize it as a natural source of antimicrobial agent in pharmaceutical industries. Utilizing the peels as antimicrobial agents can help to recover the existing environmental waste problems and at the same time offer enormous benefits to mankind.

Recycling of Waste:

Peels obtained from fruits are usually discorded or disposed in an improper manner that leads to several environmental problems. Peel that are considered to be by products from industrial processing or consumption of the fruit contributes about 15-20% of the fruit which is usually discorded as waste (Beerh *et al.*, 1976). Globally, India is the leading producer of fruits. Fruits after consumption leave a peel which is a nuisance to the environment of a solid waste. Fruit peels are considered to be novel, easily available, efficient, affordable eco-friendly, natural and economic source for antioxidants and antimicrobial agents and banana peel is one among them. Banana being a tropical fruit grown worldwide in about 122 Countries having its production on metric ton. Every part of banana has medicines properties similarly banana peel also has its own properties Which is not consumed by humans leading to waste. Currently utilization of fruit in different ways would gain attention due to their nutritional and pharmacology properties. This is not only handles waste management but molds to every aspect of manufacturing new products significantly as an antimicrobial agent.

AIM AND OBJECTIVE

AIM & OBJECTIVES:

- To explore possibility to produce a plastic which is biodegradable.
- To reduce the production cost of bioplastic.
- To find out cheap substrate for the production of bioplastic.
- To decrease the environmental pollution, litter or debris caused due to conventional or petroleum-based plastics, by using bioplastics as an alternative source.
- To have idea about the production and process development for bioplastics production from banana peel.
- To access the antimicrobial activity of ethanolic extraction of banana peel (*M.acuminata*)

REVIEW OF LITERATURE

Wanjun Liu., *et al.*, (2005), explained that the Soy Based green Composites had been fabricated by extrusion & Injection Molding.

Matook Saif Mokbel., and Fumio Hashinaga., (2005), illustrated that ethyl acetate & water soluble fructions of green banana peel displayed high antimicrobial & antioxidant activity.

Noor Fatimah Kader., *et al.*, (2007), mentioned that combination of two biopolymers corn-starch, banana peel could be used as an alternative in the production of Bioplastic.

G.A. Ayoola., *et al.*, (2008), stated that Volatile oil of Tangenine fruit peel was active against many gram positive and gram Negative Bacteria. Where it had possessed their Antibacterial & Antifungal Properties. This had the Potents of antimicrobial Agent.

N.S.AL. Zoreky., *et al.*, (2009), Speculated that *Listernia monocytogenes* showed Sensitivity to Pomegranated Peel extract in agar diffusion method. Which could be employed in food preservation & pharmaceutical Purpose.

Maruti. J. Dhanavade., *et al.*, (2011), painted that the Lemon acts as antimicrobial. Agent which helped in the Presentation of skin Infection.

B Singh and Deepanish Sharma., (2012), pointed plastic available in market is very dangerous and non-biodegradable. So that biodegradable plastics should be produced and used day by day. He also highlighted the applications, production, types, challenges, sustainability, process development of bio plastics.

B.S.Saharan., *et al.*, (2012), elucidated that Bio plastic preparation substance have Increased as means to Save fossils fuels, Reducing carbondioxide, emission and plastic Waste. In future mineral oil price will increase. Hence Bio plastic depends on effort towards performance equipment towards fulfiling price.

Zuvairea Nazren Mohd Sirajudin., *et al.*, (2013), highlighted that ethanol extract of *Musa paradisiaca* showed the high antimicrobial agent. Regarded as a Natural antimicrobial agents.

Zaienab Adil Ghani Chabuck., *et al.*, (2013), explained that Aqueous extract of yellow banana peels could be a good antibacterial Agent against gram +ve gram -ve Bacteria.

Manta Arora and Parminder Kaur., (2013), mentioned that the orange pulp had more microbial & activity than its peel. This could be used as an alternative in food, pharmaceutical & cosmetic Industry.

Ying Jian Chin., *et al.*, (2014), corroborated that Bioplastic are growing rapidly & have many applications, these plant Based Bioplastic can be recycled & would have sustainable world economy.

Sonia Parashar., *et al.*, (2014), mentioned that the arrival of antibiotic Resistant strains & adverse effects of synthetic Antioxidants had led to adverse search of Natural Antimicrobial &

Antioxidants had led to adverse search of Natural Antimicrobial & Antioxidant sources. Many Fruits & Vegetable Peels holds a Potential for a New, safe & effective Antimicrobial & Antioxidant Agent.

Pudyii Astuti., *et al.*, (2014), described that antimicrobial edible film from banana was prepared many due to a compound hydrocolloid & Application as food Packaging material was Supported by addition of clone oil by prevention of bacteria and fungi.

Ying Jian Chen., *et al.*, (2014), concluded that bio plastics are growing rapidly and has advantages in many applications their carbon foot print is lower and helping the world with the increasing problems of waste and debris.

Melissa B Agustin., *et al.*, (2014), explained that bio plastic based on starch being the matrix angle cellulose Nanocrystals from Rice straw as reinforcing filer were prepared Cellulose Nanocrystals had been the key Role for the Moisture Resistance of Bio plastic.

Suraj Premal Kapadia., *et al.*, (2015), Explained banana peel had antibacterial activity against *Pseudomonas gingivalis_& Aspergilus actinomycetemcomitans*.

Nafisa Jabeen., *et al.*, (2015), illustrated that applicable Nature of Bioplastic had been good for short long- shelf-life products, advancements in them could led to exploitation of Biobased packaging materials.

Ezgi Bezirhan Arikan and Havva Duygu Ozsoy., *et al.*, (2015), stated that bio plastics are one of the most innovative environmental friendly materials developed and he looked at the aspects of bio plastics from the perspective of sustainability, advantages, disadvantages and standards.

Nafisa Jabeen., *et al.*, (2015), concluded that bio plastics are for packaging of both short and long shelf life products and also offers versatile potential in packaging industry.

Deeneshwaran S Manimaran., *et al.*, (2016), the film were successfully prepared from banana peels by casting methods. The characteristics of the films were evaluated with different glycerine content (20ml, 25ml, 30ml) using soil burial degradation test.

Jayachandra Yaradoddi., *et al.*, (2016), shows the importance of synthesis of bio plastic material by using banana peel and the degradation tractability of the developed production

Priyanka Asai Thambi., *et al.*, (2016), stated that the Mango Peel Powder possessed a great Nutraceutical Potential Which was exhibited by its effective antimicrobial activity against various food borne Pathogens.

Pratik. B. Kamble., *et al.*, (2017), explained that pectin was extracted from solid waste by extraction process and it was by the parameter's moisture, ash content methoxyl content anhydrouronic acid content degree of esterification and equivalent weight.

Noor Fatlman Kader Sultan and Wan Lutfi Wan Johari., *et al.*, (2017)., stated that the composite of banana peel was successfully formed they were able to provide a tensile strength of 34.72 N/m2 concentration and characterized by FTIR analysis.

Yaseen M. Galali, *et al.*, (2017), compared that the Seed & peel extract of red grapes antibacterial Nature and Inhibited the growth of Microorganism But fungal Species resisted the seed & Peel extract of Red grapes.

Pratik.k.kambk., *et al.*, (2017), emphasized that the pectin extraction was of lower Quantity but their yield was high with low purity. The high Quantity Pectin could be obtained by Purification.

Bharathi Prakash., *et al.*, (2017), mentioned that the antifungal properties of Banana peel extract was effective against *Aspergillus flavus* & *Penicillium* Sp.

Isroi., *et al.*, (2017), reported that the bioplastic was obtained from cellulose of oil palm empty Fruit bunch with Cassava starch as a matrix and glycerol as a plastisizer.

M.R. Gaonkar., *et al.*, (2018), reported that bioplastic obtained from banana peel could be used as a carrying Bag and to prevent growth of Bag & Fungi Sodium Metabisulphite is used. The degradation period depends on the atmospheric condition.

Gajendra kumar Rana., *et al.*, (2018), Concluded that Cethole Banana plant such as peel, stem could be used in Packaging & Pharmaceutical fruit could be used in production of high value cellulose products the Papers, Fiber.

Norhafezah kasmuri., *et al.*, (2018), elaborated that Potato – starch-based bioplastic with egg shells & Chitosan as filler had been produced. This filler had Improved this Bioplastic & its biodegradation is faster too decompose quickly.

Professor Manasi Ghamade., *et al.*, (2018), pointed that the plastic produced from banana peel could bear the weight one and half times degradation of bio plastic starts after 3 to 4 months from the date of production when compared to petroleum plastic.

M.R. Gaonkar., *et al.*, (2018), pointed than Bio plastic film can sustain the weight near 2kg. To prevent the growth of fungi and bacteria sodium meta bisulphite is used. The degradation starts after.

Gajendra Kumar Rana., *et al.*, (2018), stated that waste management has been increased worlwide at present the pollution by various means day by day. To overcome these pollutions, he has noted that it is possible by utilizing waste.

Maulid Lubis *et al.*, (2018), that Avacado Seed starch is a natural Biodegradable polymer that had potential to replace synthetic polymer which was used in Packaging Section. Hence Bio plastic from Avacado Seed starch was developed.

N.A. Azieyanti., *et al.*, (2019), stated that natural-based materials were much less costly when compared to chemical based, natural based materials produce strong and durable bio plastic composites rather than chemical based.

Nur Athirah Huzaisham., *et al.*, (2019), works towards to develop and produce biodegradable plastic to substitute non-biodegradable plastic.

May Zon Kyawt., *et al.*, (2019), suggested that, research into bio plastic development should be further continued so that bio plastic with more diverse application can be created from fruit waste.

Muthusamy Selvamurugan and Sivakumar Pramasivam (2019), suggested bio plastics are not the only solution to the problem of plastic pollution. Control of plastic pollution comes in the behaviour of consumer in buying, consuming and disposing plastics.

S.Chodijah., *et al.*, (2019),focused to determine the effect of pectin produced from on banana peels the characteristics of biodegradable plastic film.

Selvamurugan & Sivakumar., (2019), pointed that Intensificating Research of large-scale Bioplastic production alone could not be a solution to plastic waste depends on consumer behaviour on buying a disposal of plastic, The Public awareness of Bioplastic.

S.Chodijah., *et al.*, (2019), stated that bioplastic could be obtained from the usage of pectin from Kepok banana peel.

Mohammed Saleem., *et al.*, (2019), Compared the Antimicrobial Nature of lemon, Orange & Banana Peel where Lemon had highest & Banana peel lowest, but all had capacity to acts as a antimicrobial drugs which had safer disposal when analyzed with Conventional drugs.

Nur Athirah Huzaishm and Noraini Marsi., (2020), demonstrated that banana peel-based bioplastic had shown good physical & mechanical performance, it degraded much faster when compared with commercial Biodegradable plastic.

Masanori Yamada., *et al.*, (2020), explained that the Production of Bioplastic from soybean was stable in water and also had a good bending strength value with a necessary Biodegradable property.

M.O.Ramadhan., *et al.*, (2020), mentioned that The Bioplastic could be prepared by the waste From Food Processing Industry or household Consumption. This type of Bioplastic production would be a double benefit. Which solved two problems. Reduce plastic food waste.

Jongnin lee., *et al.*, (2020), demonstrated that the Banana based plastic suited well for packaging Materials than Styrofoam. They also found the feasibility of sample product better secured with high protection.

Indhu Singh Sankhla., *et al.*, (2020), elaborated that many fungal strains such as Penicillium, Fusarium, Aspergillus had the ability to degrade Bioplastic confirmed as strong degrades.

Hence, development of efficient strain could also help in degrades. Hence, development of efficient strain could also help in degradation of Bioplastic.

Monica loyago – Castillo., *et al.*, (2020), expounded that hydroethanolic extract of M.X Paradisiaca Peel had a Broad spectrum of Biological Activities such as a good antifungal & antibacterial agent.

Ashok Bankar., *et al.*, (2020), investigated that Banana Peel Extract mediated golden Nanoparticles could be used Antimicrobial Agent as it showed Antibacterial & Antifungal activity against different strains.

M O Ramadhan and M N Handayani (2020), showed that many food waste have the potential to be developed or recycled into bio plastic if they have contain bio polymers such as starch, cellulose or other bio polymers and concludes bio plastics promote environmental sustainability.

Nur Athirah Huzaisham and Noraini Marsi (2020), aims to utilise banana (*Musa paradisiaca*) peel to be incorporated into biodegradable planting bag and to evaluate its mechanical and physical properties to compare with commercial biodegradable planting bag.

SNA Razak., (2020), focused on synthesis of bio plastic material, the properties of bio plastic, its strength, chemical composition, physical properties.

Pratiksha and Dr.Saswati Datta (2020), they concluded that the films produced from banana peels had potential application and it can be used for food packaging to enhance the quality and to protect the environment at same time.

Bhupender Kumar., *et al.*, (2020), expounded that plastic had become the Part and Parcel of life. Thus, the Separate Collection of Plastic the uptake of Recycling Industry to recycle plastic to be done to reduce the environmental issue.

Safynaz Magdy Hanafy., *et al.*, (2021), reported the ethanol extract of *Musa sapientum* peel could control Infections caused by *Salmonella typhi* & *E.coli, A.niger, A.flavus*.

S. Divya., *et al.*, (2021), highlighted that banana peels, shells of eggs and prawn was used for production of Bioplastic which was in large quantitates. This could lower cost and be an alternative for conventional plastic.

Nor Izaida Ilbrahim., *et al.*, (2021), illuminated that bioplastic are the well- known examples of Biomass green materials which had ability for commercialization of Bio-Based packaging materials.

Khadiga Mohammed Ahmed Badri., *et al.*, (2021), elucidated that the banana peels had the capacity to only Bioplastic but fuel which is commonly known as Green Engineering.

Fating Zahra., *et al.*, (2021), described that banana are highly beneficial fruit for human health economically affordable by all sorts of people.

David Neglo., *et al.*, (2021), explained that the peel of Watermelon had its highest antimicrobial activity whereas from its pulp or seed.

Nor Izaida Ibrahim., *et al.*, (2021), evaluated more than 330 million tons of plastics are produced worldwide per year, where 40% for packaging, 20% building, 8% for automotive industries. He also focuses on bio plastic packaging applications such as food beverage, healthcare and cosmetics etc.

Yusuf Sarkingobir., *et al.*, (2021), illuminated that Bio plastic have potential promising features where it can be degraded by Microbes, similarly reduce plastic waste, Bio plastic had the ability to dissipate toxins.

Fatima Zahra., *et al.*, (2021), explained the benefits of nutrients present in banana like phenols, flavonoids, phytochemicals, potassium, vitamins fiber on human health. Beneficial for human health, economically affordable.

Khadiga Mohammed Ahmed Badri and Kamal Eldin Eltayeb Yassin (2021), concluded that bio plastics could be practically obtained from banana peels using batch reactor based on spectra standard range of cellulose.

Jaikishan Chandarna and P.L.V. N Saichandra., (2021), focused to produce biodegradable plastic from banana peels as a substitute for conventional plastic, the produced bio plastic film can sustain the weight near about 2kg and have enough tensile strength.

Jerlin Vinodh., *et al.*, (2021), explains that the production of bio plastic from banana peel through various methodologies and its applications, and the additives used for its production and the end product revealed m*et a*l the standard requirements when tested.

Shradha Govind Pille., *et al.*, (2021), studied the various methods available for extraction of pectin from banana peels and the productivity is compared.

Maura Gabriela Aleiver-Gavilanes., *et al.*, (2022), pointed that banana residues were used to obtain bio plastic and it was characterised according to its thickness, water vapour permeability, tension force, break time and biodegradability.

METHODOLOGY

METHODOLOGY

➢ MATERIALS

Banana peels, citrate acid, chitosan, aquadest.

➢ EQUIPMENTS:

Magnetic stirrer, thermometer, beaker glass, filter paper, measuring pipette.

➢ Collection of Plant Material:

Fresh banana was bought from the market. (*Musa acuminata*). The peel and edible part was carefully separated which is the basic need for this experiment. Certain amount of the fresh peel was taken for the pectin extraction of bioplastic production. The remaining amount was dried for 3 days under sunlight then they were finally blended by using mechanical blender. The grated powder was stored in an air tight container for further analysis.

Fig: 14 Dried Banana Peel and its Powder

Pectin extraction:-

500gms of banana peels are cleaned for each treatment and blending. Then the filtrate is filtered the results are extracted by boiling in 2% citric acid solution (20gms of citric acid/ litre of water) at 90° C for 3hrs.Then filtered, the extracted liquid is mixed with ethanol (1:1) and filtered again. The pectin extracted was dried in an oven at 50° C for 2hrs.then they are packed, analysed, applied for making bioplastics. (S.Chodijah., *et al.*, 2019)

Fig: 15 Pectin extract

PECTIN EXTRACTION:

- 500 gm of Banana peels were cleaned & Blended using a Blender.

Fig: 16 Banana Peel blended using mechnical blender.

- Then the Blended Banana peels were filtered.

Fig: 17 Filtered Solution

- The extract filtered was by boiled by adding 2% Citric Acid / Liter of water at 90°C for 3hours in a Water Bath.

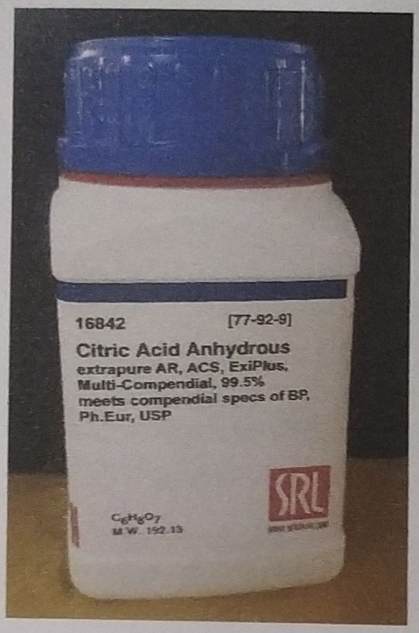


Fig: 18 Citric acid

- This was filtered the extract was obtained .

Fig: 19 Banana peel extract

- The extract was mixed with ethanol.

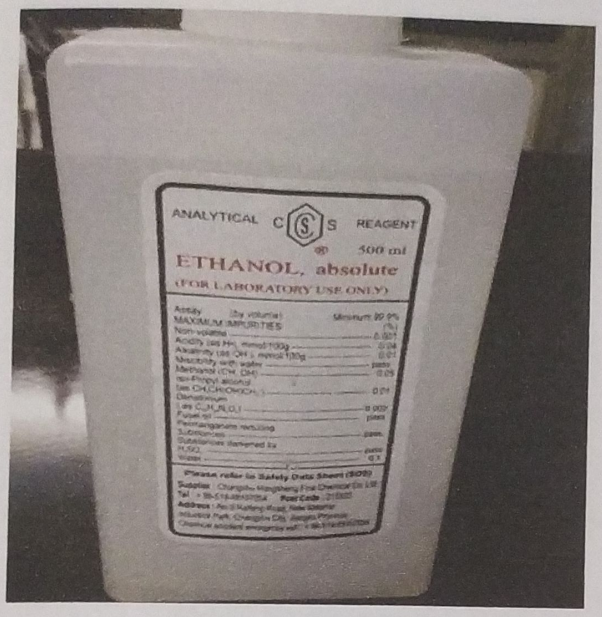


Fig:20 Ethanol

- The extract was dried in hot air oven at 50°C for 8hrs.

Fig: 21 Hot air oven

- The pectin obtained was analyzed.

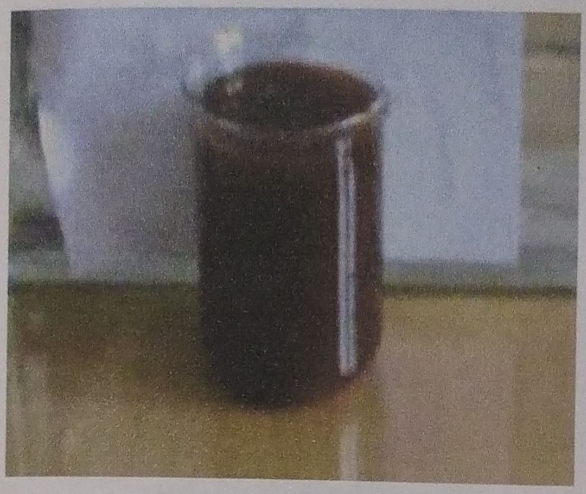


Fig: 22 Pectin extract

Making plastic film biodegradable:

The extracted pectin was dissolved in distilled water and added with 5gms of banana peel extract and then heated at 60 C. At the same time, chitosan was dissolved with 3% citric acid, then pectin solution was mixed with chitosan and stirred until homogenous. Then 2% sorbitol is added, the solution was heated to a temperature of 80°C and maintained for 10mins. Then the mixture is put into a mold and dried at temperature of 50 – 60° C. (S.Chodijah., ***et al***., 2019)

PREPARATION OF BIOPLASTIC:

- The 5 grams of Banana peel extract was added & heated at 100°C.

Fig: 23 Banana extract

- The Pectin obtained was dissolved in distilled water.

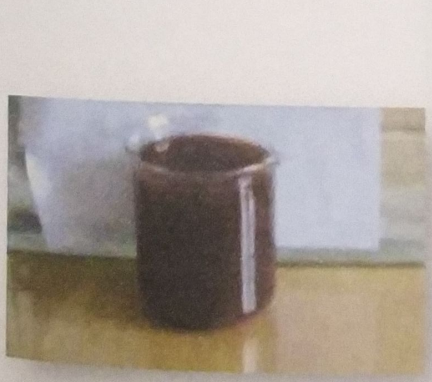


Fig: 24 Pectin extract dissolved in Water

- At the same time Chitosan was dissolved with 3% Citric Acid.

Fig: 25 Chitosan mixed with citric acid

- This mixture was dissolved with pectin extract. Then 2% Sorbital was added & heated at 80°C for 10 minutes.

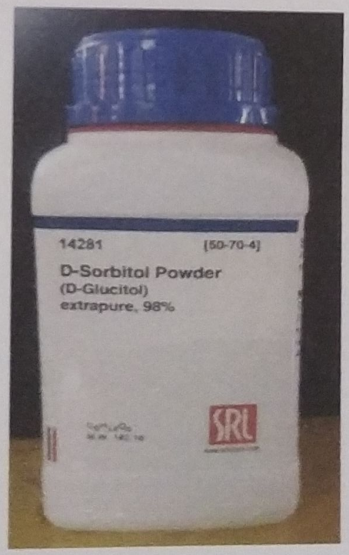


Fig : 26 Sorbitol was added to the pectin extract.

- Finally, the mixture was dried at 50 – 60°C in Hot air oven. Bioplastic was obtained.

Preparation of Banana Peel Extract:

In this method, ethanol is used as a solvent. Soxhlet apparatus was used for the ethanol extraction ground banana peel sample is placed in thimble chamber of the Soxhlet apparatus 50g of banana peel was powder was extracted with 500ml ethanol at 78.37^{O} C. Now the extracts were evaporated to dryness.

Collection of sample :-

Microorganism are cosmopolitan in nature. We collected sewage sample near sacred Heart Hospital & Waste water from Thermal Power Plant.

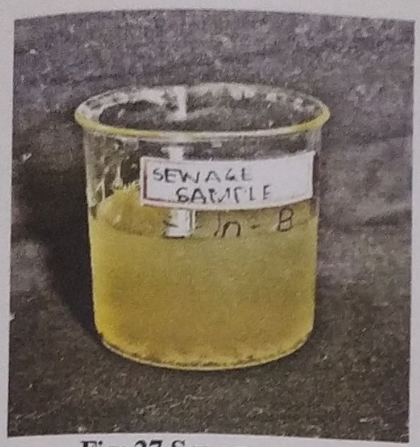


Fig: 27 Sewage

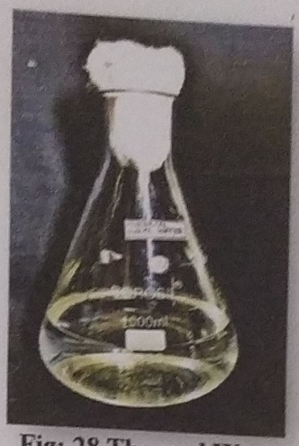


Fig: 28 Thermal Water

Isolation of Pathogens:

We isolated *E. coli* from sewage sample by culturing it in EMB Media and *Staphylococcus aureus* from the waste water of Thermal Power plant and was cultured in Mannitol Salt Agar and *Vibrio cholerae* was isolated from the sewage sample by using TCBS agar. The liquid Culture was prepared by inoculating a loop full of bacterial culture in the nutrient broth and incubated at 37°C for further procedure.

Antimicrobial activity:

Well diffusion assay was carried out to check the antibacterial activity of banana peel extract against *Staphylococcus aureus, E. coli, Vibrio cholerae.* A loop or swab was used to transfer the colonies to the Muller Hinton Agar. The sterile cotton swab was then dipped into the inoculum and the surface of the agar plate was swabbed 5mm diameter hollow tube was used to prepare the wells on each plate, and then the ethanolic banana extract was added into the respective wells. The plates were then incubated for 18-24 hr at 37°C in an incubator.

Three wells were made, one is for sample, ethanolic extract of banana peel, the second well is for positive control (antibiotic) and the last well is for negative control (DMSO).

RESULT AND DISCUSSION

RESULT AND DISCUSSSION:

Bioplastic was obtained by pectin extraction from banana peel.

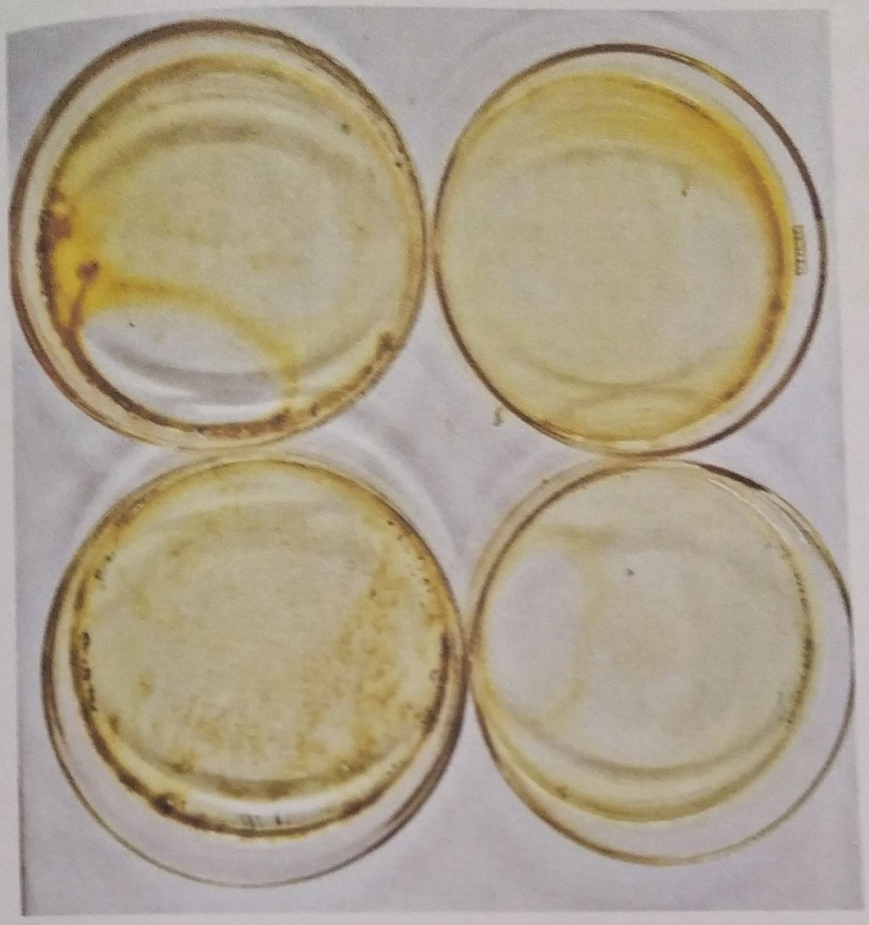


Plate 1 : Bioplastic

TABLE: 4 PRODUCTION OF BIO-PLASTIC

TOTAL BANANA PEEL (IN GRAMS)	PECTIN EXTRACT (IN GRAMS)	BIOPLASTIC OBTAINED (IN GRAMS)
500	50	27.9

S.Chodijah *et al.*, 2019 determined that pectin extracted from banana peel (*Musa paradisiaca* fomatypic*)* was further processed for the preparation of Bioplastic. In our study we extracted the Pectin from *Musa acuminata* peel & obtained Bioplastic.

M.O. Ramadhan *et al.*, 2020 explained that development of Bioplastic from food waste would show double benefit that reducing plastic and food waste thereby promoting environmental sustainability. Hence, bioplastic produced from banana peel would definitely reduce banana peel waste and it would be alternative for plastic waste.

Isolation of Pathogens:

After incubation of plates, Sewage sample which was inoculated in selective media – EMB. This showed metallic sheen growth which confirms that it is *E. coli*. Thermal waste water which was inoculated in Mannitol Salt Agar which showed yellow colonies that indicated the presence of *Staphylococcus aureus*. From sewage sample, flat yellow colonies on TCBS plate confirmed as that the pathogens was *V.cholerae*.

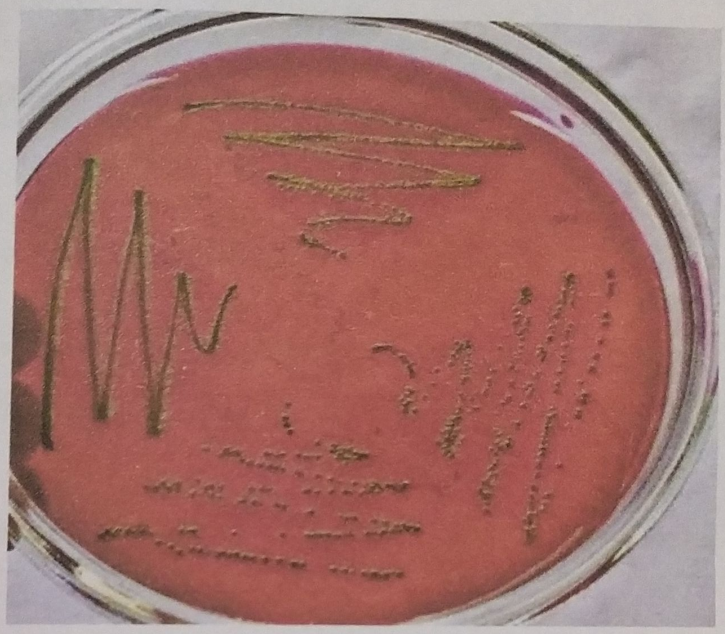


Plate 2 : Metallic Sheen on EMB

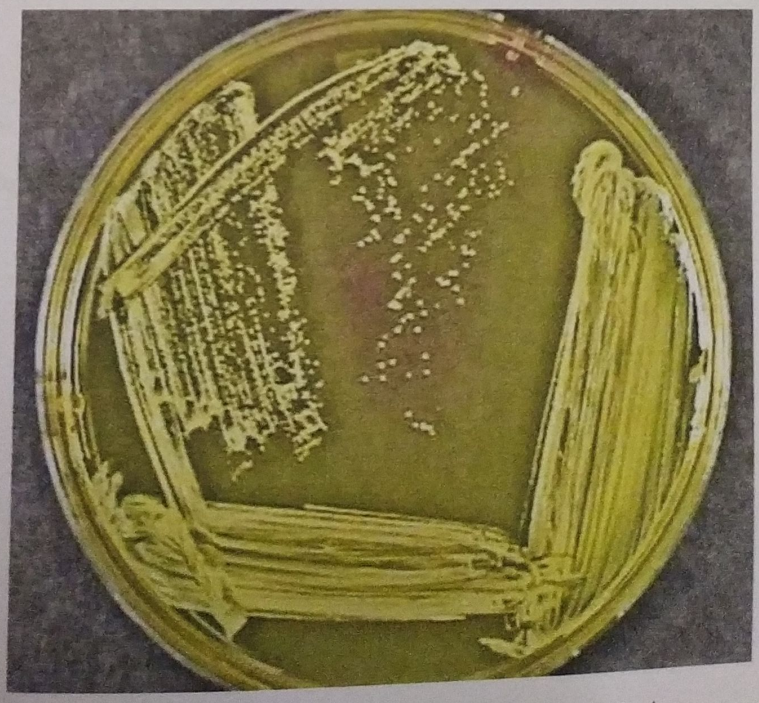


Plate 3 : Yellow colonies on Mannitol Salt Agar

Plate 4 : Vibrio cholerae grown on TCBS Medium.

Antimicrobial activity – Well diffusion assay:

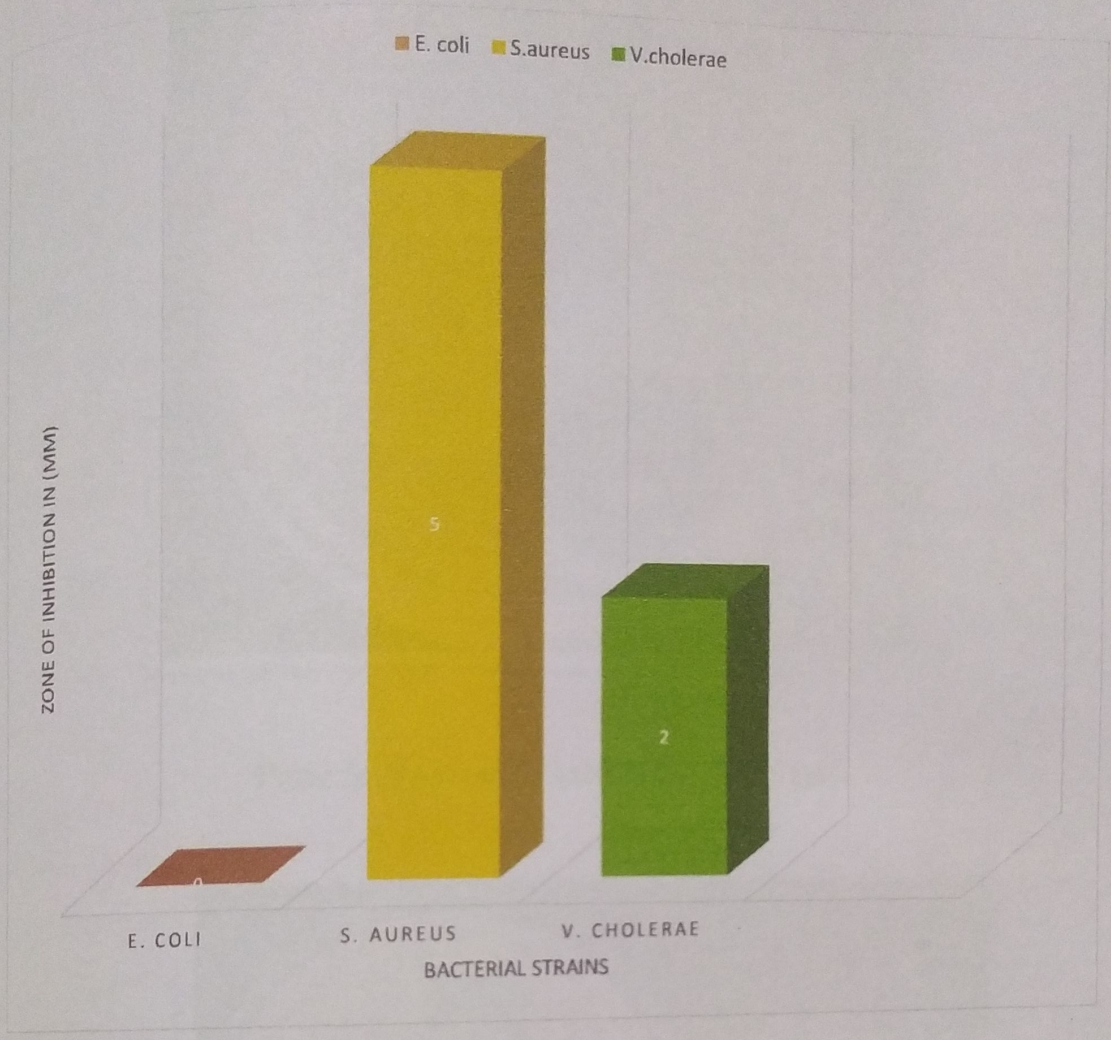
After incubation of Muller Hinton agar plate zone of inhibition was shown by ethanolic plant extract against *Staphylococcus aureus* and *Vibrio cholerae*.

TABLE: 5 Zone of Inhibition:

Organism	Zone of Inhibition
E.coli	No Zone of Inhibition
Staphylococcus aureus	5mm
Vibrio cholerae	2mm

Graph 1:

Antimicrobial activity of Ethanolic extract of Banana Peel against Bacterial Strains.



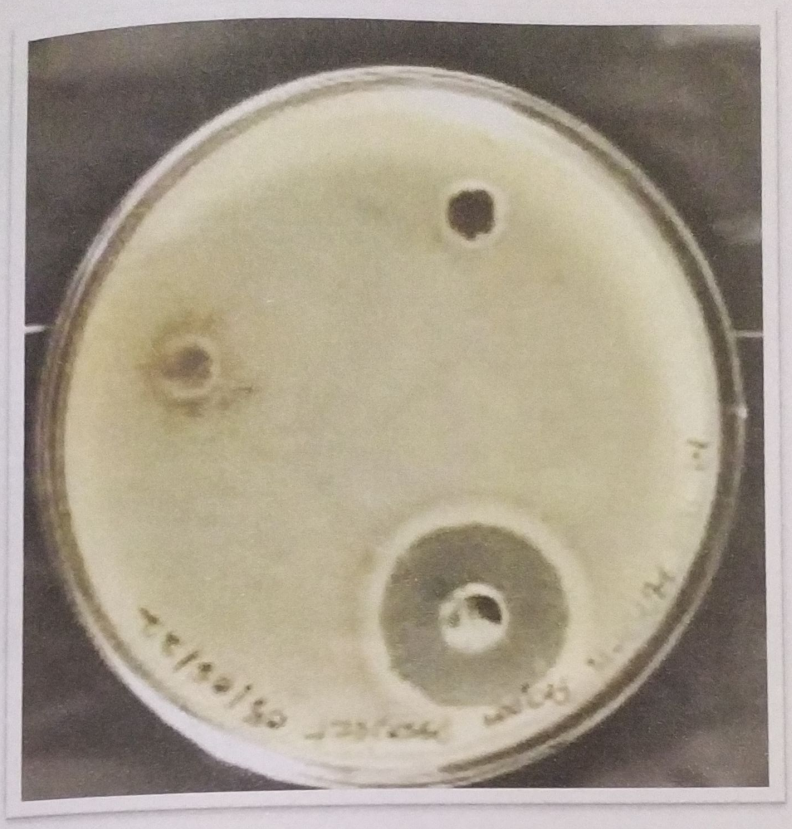


Plate 5: No zone of inhibition on *E. coli*

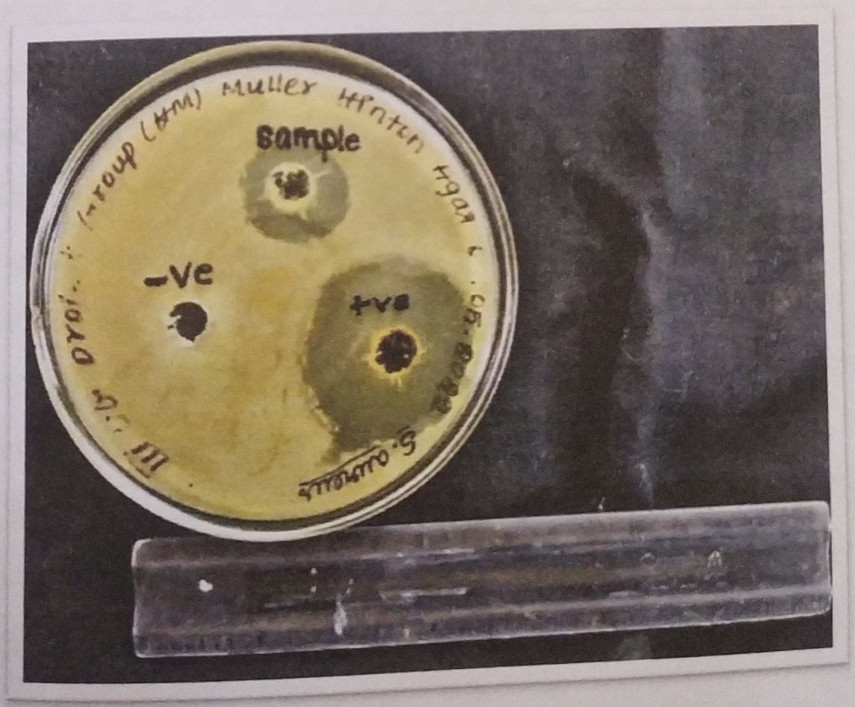


Plate 6 : Zone of inhibition in *S.aureus*

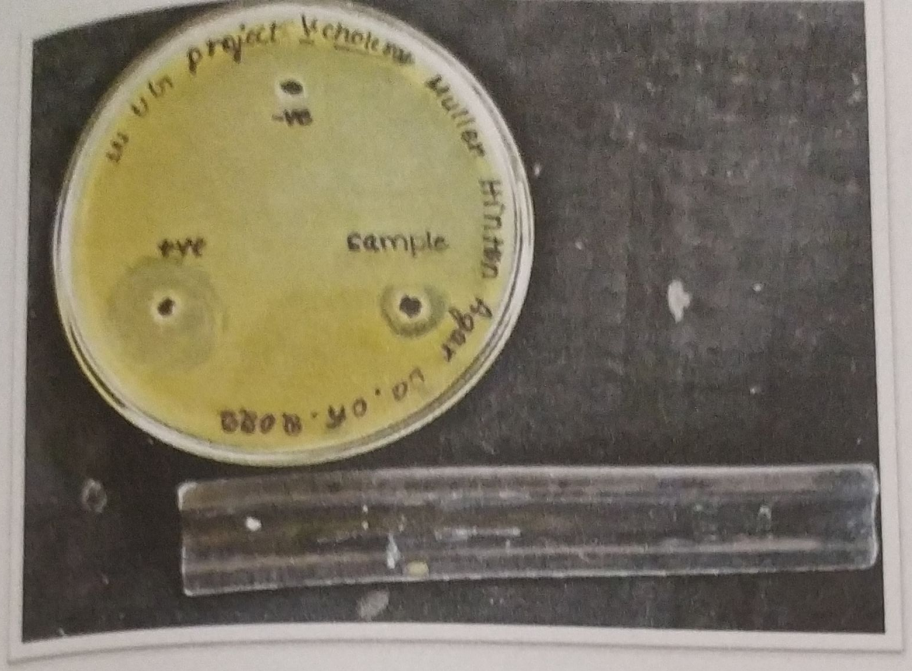


Plate 7 : Zone of inhibition on *V.cholerae*

Zainab Adil (Ghani chabuck *et al.*, (2013) concluded that aqueous extract of fresh yellow banana peel could be considered as good antibacterial agent against both gram +ve & gram -ve bacteria which are used to replace synthetic drugs in the treatment of diseases.

Ehiowemwenguan I *et al.*, (2014) affirmed that the invitro antibacterial activity of ethanolic extract of banana peels was investigated on both gram positive and gram negative bacteria using agar well diffusion technique, he concluded that ethanol showed to be a better solvent for the extraction of the bioactive agents in banana peels which include glycosides, alkaloids, saponins, tannins, flavonoids, volatile oil.

Wafaa M. Hikal (2021) stated that the methanol extract shows a greater antibacterial activity than that of ethanol water and chloroform extract. In our study we experimented it with ethanolic extract of banana peel it shows antibacterial activity against *s.aureus* and *V.cholerae*.

SUMMARY

SUMMARY

Plastic is an attractive material which are under daily usage where its activity has been destructing the natural environment. Hence, the bioplastic could be an alternative for the conventional plastic. These bioplastics had been obtained literally from a waste banana peel. Our study presents the preparation of bioplastic from banana peel where pectin - a natural polysaccharide was extracted from the peel which acts as a biopolymer. Biopolymer is a good agent which has a complete nature of Biodegradability when analyzed with chemical-based polymer. The intermediate product in our process aids the complete preparation of bioplastic. The bioplastic obtained would be eco-friendly to environment. Hence, the waste used as a raw material for the production purpose denotes us that food waste and plastic pollution can be reduced simultaneously. Bioplastic obtained from banana peel could be further transformed into packaging materials, toys, bottles as antimicrobial activity had been detected which made is confirm us that the product made out of this banana peel would be non- toxic and had the ability to attack pathogens entry. The degradation aspects would definitely help the environment. The industrial production of bioplastic would be really enriching as bioplastic are frequently being increased by consumer statistically indicating that global bioplastic market had been gradually developing. We can assure that there will be a day where we all will definitely utilize bioplastic reducing the perils of plastic pollution and manage food waste disposal.

CONCLUSION

CONCLUSION :

- The results showed that the banana peels based bioplastic is able to achieve the main grand challenge of increasing the industries efficiency and its supports.
- And the general economy in many other products such as bags, toys, drinking water bottles etc. that the plastics plays a major role and an important factor in the process of their manufacturing.
- Ethanolic extract of banana peel was carried out well diffusion assay to determined the antimicrobial activity and it was considered as good antimicrobial agent against *S.aureus* and *V.cholerae* and can replace synthetic medicine in the treatment of diseases caused by these bacteria.

FUTURE PLAN

FUTURE PLAN

- The obtained bioplastic can then be further processed for making toys that are safe for children as they are prepared from non-toxic based materials.
- It plays a key role in the production of bags.
- Bioplastic are used for the manufacture of drinking bottles which are safe to consume as it has antimicrobial properties.

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A Novel Approach On Pharmaceutical Activities of *Borassusflabellifer's* Leaves and Roots.

A PROJECT SUBMITTED TO

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Affiliated by ManonmaniamSundaranar University

In partial fulfillment of the requirements for the award of the degree of

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SUBMITTED BY

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May– 2022

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May– 2022

DECLARATION

I hereby declare that the project work entitled " **A Novel Approach on Pharmaceutical Activities of *Borassusflabellifer's* Leaves and Roots** "is a bonafide record of the work completed by us during the academic year 2021-2022 in St. Mary's College (Autonomous), Thoothukudi and submitted as a partial fulfilment of requirements for the award of the Degree of Bachelor of Science in Microbiology prescribed by the ManonmaniamSundharanar University. We also affirm that this is a original work done by us under the supervision of Dr. **Joys Selva Mary Albert M.Sc., M.Phil., Ph.D.,**Head and Assistant Professor of Department of Microbiology, St. Mary's College (Autonomous), Thoothukudi.

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SIGNATURE OF THE STUDENTS

SIGNATURE OF THE GUIDE

PLACE : THOOTHUKUDI
DATE : 25-05-2022

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ABBREVATION

1.	mm	-	Millimetre
2.	dm	-	Diameter
3.	gm	-	Gram
4.	ml	-	Millilitre
5.	kg	-	Kilogram
6.	dL	-	decilitre
7.	^{0}C	-	Degree Celsius
8.	µg	-	Microgram
9.	MHA	-	Muller Hinton Agar
10.	DMSO	-	Dimethyl Sulfoxide
11.	DPPH	-	2,2-Diphenyl-1-picrylhydrazyl

CHAPTER 1

1.1.INTRODUCTION

Plants play an important role in health as medicine from the beginning of human era. They have been used partially or as a whole plant or trees for many medicinal properties. Some of the medicinal trees Neem ,Papaya , Calihariare and plants such as Tulsi , Ginger , Garlic . Coriander and they also play a vital role in health care system.

The spread of multidrug- resistant (MDR) strains of bacteria require the discovery of new classes of anti bacterials and compounds that inhibit these resistance mechanisms. First, plants save exceptional ability to produce cytotoxic agents and second there is an exceptional rationale that antimicrobial natural products should be present or synthesized denoted in plants following microbial attack to protect the producer from pathogenic cases in its environment (Simon Gibbons, 2005).

One of the most widely known species of bacteria responsible for many hospital acquired infection is methicillin-resistant *Staphylococcus aureus* (MRSA) which emerged in the early 1960s only after one year after methicillin was introduced. This species is a gram- positive "grape" shaped coccus like bacterium which derives its specific epithet of aureus due to this golden colour of colonies grown on solid media. This bacterium is a highly talented organism, capable of causing skin infections and food poisoning through to clinical problems such as medical device colonization. It is commonly -responsible for wound-related infections and most worrying for life threatening conditions Such as bacteremia, necrotising pneumonia and endocarditic. This species is characterized by acquisition of β-lactam resistance, in particular resistance to methicillin and oxacillin via expression of altered penicillin-binding protein - MRSA Strains also produce β-lactamase enzymes which are responsible for the degradation of these antibiotics. The bacterium also has exceptional with the ability to thrive on skin and hair and has a high tolerance to salt. The bacterium also has exceptional ability to acquire resistance with many examples of resistance to tetracycline , macrolides, aminoglycosides and fluoroquinolonrs (Simon Gibbon., 2008).

The selective action and the great activity of antibiotics are of much interest to the plant pathologist, who deals primarily with diseases indicated by fungi, bacteria, and viruses. Members of each type of causal agent have been inhibited by antibiotics under experimental conditions. Successful control of plant disease in field tests has been reported using antifungal and antibacterial antibiotics for foliage sprays or for seed treatments, purposes for which most of the plant disease chemicals are now employed. Certain antibiotics have been shown to control disease by acting systemically in the plant, a property with inherent advantages over the non-systemic type of surface toxicant now used extensively. Factors that will govern the use of antibiotics on a wide scale include effectiveness in given disease situations, freedom from toxicity to plants and animals, cost and availability, mode and cost of application, compatibility with insecticides, and stability with time and to such weathering agents as light or the washing of rain (Curt Leben, GW Keitt.,1954).

Attempts to control plant diseases by the use of antibiotics have been made by plant pathologists all over the world since the discovery of penicillin. One of the greatest needs in the world is the production of food for billions of people. Such production requires the use of pesticides; however, their use generates the possibility of environmental pollution. Environmental hazards caused by conventional agricultural chemicals are classified in two categories: a non-selective toxicity; concentration and accumulation of toxic compounds in the environment (TomomasaMisato.,*et al.*, 1977).

Commonly found traditional chemicals in health management included lime, salt, potassium permanganate, sumithion, melathion, formalin, bleaching powder and malachite green. Major active ingredients of these antibiotics were oxytetracycline, chlorotetracycline, amoxicillin, co-trimoxazoie, sulphadiazine and sulphamethoxazole. Among the available antibiotics, oxysentin 20%, renamox, renamycin and orgamycine 15% was being used widely by the freshwater aqua farmers in Jessore area. (Taposh Kumar, *et al.*,2012)

2

Food safety is a terms broadly applied to food quality that may adversely affect human health. These include zoonotic diseases and acute and chronic effects of ingesting natural and human-made senobiotica.

There are two major areas of concern over the presence of residues of antibiotics in animal derived foodstuffs with regard to human health. The first is allergic reactions. Some antibiotics, such as penicillin can evoke allergic reactions even though small amounts of them are ingested or exposed by parenteral routes. The second is development of antibiotic resistance in gut bacteria of human recently multi-resistant pneumococcal, glycopeptide-resistant enterococci and gram negative bacteria with extended-spectrum &-lactamases have spread all over the world, and are now a serious therapeutic problem in human. Although it is evident that drugs are required in the efficient production of meat, milk and eggs, their indiscriminate use should never be substituted for hygienic management of farms. Drug should be used only when they are required. In addition to veterinary drugs, environmental contaminants that were contaminated in feed, water and air can make residues in animal products. Mycotoxins, heavy metals, pesticides, herbicides and other chemicals derived from industries can be harmful both animals and human health. Most of organic contaminants, such as dioxin, PCBs and DDT, and metals are persistent in environment and biological organisms and can be accumulated in fat and hard tissues. Some of them are suspected to have endocrine disrupting, carcinogenic, teratogenic, immunodepressive and nervous effects. The governmental agencies concerned make efforts to prevent residue problems; approval of drugs including withdrawal times of each preparation of drugs, establishment of tolerances, guidelines regarding drug use and sanitation enforcement of livestock products. National residue program is conducted to audit the status of the chemical residues in foods recently HACCP has been introduced to promote food safety from farm to table by reducing hazardous biological, chemical and physical factors. Animal Production Food Safety Program, Quality Assurance Programs, Food Animal Residue Avoidance Databank are para or non-governmental activities ensuring food safety. This topic will cover classification and usage or sources of chemical residues, their adverse effects, and chemical residue status of some countries issues are expanded to residue detection methodologies, toxicological and pharmacokinenc backgrounds of MRL and withdrawal time establishments, and the importance of son-governmental activities with regard to reducing chemical residues in food (Asian-Aust J. Anim, 2001).

Although the liver is particularly exposed to drugs and their metabolites, hepatic side-effects of antibiotics are far less frequent than other adverse effects such as gastrointestinal disorders or cutaneous reactions. However, the potential severity of hepatic side-effects for some drugs is stressed. Antibiotic related liver injuries cover most of the clinical and pathological expressions of hepatic dysfunction, including cytotoxic hepatitis-isoniazid, intrahepatic cholestasis- macrolides, penicillins, clavulanic acid, mixed hepatitis-sulphonamides, chronic active hepatitis-nitrofurantoin, or microvesicularsteatosis-tetracycline. In most cases, toxicity is idiosyncratic, reactions occurring only in some susceptible individuals. The mechanisms underlying toxicity may be primarily metabolite-dependent-isoniazid, hypersensitivity-mediated-β-lactams, or result from both processes-sulphonamides, erythromycin derivatives. In some cases, the liver is not the primary target organ for toxicity but appears to mediate the clinical expression of some adverse effects induced by antibiotics. The most significant example of this is hypoprothrombinaemia due to the inhibition of hepatic γ-carboxylation of vitamin K-dependent clotting factors by sulphydryl group-containing cephalosporins. Inhibition of bilirubin conjugation or transport by rifampicin or fusidic acid may also be viewed as hepatic side-effects of antibiotics. Ascertaining the casual relationship of a given drug to an hepatic adverse effect may prove particularly difficult, because of the potential contribution of host status and concurrent medications. Diagnosis is based mainly on circumstantial evidence, i.e. the temporal relationship between drug administration (or withdrawal) and the time-course of liver dysfunction. Improving morbidity related to drug hepatotoxicity relies on a free flow of information between manufacturers and practitioners in order to optimize detection of potentially serious liver damage, and advances in pharmacokinetics' toward a better identification of those at particular risk for developing drug-related liver toxicity (JF Westphal,*et.a.l*, 1994).

Antibiotic use in the early 1900 vastly improved human health but at the same time started an arms race of antibiotic resistance. The wide-spread use of antibiotics has resulted in ubiquitous trace concentrations of many antibiotics in most environments. Little is known about the impact of these antibiotics on microbial processes or"non-target" organisms. the effects of antibiotics on biogeochemical processes should involve environmentally relevant concentrations. Instead of therapeutic, chronic exposure-versus acute and monitoring of the administered antibiotics. Furthermore, the lack of standardized tests hinders generalizations regarding the effects of antibiotics on biogeochemical processes. We investigated the effects of antibiotics on biogeochemical N cycling, specifically nitrification, denitrification, and anammox. We

found that environmentally relevant concentrations of fluoroquinolones and sulfonamides could partially inhibit denitrification. So far, the only documented effects of antibiotic inhibitions were at therapeutic doses on ammonox activities. The most studied and inhibited was nitrification (25-100%) mainly at therapeutic doses, and rarely to environmentally relevant (Celine Rouse Amsaleg, *et al*.,2015).

In recent years, there has been accumulating evidence that antibiotics, besides their antimicrobial action, potentially have a number of undesired side effects that can at least in some cases, promote genetic variability of bacteria. In addition to resistant variants, antibiotics have also been shown to select mutator clones, thus stimulating evolution towards further resistance. Furthermore, mutations, recombination and horizontal gene transfer have been reported to be somehow affected when bacteria are exposed to sub inhibitory concentrations of certain antibiotics. These findings may have implications for the use of antibiotics, because they may have undesired side effects, such as enhancing antibiotic resistance evolution (Alejandro Couce,*et al*.,2009).

Human consume a wide range of foods drugs and dietary supplements that are derived from plant, which modify the functioning of the central nervous system (David O Kennedy and Emma L Wight man 2011) . Biochemical function and minerals are important in health and disease conditions of human , animals and plants as well as based on the plant food , it assist in the prevention of nutrition related disease and maintenance of good health of humans and animals (K.O Soetan*et al*.,2010) . Phytochemical /extracts can be grouped by the chemical nature of their potentially active secondary metabolite constituents into alkaloids-caffeine, nicotine-,trepene-ginko ,ginseng, valerian, melissaofficinalis , sage and phenolic compounds-curcumin, resveratol, epigallocatechin-3-gallate, Hypericumperforatum, soy isoflavone (David O Kennedy and Emma L Wightman 2011).

Plant natural ability : Hypercholesterolemia and arteriosclerosis are treated and prevented with mainly Garlic . Clinical trials on garlic shows the result in allergic reaction, alteration of platelet function, coagulation and burns-particularly applied on occlusive dressing . It increase the pharmacological effects of anticoagulants and reduce the efficacy of anti -AIDS drugs (Francesca Barrelli *et al*., 2007) . Soybean is a good source of protein feed for human and animal. Heat treatment and some other form of processing applied on soybean. Certain heat-labile factor that exert an adverse effects on the nutritional value of the protein-protease inhibitors. It causing pancreatic hypertrophy and hyperplasia in the rat underlaying cause for

Supportive measures against plant disease : The reason behind world many economic losses and major production is a plant disease. Disease control and monitoring health of plant is critical in agriculture because there no sensor commercially available to check plant health condition using molecular technique to identify plant disease. It need only detailed sampling and processing procedure. Health and disease detection through proper management strategies such as vector control through pesticide and fungicide applications and disease specific chemical applications and which can improve productivity (Sindhuja*etal*., 2010) .

Plant source as a natural preservative :

Preservation of food spoilage and food poisoning pathogens are usually detected which is mainly due to the usage of chemical preservative that have more side effects and microbial resistance . We should consider health safer and so that natural alternative preservative has increased . Preserve food stuff and food poisoning disease has been control by use of plant extract. Plant extract of variant solventis used in agar disc diffusion of five organism -Food poisoning causing organism *S.aureus*. Plant extract-ethonolic extract effective plant extract showed bacteriostatic and bactericidal activities against food borne pathogens or bacteria- *S.aureus* , *P.aeruginosa*. These plant extract was potentially effective can be used as the alternative for chemical preservative to control food poisoning disease and food stuff (Ashraf A.Mostafa*etal*., 2018) .

Role of plant in Human physiology :

The psychoactive properties of these substances are attributable to the presence of plants secondary metabolites, chemical that are not required for the immediate survival of the plant. But that are synthesized to increase the fitness of the plant to survive by allowing it to interact with its environment including pathogens and herbivorous and symbiotic insects. The effects of phytochemicals on the human CNS might be liked either to their ecological roles in the life of the plant or to molecular and biochemical similarities in the biology of plants and higher animals. (David O Kennedy and Emma L. wight man .,2011)

Plant chemical effects on human :

Plant chemical such as pyrrolizidine alkaloids it cause diseases in human and animals . After absorption of from gut this compound convert electrophillicphyrroles in the liver ,it cause damage to the organ. It also cause injury to extraheptic tissues such as lungs, heart and kidney(Alan A. A.Seawright., 1995).

Humans and animals utilise their senses to identify food, water, mates, shelter, and hazards such as predators, as well as to communicate with other members of their species. It's worth noting that wild mammals don't appear to be able to detect many of the same environmental stimuli that humans in industrialised civilizations can do with the help of various manmade gadgets. Human senses are limited, which can be seen as a disadvantage or as a way to free up neurons and allow the brain to be used for other tasks, such as higher-level thinking. The concept of human health is difficult to describe. It's not only about whether or not there's a disease (HadilteJR Sor Med ., 1995).

To optimise the benefits of companionship, including positive psychological and psychological changes, increased social growth, improved physical health, and the usage of service animals, a good mind-set is required. With continued efforts to reduce the negative features of infectious disease, aesthetic offence, bites, scratches, and pollution, a satisfactory balance can be achieved, contributing significantly to a higher quality of life for animal owners and those who come into touch with animals (JakobZinsstage*et al., 2002)*)

SOCIAL DEVELOPMENT:

A number of animal programmes have been carefully introduced into prisons and similar institutions (often with birds and ornamental fish, but also with certain dogs).These have been proved to help offenders get back on their feet.(David A HennebychristopherA wolf., 2002)

ASSISTANCE ANIMALS:

Dogs for disabled persons are particularly beneficial for those who are confined to wheelchairs or are bedridden. The trained canines can pick up fallen items, carry a portable phone, or bring any other object on demand. They orderly open doors, turn on water faucets, turn on light switches, and press lift buttons. They offer physical assistance and are even trained to stand still in the event that a patient falls over. Many individuals are concerned about the 'items they can catch from their pets. While many communicable diseases are common throughout species, the majority of infectious diseases are species specific. (Animaldiscaskkroschewsk*et al* 2006).

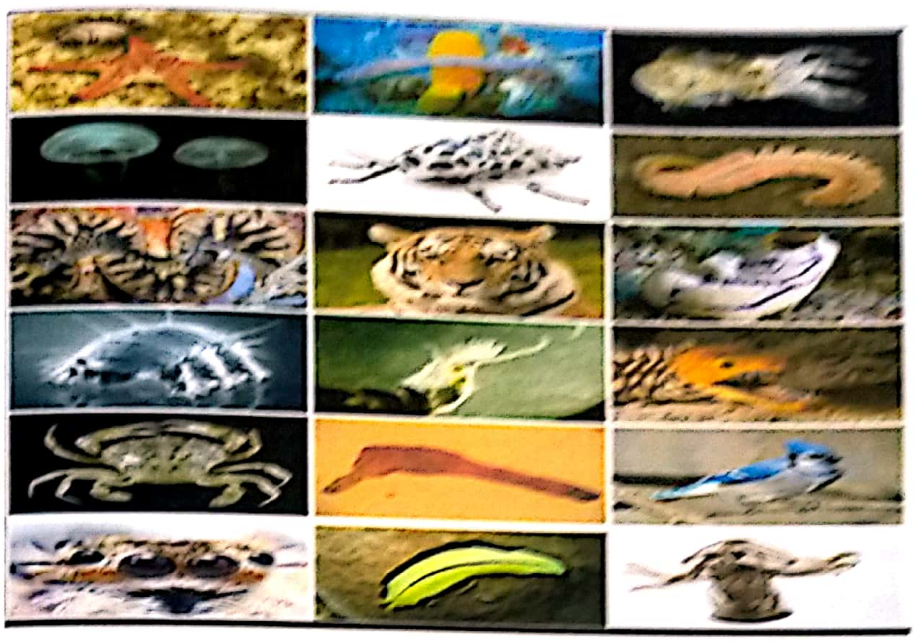


Plate1.2. Different classes of animal -Kingdom

DISEASES:

A tiny child is significantly more likely to contract an infection from another small child than from its dog or cat. The risk that does exist can be mitigated to a very low degree using common sense procedures. While industrialised countries have been able to suppress recent zoonotic disease outbreaks, many resource-constrained and transitioning countries have been unable to respond appropriately. Focusing on the animal reservoir is critical for controlling zoonosis like rabies, echinococcosis, and brucellosis. (Wilfried a kues and heinerNiemann .2004).

AWARENESS ABOUT ZOONOSES:

Many nations, particularly those with little resources and those in Sub-Saharan Africa, lack information on zoonotic disease dispersion. Because the social effects of zoonosis are not acknowledged by individual sectors, the risks for zoonosis are regarded minor when compared to those for diseases of greater relevance. Only when public health services contacted livestock services, who told them of cattle abortions, was the proper diagnosis made (Ponnie M. Marshalland Stuart Blevy Call., 2011)

PREVENTION:

Farmers behaviour is influenced by incentives, and understanding farmer behaviour aids in the control and prevention of contagious cattle disease. The people and logistical resources required to contain the outbreak will be planned based on the outbreak data, as well as the number of infected animals, farms or holdings, and products (e.g. milk, eggs, slaughter pigs).

Farm animals and their products have a long history of making major contributions to human nutrition, clothing, labour facilitation, research, development, and medicine, and have thus been critical in extending human life expectancy and enhancing human health. Recent advances in reproductive technology, such as somatic cloning and in vitro embryo generation, as well as their integration with molecular genetic tools, are expected to accelerate progress in this sector. Antibiotics are given to food animals for a wide range of nontherapeutic objectives, including growth stimulation. Concerns about the formation of resistance and its transmission to individuals as a result of nontherapeutic antimicrobial usage have resulted in a slew of contradictory behaviours and viewpoints. However, the link between drug-resistant microorganisms in humans and antibiotic use in food animals persists.

ANTIMICROBIAL USE IN ANIMALS: EFFECTS ON ANTIBIOTIC RESISTANCE:

Animals receive antimicrobials for a variety of purposes, including disease treatment, prevention, control, and growth promotion/feed efficiency. Antimicrobial growth promotions (AGPs) were originally promoted in the mid-1950s, when it was discovered that administering modest, sub therapeutic doses of antibiotics like procaine, penicillin, and tetracycline to animals in feed might improve feed-to-weight ratios in poultry, swine, and beef cattle.

IMPACTS OOF NON-THERAPEUTIC USE:

Because of their short-term application and small number of animals treated, therapeutics used effectively for the treatment of individual animals likely to control the formation and proliferation of antimicrobial-resistant strains. We reviewed over 407 global seaweed introduction even stand have increased the total number of introduced seaweed species to 277. Using binomial tests we show that several algal families. The ecological effects of introduced seaweeds have been studied in only 6% of the species, but these studies show mostly negative effects or changes to the native biots.

Seaweeds are microalgae can be of many different shape, size, colours and composition. They include brown algae, green algae, and redalgae. Seaweeds use as "livestock feed". Seaweeds have a highly variable composition depending on the species, time of collection and habitat, and external condition such as water temperature, nutrient concentration in water, and light intensity. Seaweeds contain non protein nitrogen (Paul Macartain., *et al.*, 2007).

Seaweeds have many mineral content. Seaweeds contain elevated proportions of ash 21.1 - 39.3% and Sulphate 1.3 - 5.9%.In brown algae, ash content 30.1-39.3 % was higher than in red algae 20.6-21.1%.Atomic absorption spectrophotometry of the ashes designated the marine seaweeds contained higher amounts of both macro minerals 8.083 -17,875mg/100g; Na, k, Ca, Mg and detect elements 5.1 -15.2 mg/100g; Fe, Zn, Mn, Cu than those described for edible land plants. Edible brown and red seaweeds can be used as a food supplement to help meet the recommended daily intake of some essential minerals and elements. Seaweeds and Their Neurophysiological Activities (Britta schaffelk. *et al.*, 2007)

Microalgae-derived compounds with neuroprotective activity may provide some important nutrients for the prevention and treatment of neurodegenerative diseases such as AD, PD, and other neurodegenerative diseases. Much of these bioactive compounds are derived from Phaeophyceae, brown algae (57.6%), followed by Rhodophyta, red algae (28.3%) and Chlorophyta, green algae (14.1%).Among the various components, carbohydrates are the most abundant constituents of seaweed. In addition, polysaccharides are generally the main component of red, green, and brown algae and monosaccharides and oligosaccharides are also present. The reserve polysaccharides are laminarin in brown algae, Floridian starch (more branched than amylopectin) in red algae, and starch in green algae (PilarRuperez 2008)

Plate. 1.3.Sea weed

The algal cell walls are characterized by the presence of unusual polysaccharides that can be sulfated, acetylated, etc. Thus, seaweed carbohydrates are promising compounds in several fields, such as food, pharmaceutical, and biomedical. The therapeutic applications of these notable polysaccharides are, among others, antiviral, antibacterial, and antitumor activity, antioxidant, antidiabetic, antilipidemic properties, anti-inflammatory, and immunomodulatory characteristics. Studies related to the absorption of bioactive compounds extracted from algae, namely polysaccharides, provide vital information on the most appropriate administration routes (HarindenpsMakkar *et al.*,2016).

If a drug formulation has a high absorption rate, it can be used for the controlled release of an active ingredient. Understanding the pharmacokinetics of polysaccharides derived from seaweeds (alginates, laminarins, fucoidans, etc.), may lead to their extensive use not only as drugs, but also to improve the bioavailability of certain poorly soluble compounds in pharmaceutical formulations . Laminarin, a polysaccharide composed of (1, 3)-β-D-glucan with some β (1, 6) branching, particularly abundant in species of the genus Laminaria, has been shown to have antibacterial and chemo-preventive activities, along with pre-biotic, important in the modulation of the intestinal micro biota, which in turn can regulate neuro-inflammation. PD is generally characterized, as we have seen previously, by the loss of dopaminergic neurons, and the presence of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) can induce PD. The administration of this substance may result in motor dysfunction, such as occurs in PD, which makes it a suitable experimental model for this disease. A compound (fucoidan) has been found to attenuate the neurotoxicity of MPTP activity. This sulphated polysaccharide, derived from Saccharina japonica (Phaeophyceae), has been demonstrated in mice models to be effective at a dosage of 25 mg kg−1 in protecting the cells from MPTP-induced neurotoxicity by reducing the behavioural deficits and cell death and increasing the level of dopamine. Tau is a microtubule-associated protein (MAP) found in axons, and this protein is responsible for regulating the stability of microtubules. Hyper-phosphorylation of tau results in its dissociation from microtubules and aggregation in the form of neurofibrillary tangles. Hyper-phosphorylated tau protein is a major component in neurofibrillary tangles, which is a hallmark of AD, and dysregulation of kinases and phosphatases has been found to increase tau hyper-phosphorylation levels. Only three compounds (Spiralisone A, B, and Chromone 6) from seaweed have kinase inhibitory activity, and these compounds have been isolated from brown algae (Phaeophyceae). Besides, these compounds were isolated from a single species of brown

algae, the Zonariaspiralis harvested in Australia, and all of them are phloroglucinols. The most active compound is spiralisone B, inhibiting the kinases-cyclin-dependent kinase 5 (CDK5/p25), casein kinase 1 (CK1δ), and glycogen synthase kinase 3β (GSK3β), with IC50 values of 3, 5, and 5.4 μM, respectively. Polysaccharide extracts from seaweed have very significant neuroprotective and repairing activities. These polysaccharides could be the next great advance in the treatment of neurodegenerative diseases. Fucoidan, ulvan, and their derivatives are potential agents to treat Alzheimer's disease, according to a recent review made by Bauer *et al* (Susan and williams, Jennifer and Smithia., 2007).

Sea grasses are submerged marine angiosperms. They produce flowers, fruits and seeds and have roots, leaves and underground stems (rhizomes), which enable them to form an extensive network below the surface of the water. They are primary producers and play a key role in the marine ecosystems. This, in turn, helps to slow down the effects of global warming. On the other hand, sea grasses provide food for a wide array of species, including, manatees, sea turtles, sea urchins, waterfowl, gar and pinfish. They are also considered as a source of food production for man as they serve as a nursery ground to juvenile stages of economically important species of finfish, oysters, clams and shellfish. Sea grasses have a positive influence on the health of marine animals that feed them. Turtles consuming sea grasses grow faster, attain sexual maturity earlier and the females produce more eggs compared to that of other turtles present in the wild. This is attributed to the high protein content and easily digestible nature of sea grasses. Thus, sea grass would play a major role in the long-term viability of the species (Immaculate JK, *et al.*, 2018).

PROTEIN CONTENT:

Normally protein content of sea grass is lower (12-19%) than that of the animal (23%). Hence, they cannot realistically be regarded as a significant source of dietary protein for marine animals. The dietary protein requirement of marine animals is met by some species of seaweed that contain high protein levels. (Suberkropp et al.,) reported that sea grasses are mostly low in protein content. Augier et al., 16 reported protein content is high in the whole sea grass plant compared to that of the leaves alone.

PIGMENT :

Pigment (Chlorophyll) composition in sea grass is similar to that of any other angiosperms and includes chlorophyll a and b, which are directly involved in photosynthesis. Chlorophyll content of the sea grass species can be largely influenced by the availability of the

light and morphology of the sea grass leaves. The present results indicate that there is no variation in chlorophyll concentration between the three sea grasses by registering the similar value.

ASH CONTENT:

Ash usually represents the inorganic part of the plant. This is because ashing destroys all the organic material present in the sample. The ash content of sea grasses in the present study varied from 18.2 to 28.7%. The percentage of ash content is highest in Halodule uninervis (28.7%).The chemical analysis of the three marine plants in the present study showed high ash content. The high ash content is a general feature of sea grass and these values are generally much higher than those of terrestrial green leaves (15%).50 High ash content invariably indicates the presence of appreciable amounts of diverse mineral components. The amounts of ash vary with phylum, season, environmental, geographical and physiological variations.51 The amount of ash in plant material varies considerably according to the part of the plant, age etc. The constituents of the ash vary with time and from organ to organ.

FIBER CONTENT:

Plant sources of dietary fibre are often classified into the soluble or insoluble fibre. Plant foods contain both types of fibre in various degrees, according to the plant's characteristics. The advantages of fibre in sea grass are the production of healthy compounds during the fermentation of soluble fibre and insoluble fibber's ability to increase bulk, soften stool and shorten transit time through the intestinal tract. The disadvantage of high fibres diet is the potential for significant intestinal gas production and bloating. Sea grass is regarded as a potentially rich source of polysaccharide carbohydrates for ruminants.

MINERALS :

In all the three sea grass species investigated, the average concentrations of sodium, calcium and magnesium were higher compared to that of phosphorus and potassium. (Kannan Rr Rengasamy.,2013).

MEDICINAL VALUE:

Halophila spp. is a strong medicine against malaria and skin diseases and is found to be very effective in early stages of leprosy. Sea grasses are nutraceutical in nature and therefore of importance as food supplements.

The antibacterial, antioxidant, and anti-inflammatory activities of Halophilaovalis R. Br. Hooke (Hydrocharitaceae) methanol extract were investigated and the chemical constituents of purified fractions were analysed. (N Yuvaraj*et al.*, 2012).Sea grasses were used as food, medicine, fertilizer and as livestock feed. As a contribution to the nutrition value of sea grasses, the present investigation provides data on the biochemical composition, essential elements and photosynthetic pigments of six tropical sea grasses. The multivariate analysis was performed with the aim to know the relationship among the nutrients in the sea grasses. The development of functional foods could be a new possible use of these sea grasses, particularly as a rich source in protein, fibre and lipid and for their beneficial effects that have been attributed to diseases, such as obesity and diabetes. The high amount of chlorophylls and carotenoids in these sea grasses is beneficial as they can act as vitamins and antioxidants.

FERTILIZERS :

Beach-cast sea grass litter deposits are common in many coastal areas with extended sea grass meadows. Sea grass litter abundance on the shoreline is a contentious issue on the aesthetics of the beaches and as an environmental hazard to beach revelers. To change this perception and view the sea grass litter as a potential asset, this study assessed the sea grass litter accumulation patterns at Mombasa, Kilifi and Malindishorelines. Further, the efficacy of composted sea grass fertilizer products was evaluated using amaranth (Amaranthusalbus) as a test vegetable. Beach-cast sea grass was collected, weighed and returned insitu at Kenyatta beach in Mombasa, Bofa beach in Kilifi and marine park area in Malindi. The weights were translated to T/ha. Two samples of sea grass, each weighing 500kg, were composted in separate chambers, one with saline composite while the other, with desalinated composite. The litter was composted for 4 months, sifted and packaged as organic fertilizer for field trials. The performance of the sea grass organic fertilizer was compared to farmyard manure and inorganic fertilizer (DAP) in the field trial. New leaf development, leaf size, height, dry matter and leaf yield of Amaranths plant were used as growth parameters to assess the efficacy of sea grass organic fertilizer. Highest accumulation of sea grass litter was noticed in Kilifi at 72.4T/ha which was significantly different ($P<0.005$) from Malindi at 26.5T/ha and Mombasa at

24.75T/ha. There was no significant difference in litter accumulation between Malindi and Mombasa. After four (4) months of composting sea grass, the saline setup achieved 85% decomposition, while the desalinated setup decomposed at 75%.(Thangaradjon T., *et al.*, 2011).

CORALS:

The large expanse of seas and oceans hides many mysteries. Most of marine ecosystem remains unexplored despite several new advances in technology. While the ocean may house several living and non-living matter that could be dangerous to humans, it also has several bioactive compounds that could be immensely useful to mankind.Images from under the sea in tropical regions brought to us by deep-sea divers often reveal crystal-blue water with a beautiful and colourful seabed formed by corals. Recently, five novel fluorescent proteins have been isolated from non-bioluminescent species of reef-coral organisms and have been made available through ClonTech. They are AmCyan, As Red, DsRed, ZsGreen and ZsYellow. These proteins are valuable as reporters for transformation because they do not require a substrate or external co-factor to emit fluorescence and can be tested in vivo without destruction of the tissue under study. We have evaluated them in a large range of plants, both monocots and dicots, and our results indicate that they are valuable reporting tools for transformation in a wide variety of crops. We report here their successful expression in wheat, maize, barley, rice, banana, onion, soybean, cotton, tobacco, potato and tomato. Transient expression could be observed as early as 24 h after DNA delivery in some cases, allowing for very clear visualization of individually transformed cells. Stable transgenic events were generated, using mannose, kanamycin or hygromycin selection. Transgenic plants were phenotypically normal, showing a wide range of fluorescence levels, and were fertile. Expression of AmCyan, ZsGreen and AsRed was visible in maize T1 seeds, allowing visual segregation to more than 99% accuracy. The excitation and emission wavelengths of some of these proteins are significantly different; the difference is enough for the simultaneous visualization of cells transformed with more than one of the fluorescent proteins. These proteins will become useful tools for transformation optimization and other studies. The wide variety of plants successfully tested demonstrates that these proteins will potentially find broad use in plant biology. Reef-coral proteins as visual, non-destructive reporters for plant transformation(A Wenck *et al.*, 2003) .

Some of the pontential uses of corals are in the following conditons:

Cancer: the anticancer properties of molecules obtained from corals is particularly attractive. Certain molecules extracted from the coral sinularia have demonstrated anti-cancer effects. In addition, molecules isolated from other corals like sarcophyton, lobophytummichaelae, and nephtheachabrolii also have anticancer properties.

Dental procedures: corals could provide certain materials that are used in dental procedures but are often in short supply. However, since corals have a high dissolution rate, they need to be strengthened with other material for this purpose.

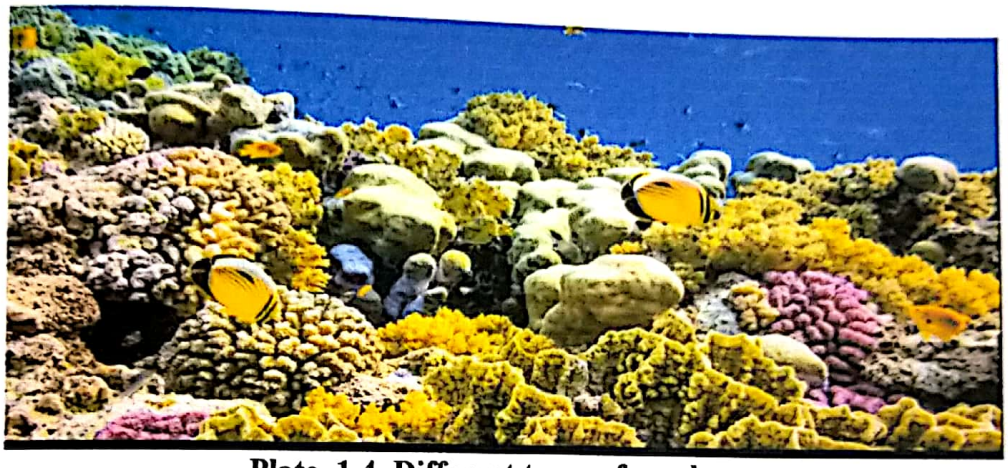


Plate. 1.4. Different types of corals

Inflammatory diseases:some molecules obtained from corals have been demonstrated to have anti-inflammatory properties. The anti-inflammatory effects of corals could possibly find its usefulness in rheumatoid arthritis. Some of the current drugs used in rheumatoid arthritis I are toxic in some patients while the newer ones are extremely expensive. Therefore, drugs obtained from corals could be useful in this condition.

Bone disease: studies in animals have shown that corals may be useful in preventing and treating bone loss in menopause. The limestone skeletons have a potential use as scaffolds in fractures around which bone can heal.

Pain: Several molecules from corals are being investigated for their use in neuropathic or nerve-related pain.

Parkinson's disease: the molecule 11-dehydrosinularolide obtained from a coral could protect the brain against the damaging effects of inflammation and apoptosis, which is seen in Parkinson's disease. Currently, the course of Parkinson's disease is downhill and in the later

stages, the patient is fully dependent on the caregiver. Thus, any drug that could be of use in prolonging the course of the disease would be of help.

Antibacterial effects: corals are sometimes attacked by bacteria. They therefore associate themselves with certain other bacteria that produce antibacterial substances against the pathogenic bacteria. These antibacterial substances could possibly be exploited and developed as new antibiotics for bacterial diseases.

Hyper tension: coral sand is a rich source of silicon and could be possibly useful in hypertension.

Cosmetic purpose: A sunscreen has been developed from molecules obtained from coral. Corals produce a substance which has a sunscreen-like effect to protect themselves from the ultraviolet rays of the sun. this substance is extracted from the corals for use in sunscreens (Hannon, H.E.; Atchison, W.D. Omega 2013)

Drugs obtain from marine source: It is interesting to note that several drugs from marine sources are already in use. These include: Carbine, is a drug that was obtained from marine sponge from the Caribbean reefs. It is used for certain blood cancers. The antiviral drugs vidarabine and azidothymidine, which are obtained from marine sponges. Azidothymidine is effective against Aids/Hiv infection (Gantar M *et al.*, 20110).Trabectedin, which is obtained from a caribbeantunicate.It is used for certain cases of advancedsoft-tissue sarcoma and recurrence of ovarian cancer (Petek, B.J .,*et al ., 2009*).Ziconotide, which is a drug that is obtained from a marine cone snail and is FDA-approved in chronic pain that does not respond to other painkillers. It is injected into the space between the spinal cord and its surrounding sheaths (Hannon, H E *et al.,2013.*)

1.2. Scientific classification of Palmyra tree

Kingdom	: *Plantae*
Division	: *Magnoliophyta*
Class	: *Liliopsida*
Subclass	: *Arecidae*
Order	: *Arecales*
Family	:*Arecaceae*
Genus	: *Borussus*
Species	: *Borassusflabellifer*

Plate 1.5.Palmyra palm (Borassusflabllifer)

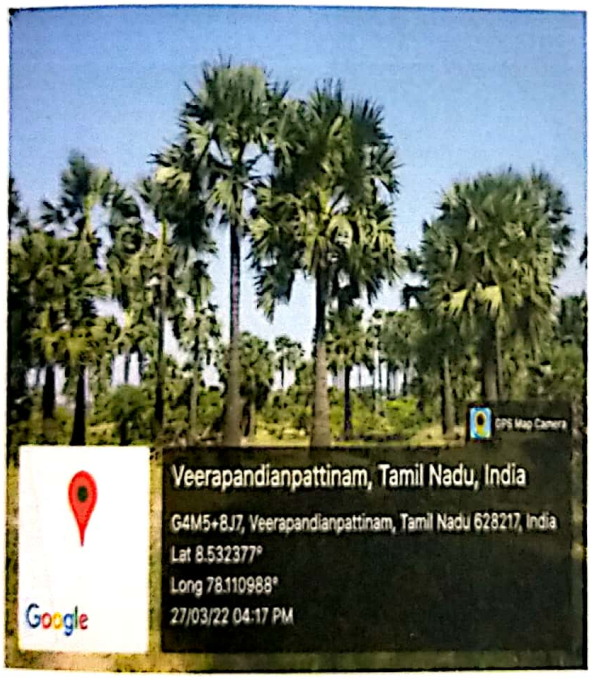


Plate.1.6.Palmyra palm root

Palmyra palm tree is a dioecious palm tree orginated in African belongs to Arecaceae family. Palmyra tree (Borassusflabellifer) can be found in tropical countries such as Thailand , Malaysia , Indonesia, India, Myanmar , Srilanka and Cambodia. *B. flabellifer* sugar was used in India during 4th century. It is a significant economic crop in kirimat district, it generates a

large quantities of husks, stalks, shells as by products and waste. The most important product of Palmyra palm is the sap, syrup and cake. The sap Of Palmyra tree is sterile but microorganisms are found in the sap during collection process. The male and female inflorescences are tapped to produce a sweet sap. Palm sap I quite nutritious and highly prone to fermentation as microorganism in the sap use sugar as a source of energy and fermentation.

Plate1.7.Palmyra sugar

Plate 1.8.Palmyra juice

Plate 1.9.Palmyra seed

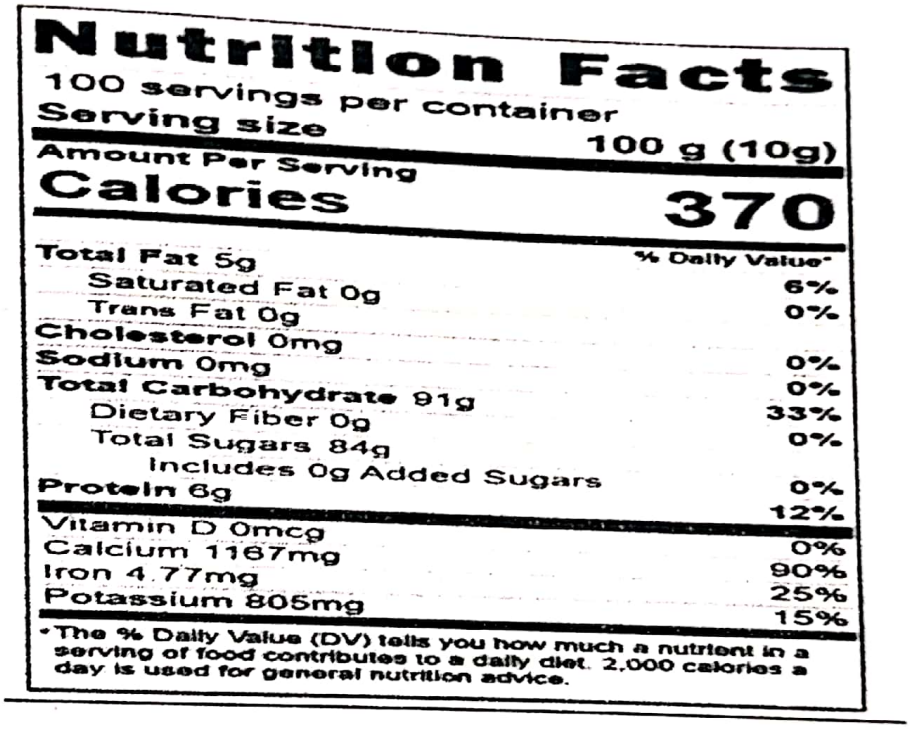
Plate1.10.Palmyra fruit

Plate 1.11.Palmyra jelly

Plate .1.12.Palmyra sap

1.3. Natural benefits:

Palmyra tree contains gums, steroidal glycosides , albuminoids, carbohydrates like sucrose and fat. The fruits of Palmyra tree are naturally reclative, laxative , stomachi and useful for the treatment of inflammatory reactions. The roots are also beneficial it has nutritional, nutraceutical properties. The nutritional analysis of the roots has shown 8.54% protein content,23.53% carbohydrates, 7.29 %crud fiber and negligible fat content. These roots are found to be high in calories. MP-AES analysis has shown these ediable roots cantain some amount of iron (1.38ppm) and traces of aluminium, arsenic , strontium , lead ,manganese , copper and zinc.



Nutrition Facts

100 servings per container

Serving size 100 g (10g)

Amount Per Serving

Calories	**370**
	% Daily Value*
Total Fat 5g	6%
Saturated Fat 0g	0%
Trans Fat 0g	
Cholesterol 0mg	0%
Sodium 0mg	0%
Total Carbohydrate 91g	33%
Dietary Fiber 0g	0%
Total Sugars 84g	
Includes 0g Added Sugars	0%
Protein 6g	12%
Vitamin D 0mcg	0%
Calcium 1167mg	90%
Iron 4.77mg	25%
Potassium 805mg	15%

*The % Daily Value (DV) tells you how much a nutrient in a serving of food contributes to a daily diet. 2,000 calories a day is used for general nutrition advice.

Plate. 1.13.Nurtional benefits of Palmyra palm (*Borassus flabellifer*)

AIM AND OBJECTIVE

- To prepare leaf and root extract of Palmyra palm using soxhlet apparatus and cold extraction methods respectively.

- To identify the Phytochemical compounds present in the root and leaf extracts of Palmyra palm.

- To isolate the bacterial strains from sewage water sample and wound sample

- To determine the antioxidant activity from the leaves and root extracts of Palmyra palm.

- To evaluate the anti-inflammatory activity from the leaves and root extracts of Palmyra palm.

- To determine the anti-ulcer nature of *Borassus flabellifer*.

- To detemine the antibacterial activity of extracts against isolated bacterial strains.

CHAPTER 2

REVIEW OF LITRATURE

ArapakornSakulsathoporn *et al.*, (2019), explained that *Borassus flabellifer-* family Arecaceae had undergone 46RNA editing. This provided an alternative set of loci for phylogenetic tree reconstruction of Arecaceae's sub families.

AyubaAuduet *al.*, (2016), carried out to activate some engineering properties, mechanical properties and statistical analysis of Palmyra palm tree.

Bishnu Joshi *et al.*, (2011), mentioned that antimicrobial test done for four plants (Tulsi, Neem, Datiwan, Clove) showed that all plants are resistant to E. coli and E. carophyllata (clove), A. indica were sensitive to S. typhi. Phytochemical analysis shows the presence of alkaloids, glycoside, terpenoids, steroids flavonoids, tannins, and reduced sugar.

ButsarakhamSingchain *et al.*, (2015), mentioned fresh palm sugar of *B. flabellifer* have natural constituents mainly antioxidant. Active phytochemicals responsible for activity was to be purified, isolated and characterized.

Chanphenchumsang*et al.*, (2014), described that pal waste could be used to produce fuel Briguettes as a substitute for wood charcoal and LPG.

T A Davis *et al.*, (1987), explained that *Borassus flabellifer*occured in Tamil Nadu is a multipurpose tree of green utility. Hence Palmyra products to be developed to maximize the economic value and produce sustained yield.

DarshanDharajiya et al., (2017), mentioned antimicrobial activity of Aloe Vera from hexane extraction which showed minimum inhibition zone against *S.Marcescens*. Phytochemical analysis resulted antimicrobial activity compound which was used for the development of antimicrobial drug against pathogenic microorganisms

Dharini S. G. *et al.*, (2021), reported that proximate analysis and traditional method results in Pok was as a highly nutritive food and had a considerable amount of fat soluble and water soluble vitamins. Phytochemical analysis shows the presence of flavonoids, alkaloids, glycosides and saponins.

Dennis Johnson (1992), illuminated that processing techniques involved with many palm products are involved with many palm products are advanced with Neotropics. The palm would be useful for other products production.

Dinesh Chand *et al.*, (2019), mentioned that Nanoparticles synthesis was easily done with palm bark, palm leaf and ghat leaf. This nanoparticles are user-friendly, energy efficient and environmentally safe for human health.

Dung Huynh Thile *et al.*, (2020), investigated the drying –solidification conditions of *B. flabellifer L* flower sap denoted that drying increased the antioxidant activity vacuum drying process had a good potential to shorten the production time and increase the BioActive phytochemical content of Palm granulated sugar.

Dung Huynh Thi Le *et al.*, (2021), established the ultrafiltration pretreatment of Palmyra palm syrup it generates a good appearance and reduced the HMF content any how it also affects the volatile compounds and physiochemical characteristics.

GarimaSinghal*et al.*, (2011), reported antimicrobial property of silver nanoparticles by chemical method. It was rapid, cost-effective and eco-friendly for synthesis of silver nanoparticles which is used for various industrial and medical applications.

Gayathri N *et al* .,(2016), reported phytochemical analysis of capsicum chinese sample showed carbohydrates, tannins, saphonins, flavonoids, phenols, alkaloids, terpenoids, and volatile oil are present so that reason it provided flavour improvement to food. Capsicum chinese fruit is very effective in the prevention of a lot of disease and used as a natural preservative in the cosmetic food industry.

HayrunnisaNadasoglu*et al.*, (2017), stated that the physical & clinical effects of antimicrobial antioxidative non-toxic nanoparticles obtained by green synthesis had significantly increased Biosensor construction is one such application.

Hamza Miohammed Ahmed *et al.*, (2020), reported phytochemical analysis showed cinnamon bark solvent extract had alkaloids, flavonoids, coumarin, tannins, terpenoids, saponin, glycoside, anthrocyanin and phenolic compound. High concentration of antimicrobial activity produced the extract's essential oil against the test organisms.

Iram C *et al.*, (2013), mentioned Lawsonialnermis extract had great significance of antimicrobial agent in therapeutics.

Jagannadha Rao P V K *et al.*, (2005), concluded that Thermal conductivity of sugarcane. Palmyra palm and date-pal granular jaggery was determined. They had a linear correlation for moisture content

Jagpreet Singh *et al.*, (2018), informed nanoparticles synthesied from plant extract are costly applied in environmental remediation and pharmaceutical industries.

Jerry.A (2018),throwed out light about the specificity of palmyra tree that it had a good capacity of antimicrobial activity, anti-inflammatory, anti-oxidant and analgesic activity. Hence, Palmyra tree is one of natural medicine, its various parts can be used for the treatment of various ailments.

KanchanaLathaChitturi*et al.*, (2018), concluded that silver and copper nanoparticles obtained from Palmyra fruit pulp has shown antitumor, antibacterial, antioxidant activity simultaneously Palmyra tree had the similar activity as of Ag – Cu nanoparticles.

KB Hebber*et al.*, (2018), suggested that there were various techniques for the improvement in quality and safety aspects of pal sap without affecting its natural flavour mainly in terms for microbial control in pal sap.

KrishnamoorthyRenuka*et al.*, (2018), reported that immature Palmyra palm fruit obtained phenolic and flavonoid content from phytochemical which analysis supported as a rich source for identification of nutracuticals and acts as a antioxidant

KrishnamoorthyRenuka*et al.*, (2020), explained that the use of immature fruits of palmyra palm in the treatment of diabetes gave out drastic change in the decreasing of blood glucose level. This showed significant antidiabetic properties.

Kwanjaipipatcharlearn Wong *et al.*, (2017), stated that *B. flabellifer* was a invasive plant successfully spreaded throughout Thailand and Southeast Asia countries. Inspite of their own diversity.

LetieleBruck de Souza *et al.*, (2020), mentioned *C. nutans* leaves extract was rich in phenolic and flavonoid compounds as well as it had low toxicity and absence of cyto and genotoxicity.

Manisha Vats *et al.*, (2011), reported antimicrobial activity of MurrayaKoenigii roots from chloroform and petroleum. Ether extraction minimum inhibitory against *A. niger, P. aeroginosa*. These result showed that roots had antifungal activity which can be used to formulate antimicrobial compostition. Phytochemical screening of root showed the presence of carbohydrates, alkaloids, steroids and flavonoids.

Mihai Babota*et al.*, (2018), mentioned *H. arewenarium* and *A. diocia* was the important sources of bioactive compounds in vitro activities. Ethanol extract is important sources of chlorogenic acid and flavonoids it also used for the treatment of oxidative stress related disorder.

Maathumai*et* S *al.*, (2018), mentioned that the effect of sodium metabisulphite on palmyra fruit pulp was that it acts as a strong preservation capacity when combined with refrigeration.

Mohamed Abdullah Aziz *et al.*, (2016), demonstrated that the extract of *Borassus flabellifer* would have numerous Neuropharmocological activities, such as Anti-depressive and anxiolytic activity. This extracts would be promising area of researches

NaganiPammi*et al.*, (2021), investigated the therapeutic and probiotic aspect of traditional Toddy Palm Nectar and also the nutritional profiling and isolation of lactic acid bacterial strains from TPN.

NutsudaSumensiria (2021), mentioned that a colourful and a protein content gummy jelly was produced from rope Palmyra fruit pulp. The adhesiveness was reduced after the incorporation of fruit juice

PhisutNahnean*et al.*, (2013), determined that pasteurized palm soap shelf life had been extended by usage of chitosan as a natural preservative.

Polamarasetty V *et al.*, (2010), stated that the Palmyra pal granules had highest glass transition temperature when compared with date-palm and sugarcane jaggery granules.

Prasad G Jamkhande*et al.*, (2016), concluded that the plant *B. flabellifer* has broad spectrum antimicrobial and antioxidant activity and a potential option for treating various infectious disease

Sandhiya and S Kalaiselvam J (2020), reported that *B.flabellifers* seed coat extract has the ability towards antimicrobial activity against bacteria and fungi. This antimicrobial activity has been synthesized

Samira *et al.*, (2020), mentioned extract of C. daetylon showed a wide range of activity against Gram-positive and Gram-negative bacteria and mold. Phytochemical compounds was used for the application of the therapeutic drug in folk medicine. Antimicrobial drugs are produced from *C. dactylon* used to treat various disease such as eye infection, pneumonia and gonorrhoea.

Sooad Al-Darihamet *al.*, (2012), reported high antimicrobial activity in methanol extract of *Cucurma longa* and *C. molmol* against *S.pyrogens* and *S. aureus*. Zingiberofficinals *and C. longa* can serve as bioactive healthy compounds in diets which helps to prevent from disease.

SrideviKrishnaveni T.R *et al.*, (2020), concluded that Palmyra tree are unique in the production of food and non-food product that is suitable for only Tamil Nadu climate. Then it also had many health products beneficial for human.

S Sushmitha*et al.*, (2013), reported antimicrobial activity of Azadirachtaindica (Neem) leaves all solvents are resistant to test organism except water. In which study suggested a number of active constituents present in the neem bark extract which controlled pathogens. Phytochemical plants showed secondary plant metabolites are alkanoids, glycosides, terpenoids, steroids, flavonoids, tannins reducing sugar.

Tawheed*et al.*, (2014), reported phytochemical screening, antimicrobial and antioxidant activities of ethanol and chloroform extracts of *Linumu sitatisimum* L showed the free radical scavenging activity using DPPH and hydrogen peroxide method. Phytochemical analysis showed tannins, flavonoids, terpenoids, phenols, protein and aminoacid were present in ethanol extraction of flaxseeds.8

TanzeelaNisar*et al.*, (2015), reported turmeric has antiparasitic, anti-oxidant, anti-inflammatory, anti-rhuematic, anti tumor, antiphlegmatic, antiviral properties which has been used in the treatment of severe disease including carcinogenesis inflammatory disorders and oxidative stress-induced pathogenesis.

Taranisen Panda *et al.*, (2021), suggest that the proper marketing of Palmyra products was to be done. As this plant does not have any expensive maintenance which would definitely support the rural livelihood.

Theivamirther C. *et al.*, (2020), mentioned that carbon–based materials produced from *Borassus flabeiilfer* had become a promising alternatives in fighting against global warming and pollution.

Vijayakumari*et* B *al.*, (2015), demonstrated palmyra fruit pulp has revealed that it had phytochemical compounds which supported various pharmacology actions. Hence, this powder promotes health when implemented in food product

VijayaBharathi S *et al.*, (2012),mentioned antimicrobial activity of aqueous extract of orithazhthamarai sensitive against all strains of microorganisms Phytochemical analysis showed the presence of the tannins which act as vitamins, flavonoids, phenols as antioxidant and terpenoids so that it was for used for the management and treatment of various diseases.

ViniyanShanmugalingam*et al.*, (2021), mentioned that the significance of *B.flabellifer* should be understood to sustain the invaluable source and this plant extract would definitely reveal discovery of new drug for various syndromes.

Wilson LamayeDanbature (2020), demonstrated phytochemical test of *Borassus aethiopum* sprout extract confirmed the presene of seven secondary compounds. These phytochemical were responsible in the production of nanoparticles which had a significant larvicidal potency.

Yash Punjabi *et al.*, (2014), reported N-nouchali flower methanolic extract was the most active and showed highest activity in Salmonella paratyphi A and the lowest activity in Salmonella paratyphi B it contains antimicrobial agent used for control of infectious disease

CHAPTER 3

EXTRACTION PREPARATION

3.1. EXTRACTION METHOD

3.1.1 .SAMPLE COLLECTION:

The root and leaves of palm were collected from the Veerapandiyapattinam , Thiruchendur,Tamil Nadu.The samples were washed and air dried under the shade.They were coarsely powdered in a electric mixer and stored in a glass bottle for further analysis.

Plate. 3.1. Root extract powder

Plate. 3.2.leaf extract powder

3.1.2. SOXHLET EXTRATION:

10 gram of powdered samples of palm leaf and root were taken and the extraction was done in soxhelet extraction. The extraction process was continued up to 8 hrs. After the stipulated time the solution was filtered and evaporated at room temperature and the extract was collected and stored in vials for further studies.

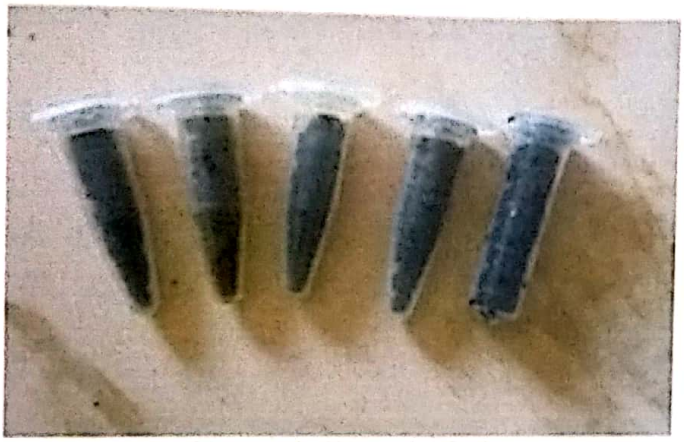


Plate.3.4. Leaf extract in Eppendorf tube

Plate. 3.5. Soxhlet extraction of leaf sample

Plate.3.6.Leaf extract powder in plates

3.1.3. COLD EXTRACTION

The root was soaked in acetone in a conical flask. Placed in rotary shaker at 120-130 rpm for 48 hrs. .Then extract was filtered in Whatman filter paper No 1.The solvent was evaporated and residue was collected

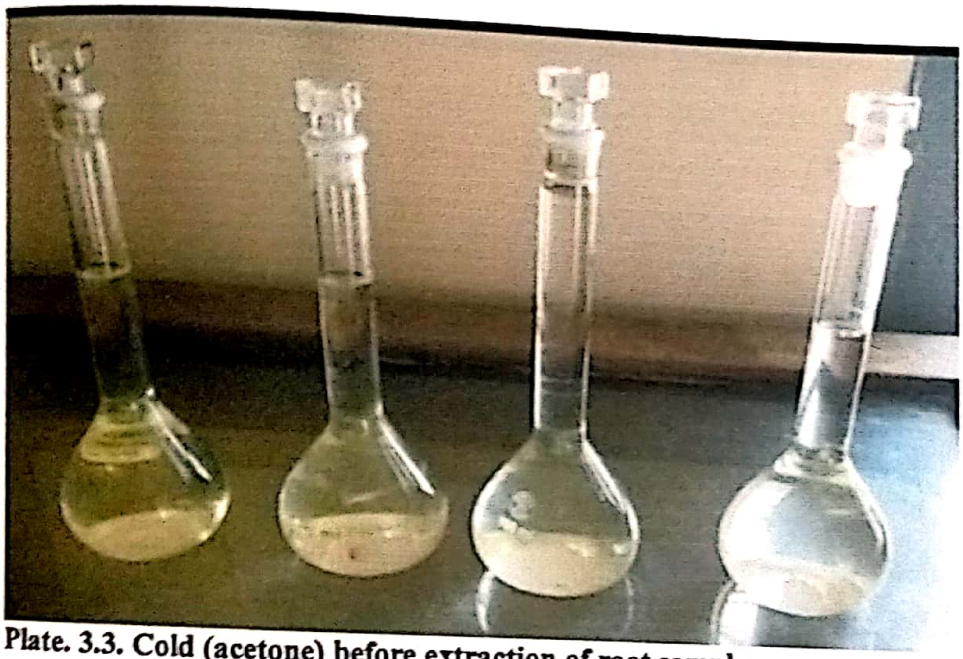


Plate. 3.3. Cold (acetone) before extraction of root sample

3.5. RESULT AND CONCLUSION:

Acetonic leaf and root extract of *Borrassus flabellifer* was obtained by hot (Soxhlet apparatus) and cold extraction respectively . These extract were evaporated and crude extracts were used for further analysis.

CHAPTER 4

PHYTOCHEMICAL ANALYSIS

4.1. INTRODUCTION:

Phytochemical analysis is a techniques commonly used in the field of phytochemistry are extraction, isolation and structural elucidation of natural products as well as various chromatography techniques. It is the preliminary screening for the detection of plant constituents by phytochemical analysis. Phytochemical are the non-nutritional bioactive compounds found in various parts of plants . These compound perform vital functions. Particularly helpful in synthesis of useful drug. Phytochemical are a wide variety of non nutritive chemical compounds found in plant food , which may have health effects.a few exampls for phytochemicals are the flavonoids , phenolic acid , isoflavones, curcumin , isothiocyanates and carotenoids etc. (Winston J Craig 1997).

The diet rich fruits and vegetable has protective effect containing huge group of compounds collectively termed "Phytochemical" It provides much of the flavour and colour of edible plants and beverages are derived from them. Some compounds that's do exert anti – carcinogenic effects at realistic may contribute to the putative benefits of plant food such as berries, Braddock vegetables and tea. Phytochemical has been conducted in vitro with little regard to the bioavailability and metabolism of the compounds studied (Ian T Johnson 2007). Phytochemicals as plant components with discrete bio-activities towards animals biochemistry and metabolism are being widely examined for their ability to provide health benefits. Phytochemicals could provide health benefits as 1. Substrates for biochemical reaction 2. Cofactors of enzymatic reaction 3. Inhibitors of enzymatic reaction 4. Absorbents / sequestrates that bind to and eliminate undesirable constituents in the intestine 5. Legends the agonize or antagonize cell surface or intracellular receptors. 6. Scavengers of reaction or toxic chemicals 7. Compounds that enhance the absorption and or stability of essential nutrients 8. Selective growth factor for beneficial gastrointestinal bacteria 9. Fermentation substrates for beneficial oral gastric or intestinal bacteria and 10. Selective inhibitors of deleterious intestinal bacteria (Cora J Dillard and J Bruce German 2000).

Phytochemicals including Phenolics, flavonoids and carotenoids from fruits and vegetable may play a key role in reducing chronic disease risk. Apples are a widely consumed, rich source of phytochemicals. It contains a variety of phytochemicals including quercetin, catechin, phloridzin and chlorogenic acid. The phytochemical composition of apple varies greatly

between different varieties of apple and there are also small changes in phytochemical during they are maturation and ripening of the fruit. The health benefits of apple are based on phytochemicals, phytochemical bioavailability and antioxidant behaviour and they are variation during ripening, storage and processing on apple phytochemicals (Jeanelle Boyer and Ruined Hair Lou 2004). Nuts,whole grains, fruits and vegetables contains Phenolic compounds, terpenoids and pigments. That have been helped with protection from and treating of chronic disease such as heart disease, cancer, diabetes and hypertension as other medical condition (Winston J Craig 1997).

Phytochemicals constituents are essential for the survival and proper functioning of plants. They provide protection against herbivores, microorganisms and competitors regulate growth and control pollination, fertilization and the rhizosphere environment. Plant producing Phytochemicals are "good. Phytochemicals are constitutive metabolites that enable plants to overcome temporary or continuous threats integral to their environment, while also controlling essential functions of growth and reproduction (Russell J Molyneux *et al.*, 2007). Phenolic Phytochemicals are the largest category of Phytochemicals and the most widely distributed in the plant kingdom. The three most important groups of dietary Phenolic are flavonoids. Phenolic acid and polyphenols. Phenolic polymers commonly known as tannins are compounds of high molecular weight that are divided in to two classes hydrolyzable and condensed tannins. (Amyking and Gloria young 1999).

There are concerns about using synthetic Phenolic antioxidants such as Butylated hydroxyl toluene and butylated hydroxyl anisole as food additives because of they are reported negative effects on human health (Ammatha Altemimi *et al.*,2017). Flavonoids constitute a large group of polyphenolic compounds with numerous effects on behaviour and cognition. These effects vary from learning and memory enhancement for the improvement of general cognition (Ioannis Bakoyiannis *et al..*, 2019). Interaction between phytochemical components often modify the pharmacological effects of botanical dietary supplements, functional foods or drugs. Two types of phytochemical interactions, 1.Exointeraction – that occur between components from different plants or between plants and synthetic drug. 2.Endointeraction – that occur between components within a plant species (Mary Anna Lillaand ILY Tasking 2005). Health promoting Phytochemicals commonly found in our daily food. These include carotenoids, Phenolics , Phytoestrogens , polyunsaturated fatty acid , conjugated linoleum acid, tools, Allicin, glucosinolates, limonene and capsaicinoids(ynakrzy zanowska *et al.*,2010). The use of phytochemicals against drug resistance microbes to treat microbial disease and used food

preparation, to inhibit methanogenic archaea in the rumen and to modulate lipid bio hydrogenating microbial populations to increase conjugated linoleic acids in animal – derived foods (Allan K Patra 2012).

Many Phytochemicals possess antioxidant and anti-inflammatory properties that may positively affect the gut micro biota (GM) including polyphenols, carotenoids, phytosterols / phytostanols, Lignans, alkaloids, glucosinolates and terpenes. Some polyphenols may act as prenuptial Phytochemicals may interact with the mucosal, another important factor for colonization and prevent it's degradation. Phytochemicals and health benefits related to the GM, emphasizing their potential as adjutant strategies for GM related disease (Giulia Dingeo*etal* 2020). The health potential of pulses, examining the bioactivity of pulse. It has isoflavones, phytosterols, resistant starch, bioactivity carbohydrates, alkaloids and saponins. The evidence for health properties is considered as is the effect of processing and cooking these potentially beneficial phytochemicals (Simone Rochfort and Joe Panozzo 2007). Being highly competitive and effective, the ideal phytochemical should possess a combination of toxic effects and residual capacity. Identification of novel effective mosquitocidal compounds is essential to combat increasing resistance rates, concern for the environment and food safety. The unacceptability of many organophosphates and organochlorines and the high cost of synthetic pyrethrodi. Larvicidal plants species, extraction processes growth and reproduction inhibiting Phytochemicals botanical ovicides , synergistic additive and antagonistic joint action effects on non-target organisms, resistant, screening methodologies and discuss promising advances made in phytochemical research (Essam Abdel - Salam Shaalan*et al.*, 2005).

4.2. METHODS

4.2.1. Test For Saponins /Foam Test:

2 ml of extract was taken in a test tube and 6ml of distilled water was added to it. The mixture was then shaken vigorously for 2 minutes. The persistence appearance of foam was observed that indicates the presence of saponins.

4.2.2.Test for Terpenoids /Salkowski test:

2 ml of extract was treated with 2 ml of acetic anhydride. Few drops of concentrated sulfuric acid was then added to this solution and observed the formation of blue, green rings that indicates the presence of terpenoids.

4.2.3. Test for Flavonoids/Alkaline reagent test:

2 ml of extract was treated with few drops of 1N sodium hydroxide solution and observed the formation of intense yellow colour. This yellow color becomes colorless on addition of dilute hydrochloric acid, indicating the presence of flavonoids.

4.2.4. Test for Phenol and Tannins/Ferric chloride test:

2ml of extract was taken in test tube and 3-4 drops of 0.1% of ferric chloride added. The addition of ferric chloride to the sample changed the colour to blue green or black, it indicates presence of phenol and Tannins.

4.2.5. Test for Steroids /Libermann Burchard Test:

2ml of extract is taken in test tube and dissolved with 10 ml of chloroform then equal volume of concentrate Sulphuric acid added to the test tube by sides. The upper layer in the test tube was turns into red and Sulphuric acid layer showed yellow with green fluorescence, it indicates presence of Steroids.

4.3. RESULT AND DISCUSSION:

Borassus flabllifer root and leaf was screened for the phytochemical constituents. It was found that are easily detected by qualitative tests. In our analysis it was clear that the *Borassus flabellifer* leaf and root are rich in terpenoids , steroids , flavonoids , sponins, tannins and phenol showed in Table 3.1 .Phytochemicals generally originated from the plant source are nothing but the bioactive compunds also known as secondary metabolites. Secondary metabolites produce by plants may have little need for them primary metabolites growth and developement.These are plant like bark , leaves , steam, root, flower , fruits , seeds .(Twinkle S. Bansode ., *et al., 2015).*

PHYTOCHEMICAL CONSTITUENTS	ACETONIC (LEAF)EXTRACT	ACETONIC (ROOT) EXTRACT
Tannins	+	+
Phenol	+	+
Steroids	+	+
Flavonoids	-	-
Terpenoids	-	-
Saponins	+	-

TABLE 4.1. PHYTOCHEMICAL CONSTITUENTS OF *Borassus flabllifer* LEAF AND ROOT EXTRACT

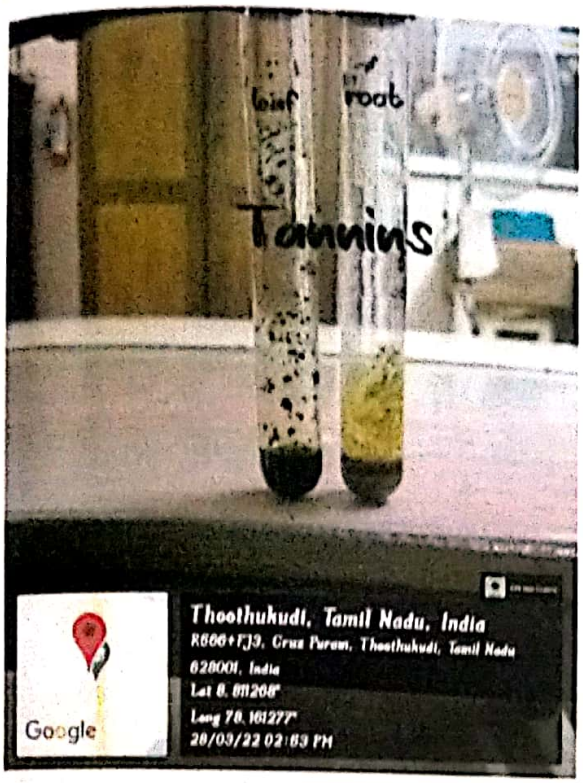


Plate.4.1. Tannin test

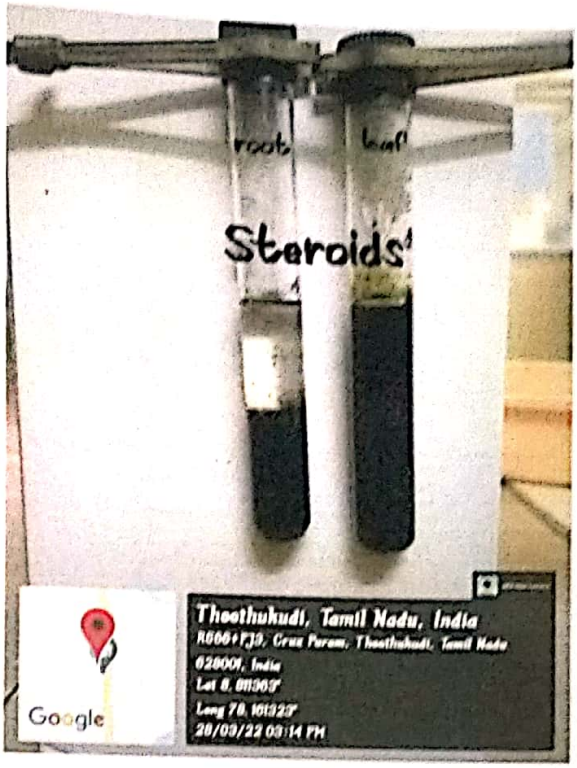


Plate 4.2. Steroid test

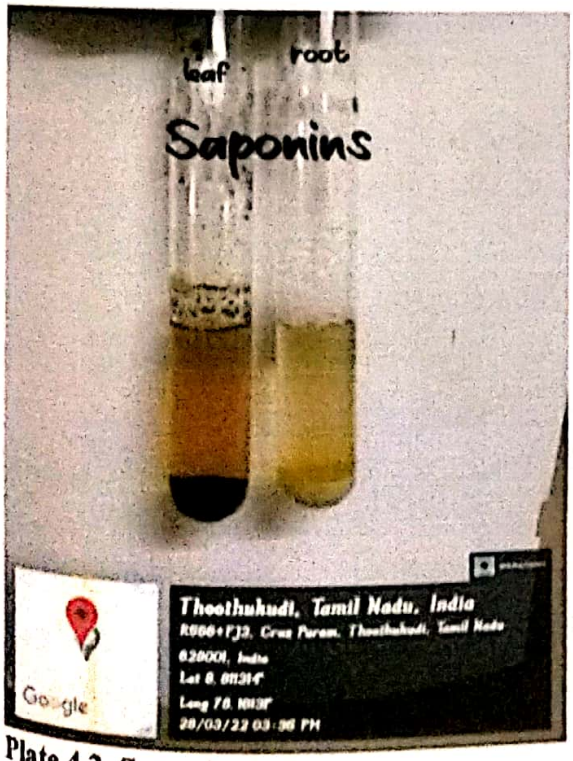


Plate.4.3. Saponin test

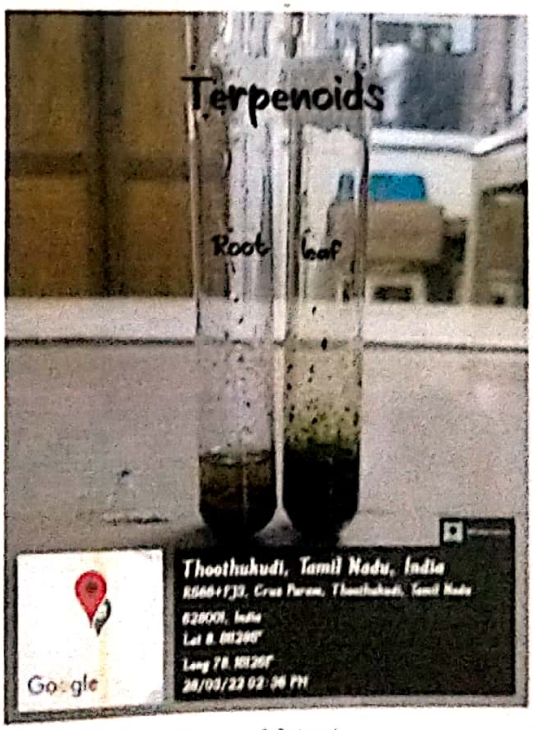


Plate 4.4. Terpenoid test

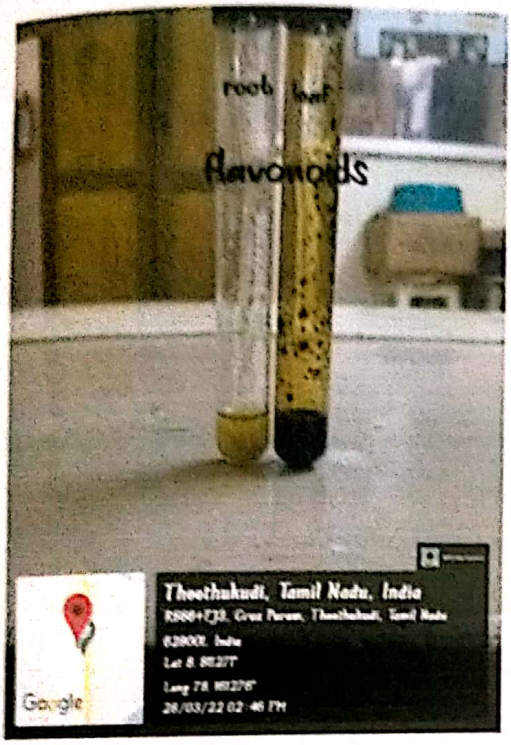


Plate.4.5. Flavonoid test

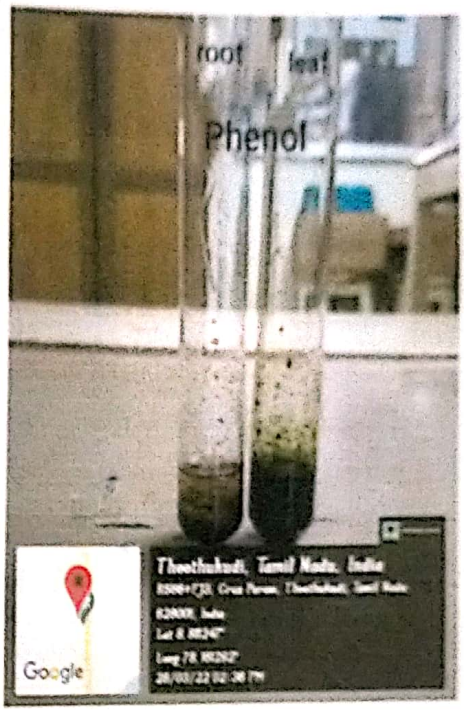


Plate 4.6. Phenol test

CHAPTER 5

ANTIOXIDANT ACTIVITY

5.1. INTRODUCTION:

Antioxidant is the substance that protects cells from the damage caused by free radicals. Unstable molecules made by the process of oxidation during normal metabolism. Free radicals may play a part in cancer , heart disease , stroke and other disease of aging. A diet rich in antioxidants may reduce the risk of many disease (including heart disease and certain cancers). Broccoli, spinach, carrot and potatoes are all rich in antioxidant. Antioxidant activity can be defined as limitation or inhibition of nutrient oxidation (especially lipids and proteins) resulting oxidative chain reaction .The important of oxidation in the body and in food stuffs has been widely recognized. Oxidative metabolism is essential for the survival of cells. A side effect of this based on dependence of production of free radicals and other reactive oxygen species that cause oxidative changes (Michael Antolovich.,*et al*.,2002).

The term antioxidant is widely used but rarely defined. One suggested definition is that an antioxidant is a substance that are present at low concentration compare with those of an oxidable substrate significally delays or prevents oxidation of the substrate (Halliwell B., 1995).Antioxidant play an important role in food preservation by Inhibiting oxidation process and contribution to health promoting rendered by many dietary supplements, nutraceaticals and functional food ingredients. Antioxidant activity can be monitored by a variety of assays with different mechanism including hydrogen atom transfer (HAT), single electron transfer (ET), reducing power and metal chelation among others (Fereidoon Shahidi and Ying Zhong., 2015).

The use of antioxidant for the treatment of human disease and the role of dietary antioxidants in the prevention of disease department .There are successes and failures in the therapeutic use of antioxidant (Barry Halliwell., 2000).The important mechanism is to understand the biological meaning of antioxidant possible use their production by organic synthesis or biotechnological methods or for the standardization of the determination of antioxidant activity. In vitro antioxidant reaction mechanism of organic compounds poly phenols, carotinites and vitamin C against radicals and pro oxidant compounds under diverse condition (Franconia Santos – Sanchez., *et al.*, 2010). The word antioxidant means different things to different people. Often (e.g. by food scientists) the term is implicitly restricted to chain breaking inhibitors of lipid per oxidant, such as Cr-tocopherol. However free radicals

generator in vivo damage many other targets including protein, DNA and small molecules (Barry Halliwell., 1995).

The uncooked whole trains are Bond with phytochemical which are major contributors to the total antioxidant activity 90% in wheat, 87 % in corn ,71% in rice and 58 % in oats (Kafui KwamiAdom and Rui Hai Liu .,2002).All the vegetables (fresh and thermally treated) shallots showed the highest total antioxidant activity followed by spinach swamp cabbage, cabbage and kale. Expect for shallots and cabbage the antioxidant activity of kale spinach and swamp cabbage were significantly decreased ($P<0.05$) after thermal treatment (Amin Ismail.*et al.*, 2004).

The use of synthetic antioxidant in food processing by the addition of natural oxidation inhibitors or by the preferential use of ingredients that naturally process antioxidant activity. The chemical structure of antioxidant are related to the synthetic antioxidant. Most phenolic antioxidant are pyrocatechol or pyrogallol derivatives, dihydrochromanols, or flavonoids. Fatty foods that are oxidatively stable without requiring the addition of antioxidant (Janpokorny.,1991).Antioxidant are present naturally or virtually in all food commodities, providing them with a valuable degree of protection against oxidative attack. Natural antioxidant are often depleted physically form the nature of the process itself or by chemical degradation. Antioxidant are applied intelligently were mechanism are complex and of anti-oxidative may rely on one or more of several alternative forms intervention (Bertram J Hudson., 2012)

5.2MATERIALS AND METHODS

5.2.1 Chemicals and reagents:

2,2-Diphenyl-1-picrylhydrazyl (DPPH) and ascorbic acid was purchased from Sigma-Aldrich. Phosphate buffer (pH- 7.4) and Methanol. All the chemicals and solvents used were of analytical grade.

5.2.2DPPH free radical scavenging activity:

DPPH solution (0.004 %), leaf and root extracts and standard (vitamin C) was prepared in methanol. Leaf and root extract and standard (vitamin C) solution were prepared in different concentrations 20, 40, 60, 80 and 100 µg/ml. 0.5ml of different concentrations of standard solution or leaf and root extracts was taken in different test tubes and then 0.5 ml of DPPH (0.004 %) solution was added and kept in dark for 30 min. and absorbance was recorded at

517 nm. The decrease in absorbance of the DPPH radical caused by antioxidant was due to the scavenging of the radical by hydrogen donation. It was visually noticeable as a colour change from purple to yellow. (Majo et al., 2008) The percentage inhibition activity was calculated using the formulae below:

% DPPH free radical scavenging

$$= \frac{\text{(Absorbance of control – Absorbance of test)}}{\text{Absorbance of control}} \times 100$$

5.3 RESULT AND DISCUSSION:

In the present study the scavenging activity was increase with the high concentration from 20- 100 mg/ml but the scavenging activity of the acetone extract of Palmyra leaf and root extract showed good result than the control which is summarized in the Table 7.1 and 7.2. The result than the experiment reduced the radical to the corresponding hydrazine exhibiting better free radical scavenging activity of standard vitamin C. In the present study, acetone extract of Palmyra palm leaf and root showed 41.73% and 71.06% respectively at the concentration of 100 µg/ml. When compared to the acetone extract of Palmyra palm leaf extract showed good result. The effect of antioxidant on DPPH radical scavenging is thought to be due to their hydrogen denoting ability (Joys Albert , Pratheta *et al.*,2011).the action of polyphenol is believed to be due to the redox potential which play an important role in adsorbing and neutralizing free radicals , quenching singlet oxygen or decomposing peroxides (Itagaki *et al.*,2009).

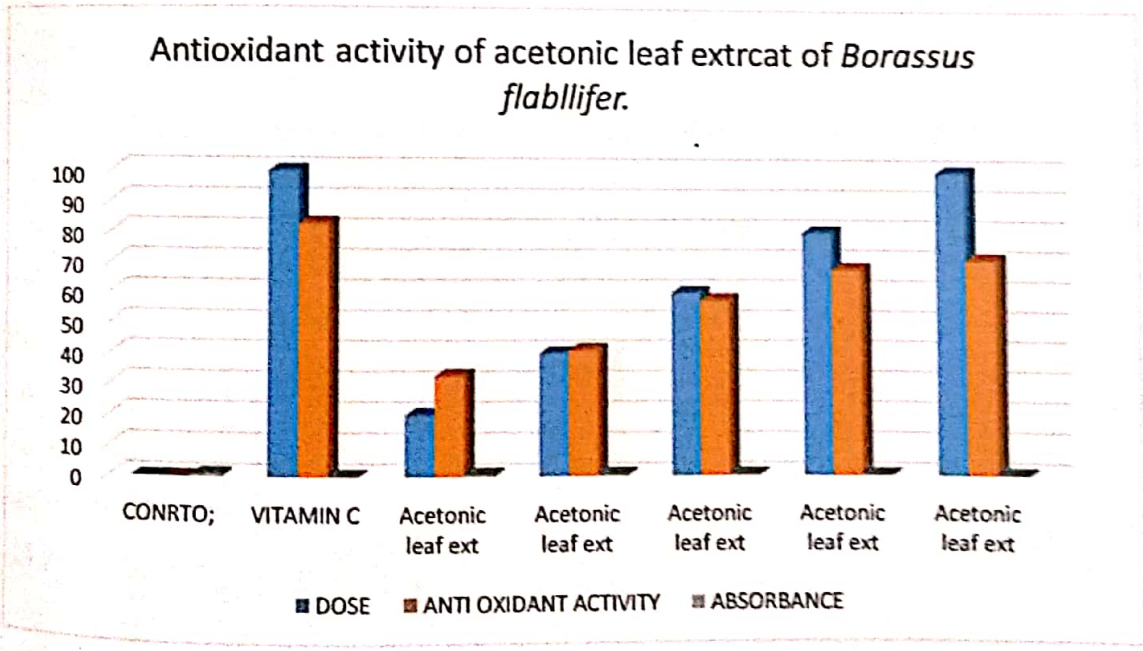
In the present study *Borassus flabllifer* showed nearly the same component as reported in other studies. The acetone extract of Palmyra palm leaf and root extract exhibited rapid scavenging activity but in varying rate of activity and its variation

4.4 CONCLUSION:

The result of the study showed that the acetone extract of Palmyra palm leaf and root and its constituents could be used as an easily accessible source of natural antioxidant and as a possible food supplement or in Pharmaceutical industry. Therefore, it is suggested that further work could be done on the isolation and identification of the anti-oxidative component of *Borassus flabllifer* root and leaf and its constituents

TREATMENT	DOSE	ABSORBANCE @ 517 nm	% OF ACTIVITY AGAINST DPPH RADICALS
Control	------	DPPH control=0.992	------
Vit C	100	0.172	82.66
Acetonic leaf ext.	20	0.816	17.74
Acetonic leaf ext.	40	0.765	22.88
Acetonic leaf ext.	60	0.684	31.04
Acetonic leaf ext.	80	0.624	37.09
Acetonic leaf ext.	100	0.578	41.73

Table 5.1 Antioxidant effect of *Borassus flabllifer leaf* on DPPH



5.1 Effect of antioxidant from acetonic leaf extrct of *Borassus flabllifer* against DPPH free radicals.

Treatment	Dose (µg/ml)	Absorbance @517 nm	% activity Against DPPH radical
Control	----	DPPH control= 0.992	----
Vit C	100	0.172	82.66
Borassus flabllifer root	20	0.672	32.25
Borassus flabllifer root	40	0.585	41.02
Borassus flabllifer root	60	0.422	57.45
Borassus flabllifer root	80	0.320	67.74
Borassus flabllifer root	100	0.287	71.06

Table 5.2 Antioxidant effect of *Borassus flabllifer* root on DPPH.

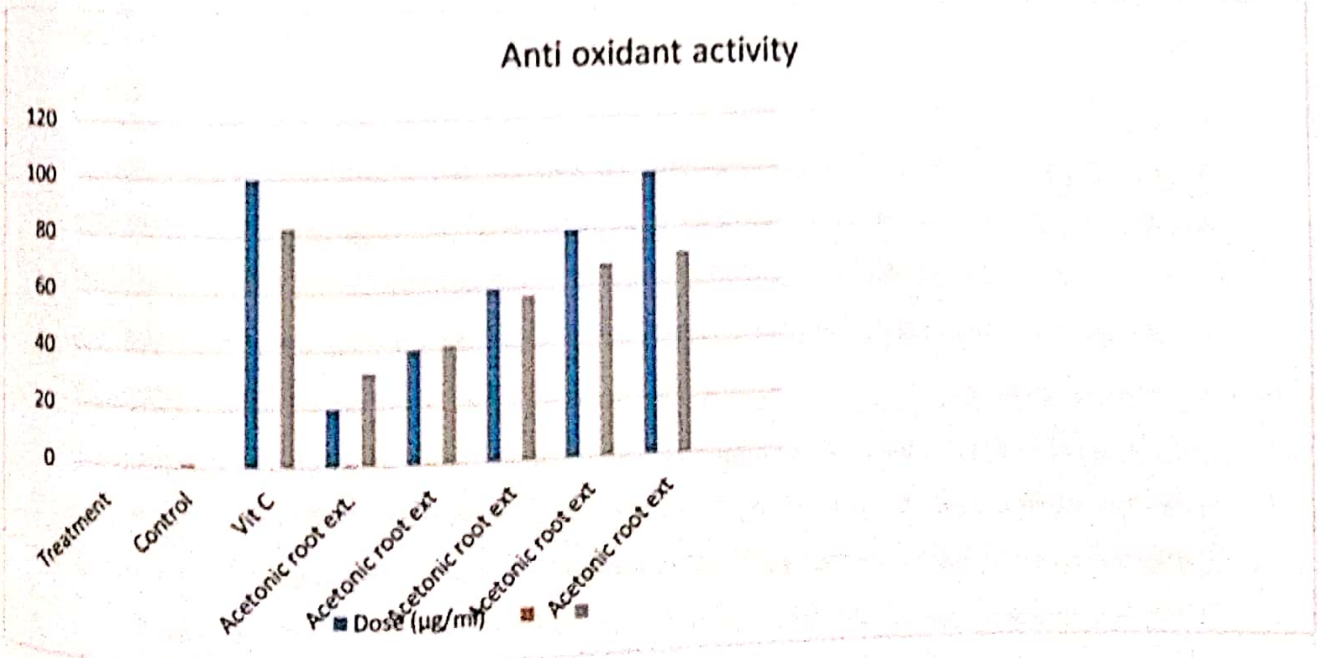


Figure.5.2.Effects of antioxidant activity of Palmyra palm (*Borassusflabellifer*) in acetone extracts of root on DPPH radicals

CHAPTER 6

ANTI – INFLAMMATORY ACTIVITY

6.1 INTRODUCTION:

A drug or substances that reduces inflammation (redness , swelling and pain) in the body . Anti-inflammatory agent block certain substances in the body that causes inflammation. Anti-inflammatory activity is important for wound healing procedure. In this phenomena, immune responsive compounds (cytokinins and interleukins) are produced by keratinocytes, B lymphocytes, T lymphocytes and macrophages.Anti-inflammatory activity used for determine whether plants have the capability to reduce the inflammation in human and animal.

The anti-inflammatory Cytokines are immunoregulatory molecules that control the proinflammatory Cytokines response. Cytokines act in concert with specific cytokine inhibitors and soluble cytokine receptors to regulate the human immune response. Physiological and pathologic role of Cytokines respectly inflammation and systemic inflammatory states are increasingly recognized. Major anti – Cytokines are interleukin (IL-1) receptor antagonist, IL-4, IL -6, IL-10, IL-11 and IL-13 (Steven M Opal and Vera A Female 2000). Inflammation disease are linked to enhanced bone loss. The effect of inflammation on bone is mediated by proinflammatory Cytokines, which regulate bone formation as well as bone resorption thereby altering bone homeostasis. Several proinflammatory Cytokines are major triggers for osteoclast activation explaining the enhanced bone loss during inflammation tissue is decisive whether inflammation triggers bone loss or not (Georg Schertt 2011).

Cytokines that is produced and released by contracting skeletal muscle fibres, exerting its effects in other organs of the body. Myokines may be involved in mediating the health beneficial effects of exercise and that protection against chronic diseases associated with low grade inflammation such a diabetes and cardiovascular disease（Anne Marie W Petersen and BenteKlarlundpedersan 2005）. Rheumatoid arthritis （RA）is a chronic systemic inflammatory disorder with its primary manifestations in the joints. Opioid drugs are not recently used in the treatment of RA because of their range of side effects and anti-inflammatory actions have been largely unrecognized . They are powerfully anti-inflammatory in a dose-dependent, time – depended, stereo selective and antagonist reversible manner（Judith S Walker 2003）. High intake of plant foods is associated with lower risk of chronic

disease. The mechanism of action and the components involved in this effect have not been identified clearly. Molecular activities of flavonoids include inhibition of transcription factors such NF-KB and activating protein -1 (AP-1) as well as activation of nuclear factor-erythroid 2-related factor 2 (Nrf2) (Ana Garcia – Lafuente.,*et al.*, 2009) .Many synthetic drugs reported to be used for the treatment of inflammatory disorders. Herbals containing anti-inflammatory activity are topics of immense interest due to the absence of several problem, which are associated with synthetic preparations. Inflammatory agents belonging to various class of phyto constituents like alkaloids , glycosides , trepenoids, steroids , polyphenolic compounds and also the compounds isolated from plant of marine origin , algae and fungi (Sarwar Beg *et al* 2011) .

Historically anti-inflammatory drugs had their originsin the serendipitous discovery of certain plans and their extracts being applied for the relief of pain , fever andinflammation. Thediscovery of the cox isoformd led to establishing their importance in many non arthritic or non – pain states where there is an inflammatory component to pathogenesis, including cancer, Alzheimer's and other neurode generative disease. Types of anti- inflammatory agents and discovery of new therapeutic targets to treat a whole range of conditions that were never hitherto envisaged (K D Rainsford 2007) .

Need of new anti inflammatory agents , great effort has been expended on the development of drugs for the treatment of inflammation. This disorder reduce the quality of life and overall average productivity causing huge financial losses (Rita De CassidaSilveriraesa 2013) . Inflammation involving the innate and adaptive immune system is a normal response to infection. Prolonged untreated auto inflammatory disorders, neurodegenerative disease or cancer .Safe and effective anti-inflammatories, with many more drugs under development. The new era of anti- inflammatory agent includes "biologicals" such as anticytokines therapies and small molecules that block the activity of kinases (Charles A Dinarello 2010) .

The past few decades , inflammation has been recognized as a major risk factor for various human disease. Acute inflammation is short- term self-limiting and its easy for host defences to return the body to homeostasis. Chronic illness characterized by infiltration of inflammatory cells , excessive production of cytokines , dysregulation of cellular signalling and loss of barrier function.

Flavonoids are widely present in the average diet in such food as fruits and vegetables . It demonstrated to exhibit a broad spectrum of biological activities for human health including an anti-inflammatory properties. Anti-inflammatory activities mechanisms of flavonoids and their implicated effects meant for the development of various chronic inflammatory disease (Min-HsiungPan., *et al.*, and 2010) .

6.2. MATERIALS AND METHODS

6.2.1. Drugs and reagent:

Diclofenac sodium (Standard for anti-inflammatory) SD fine grade and the solvents methanol of SD fine grade were used in this experiment.

6.2.2. Animals used:

Adult Wistar albino rats of either sex weighing between 150 and 180 gm were used. The selected animals were housed under standard environmental conditions (temperature of 22±10° C) in a 12 hours light- dark cycle. The animals were fed on standard laboratory animal diet (Amruth animal feed company, Sangli, Maharashtra) and the animals were fasted overnight before the experiment.

Plate 6.1.Albino rat is used for this present study

6.3. EXPERIMENTAL STUDY:

6.3.1. Anti – Inflammatory Testing

Anti - inflammatory activity was assessed by the method suggested by Winters et al., (1962) using carrageenan as phlogistic agent. The adult Wister albino rats of either sex

weighing between 150 & 180 gm were housed in groups of four animals each. They were starved overnight during the experiment but had free access to water. The volume of paw of each animal was determined before giving any drug. Animals are divided into four groups each consisting of four animals. Group I served as control which received normal saline [1ml/Kg. (p.o)], Group II received standard drug Diclofenac sodium [10 mg/Kg (p.o)], Group III received Acetonic leaf extract 400mg/kg p.o. and Group IV received Acetonic root extract 400mg/kg p.o. Acute inflammation was produced by sub plantar injection of 0.1 ml of 1% suspension of carrageenan in normal saline, in the right hind paw of the albino rats. One hour after oral administration of the drugs, the paw edema was measured with the aid of a standard plethysmometer (Inco, Chennai) at 1, 2, 3 and 4 hours after the injection of carrageenan. The difference between the readings at time 0 minutes and the different time intervals was taken as the thickness of edema volumes were given in table I. Percentage inhibition of paw edema was calculated by comparing the control for each dose at different hours as given below.

Percentage inhibition= Control OD – (Sample OD / ControL OD)* 100

6.4. RESULT AND DISCUSSIONS:

Anti-inflammation activity of leaf and root (acetone) extractions of Palmyra palm (*Borassusflabllifer*) on carrageenan induced raw edema in rats. Average given in table 6.1. and Figure 6.1.The role of test compound Palmyra palm (Borassusflabllifer)was evaluated at concentration 400mg/kg. Edema was reduced by 64.34 and 77.51% in a time depended manner due to carrageenan administration in all the animals severe swelling reached at 1 hour and the swelling was further aggregated in the control animal until the 4th hour while in the treated animal the swelling gradually reduced as the time of treatment increased. So Palmyra palm (*Borassus flabllifer*) leaf and root sample showed decreased paw edema significantly throughout the study. The swelling was constantly reduced during the 4th hour in the treated.

So that result revealed that both the test compound protected the rats from carrageenan induce inflammation and the test compounds showed the significant anti inflammation activity against the control group. (Gulgarani A R., *et al.*, 1999).The therapeutic application of flavonoids on inflammation have previously been reported and it reduced the inflammation to a control group.

6.5. CONCLUSION:

The discovery of novel drugs capable of fight inflammation particular through the origin. Bioactive constituent's terpenoids and fatty acid are as a promising source of anti-inflammatory substance. Further studies can be carried out to ensure a safe therapeutic agent against inflammatory diseases.

Statistical Analysis: All the data were expressed as mean ± S.E.

Drug Treatment	Dose mg/kg	Mean changes in paw edema ± SEM % edema inhibition			
		1 hour	2 hours	3 hours	4 hours
Control Saline	1 ml/kg	0.35±0.01	0.84±0.00	1.26±0.00	1.29±0.01
Standard Diclofenac Sodium	10mg/kg	0.20±0.00 (42.85%)	0.28±0.01 (66.6%)	0.25±0.02 (80.15%)	0.23±0.00 (82.17%)
Acetonic leaf extract	400mg/kg	0.30±0.02 (20%)	0.35±0.01 (58.33%)	0.45±0.01 (64.28%)	0.46±0.01 (64.34%)
Acetonic root extract	400mg/kg	0.28±0.02 (20%)	0.29±0.01 (65.47%)	0.28±0.01 (77.77%)	0.29±0.01 (77.51%)

Table 6.1.Effects of Palmyra palm (*Borassus flabellifer*) acetone extraction of leaf and root extract on Incision Inflammation model

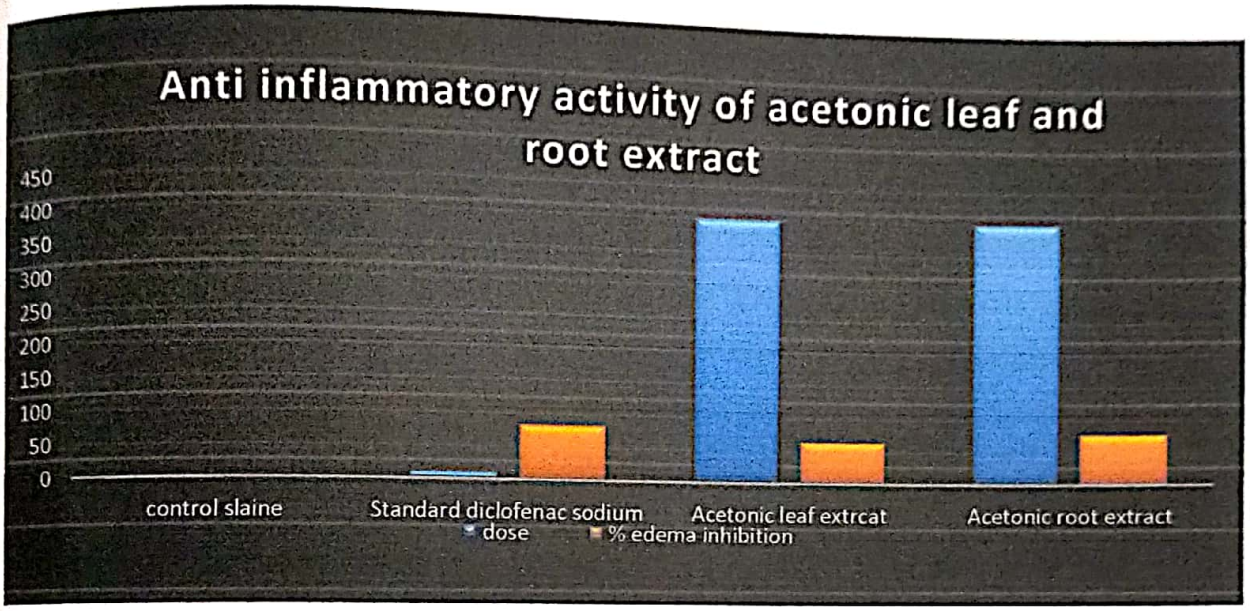


Figure 6.1 Effect of inflammation with acetonic extracts of leaf and root.

CHAPTER 7

ANTI ULCER ACTIVITY

7.1. INTRODUCTION:

Antiulcer are the agent tend to prevent or treat ulcers and especially ulcers of the wall of the stomach or duodenum. The antiulcer activity of lycopene can be attributed to different mechanisms, including inhibition of gastric acid secretion, reinforcement of gastric mucosal barrier and free radical scavenging activity.

Vitro and vivo studies of isolated phytochemical from plant extracts showed potential antiulcer genic (Mahdi Sharifi Ralf. 2018).There are two major factors that can disrupt the mucosal resistance to injury non-steroidal anti-inflammatory drugs (NSAIDS) example, aspirin and Helicobacter pylori infection. Numerous natural products have been evaluated as therapeutics for the treatment of acid various diseases including peptic ulcer (Ateeq Ahmad.,2013).The antiulcer activity of aqueous extract of roots of Tephrosiapurpurae (AETP) was identified in rats gastric and duodenal ulceration. AETP possesses significant antiulcer property which could be either due to cytoprotectiveaction of the drug or by strengthening of gastric and duodenal mucosal and thus enhancing mucosal defence (S SDeshpandaetal 2003).

Grape seed extract has a antiulcer activity were investigated using rats. The mechanism of antiulcer activity may be the protection by radical scavenging activity on the stomach surface against radical injury induced by HCl/EtoH solution and the defence action of procrastination covering the stomach surface by their strong ability to bind protein (Makoti Saito., *et al.*, 1998).

A number of substituted imidazole (1, 2-a) pyridines and related analogues were selected for analysis of their in vitro biochemical and in vivo gastric antisecretory activity using comparative molecular field analysis and hypothetical active site lattice (HASL) methodologies (James /Kaminsk and Arthur M Doweyko., 1997).Peptic ulcer are a broad term that includes ulcers of digestive tract in the stomach or the duodenum. The formation of

peptic ulcer depends on the presence of acid and peptic activity in gastric juice and a breakdown in mucosal defences (R.Gadekar. *et al.*, 2010).

Attachment of the p-aminobenzamido group at other positions on the pyridine ring or substitution of other nitrogen heterocyclic for pyridines, greatly decreased activity. P-aminobenzanilide was found to be highly active but quite toxic. The compounds were prepared from p-nitro benzoyl chloride and the requisite aromatic amine followed by reduction of the NO2 group. Gastrointestinal ulcers are commonly treated by diet, antacids, anticholinergic or surgery. Anticholinergic reduce the amount of gastric juices and its acidity and also the motility of the stomach and intestine (Robert B moffett., *et al,*. 1971). Z-benezylthio-5,6,7,8-terahydro-4(3H)-quinazolinones and evaluated in a histamine stimulated gastric secretion model. The sodium salt of the 2(dimethylamino)benzylthio derivatives showed gastric mucosal protection and gastric antisecretory activities. It was also effective against experimental gastric and duodenal ulcers induced by some ulcerogenic agents. Reduction of gastric acidity is more important factor than the reduction of gastric volume output or gastric total acid output (Kohji Terashima.,*et al.*,1995).

Antiulcer effects of pantoprazole, a proton – pump inhibitor, on water – immersion restraint stress (WIRS), alcohol (ethanol) and pylous ligation induced gastric ulcers. Including pantoprazole might reveal highly different effects according to the types of ulcer induce so that for that reason Antiulcer agents carefully selected for prescription(Dae – kwon Bae.,*et al.*,2011). Many antacids and the site protective agent sucralfate contain aluminium, Which can be absorbed, producing high level of serum aluminium. Prolonged intake of aluminium causing Alzheimer's disease. Some evidence indicates that aluminium is not a risk factors for Alzheimer's disease (Douglas W Piper .,1995).Inhibitors of gastric acid secretion and efficient drugs in the treatment of acid related disease. It reducing gastric acidity hypergastrinemia develops. Hypergastrinemia induced by potent inhibitors of acid secretion may be expected to increase the occurrence of gastric carcinomas in the future (Helge L Waldron., *et al.* 2005).

7.2. MATERIALS AND METHODS:

Antiulcer assay was carried out by following the method of Shay et al., 1945 and Suzuki et al., 1976. Albino rats (120-180gm) were starved for 48 hours with access only to water. During this time, they were housed singly in cages with raised bottoms of wide wire in order

to avoid cannibalism and coprophagy.Animals were divided into four groups of four animals each.

7.2.1. Anti-ulcer Testing:

Plate 7.1. Albino rat is used in the present study

The Group I received normal saline and served as negative control. Group II received 10 mg/kg p.o. of Ranitidine as standard drug. Group III received leaf and Group IV received root extract dissolved in normal saline, at a dosage of 400 mg/kg p.o. All the animals received test extracts daily for three days and on the fourth day, a midline incision was made under anaesthesia with ether and the stomach was exposed for pylorus ligation (Shay *et al.*, 1945). The abdominal opening was closed by suture and the rat was not allowed to sleep. The animals were deprived of water during the postoperative period and were sacrificed after 19 hours.. The stomach was carefully dissected out and gastric juice was collected and centrifuged at 3000 rpm for 30 minutes. The total volume of gastric juice, its pH, free and total acidity of gastric secretion, were estimated. The free and total acidities of the supernatant were calculated by titration with 0.1 N NaOH (Kulkarni, 1999). The stomach was incised along the greater curvature and pinned on a soft wooden board for evaluation of the ulcerated area and the ulcer index in the glandular stomach region was measured as

Normal coloured stomach-0, Red colouration-0.5, Spot ulcers-1, Haemorrhagic streaks-1.5, Surface ulcer-2, Deep ulcer-3, Perforation-4 and the mean ulcer score for each animal were expressed as ulcer index (Ganguli, Bhatnagar, 1973).

The percentage of ulcer inhibition was calculated by the following formula:

$$\frac{\text{Control mean ulcer index} - \text{Test mean ulcer index}}{\text{Control mean ulcer index}} \times 100$$

7.3 OBSERVATION:

CONTROL- Red colouration-0.5, Spot ulcers-1, Haemorrhagic streaks-1.5

STANDARD- Red colouration-0.5

Acetonic leaf extract - Deep ulcer-1, Red colouration-0.5

Acetonic root extract- Red colouration-0.5

TABLE 7.1: OBSERVATION OF ANTI-ULCERACTIVITY OF ACETONIC LEAF EXTRACT AND ACETONIC ROOT EXTRACT AGAINST PYLORUS LIGATION INDUCED GASTRIC ULCER IN ALBINO RATS

TREATMENT	DOSE(m/kg)	VOLUME OF GASTRIC JUICE (ML)	PH	ACIDITY		ULCER INDEX	% OF ULCER INHIBITION
				FREE ACIDITY (meq/l)	TOTAL ACIDITY (meq/l)		
Control (Normal saline)	2ml/kg	9.55±0.06	2.70± 0.06	94.25±1.24	121.75±1.37	3.00±0.20	-
Standard (Ranitidine)	10mg/Kg	2.60±0.22	4.71± 0.06	30.75±1.22	56.75±1.10	0.50±0.20	83.33%
Acetionic leaf extract	400mg/Kg	5.14±0.28	4.20± 0.14	36.10±1.24	18.24±0.12	1.22±0.08	59.33%
Acetonic root extract	400mg/Kg	3.04±0.16	4.40± 0.12	34.12±1.26	56.45±0.22	0.80±0.10	73.33%

7.4 RESULT AND DISCUSSION

The rat that received the root extract has ulcer index 0.80 ±0.10 and showed 73.33% of ulcer inhibition from the results of this work, it is evident that the root sample has high antiulcer activity compared to acetonic leaf extract. The leaf extract showed deepulcer-1 and red colouration 0.5 and the root extract showed only red colouration0.5.

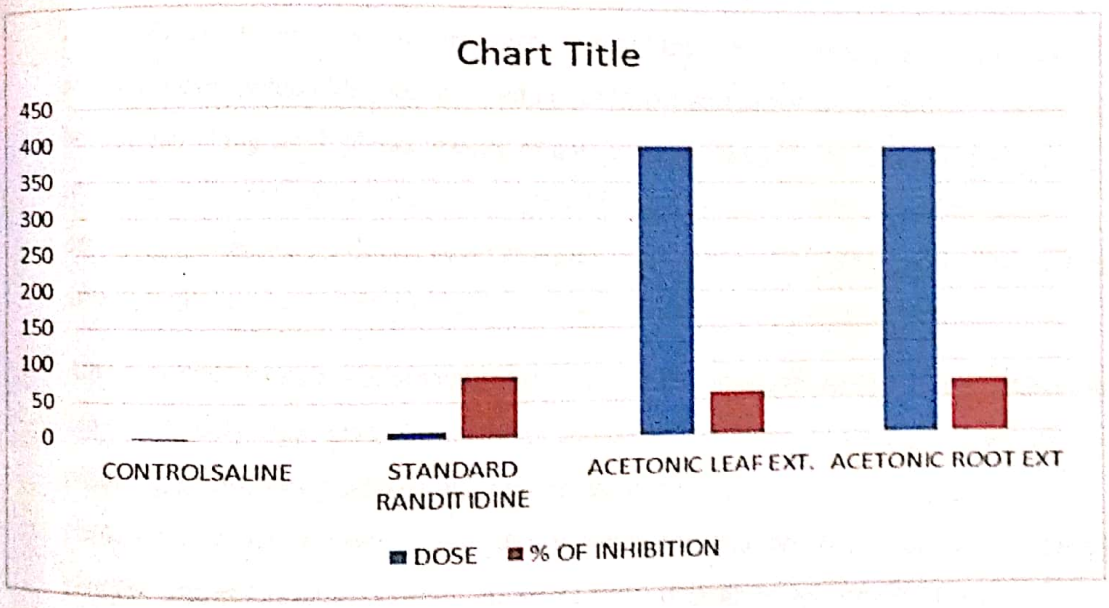


Figure 7.1.Effects of antiulcer activity of Palmyra palm (*Borassusflabellifer)* in Acetone extraction of leaf and root.

CHAPTER 8

ANTIMICROBIAL ACTIVITY

8.1. INTRODUCTION:

Antimicrobial activity can be defined as a collective term for all active principles that inhibit the growth of bacteria prevent the formation of microbial colonies and may destroy microorganism .It is the most important characteristic of medical textiles , to provide adequate protection against microorganism biological fluids and aerosols as well as disease transmission. For medicinal plants, antimicrobial activity correlates with the proportion of antagonistic entophytes. Medicinal plants are known to harbour potential entophytic microbes due to their bioactive compounds. Antimicrobial agents must share there essential characteristics in vitro and vivo. To realize their full potential for effective therapy in critically ill patients . Few antimicrobial agents are e.g. Pencillin, valacyclovir, Fluconazole, Prazi quantel.

Plants rich in a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids and flavonoids, which have been found in vitro have antimicrobial properties. Traditional healers have long used plants prevent or cure infectious conditions. Western medicine is trying to replicate their successes (Marjorie Murphy Cowan., 1999). Antimicrobial host defence peptides are produced by all complex organisms as well as some microbes and have diverse and complex antimicrobial activities (Harvard Jensen., *et al.*, 2006).

Antimicrobial agents are compounds that are in low concentrations suppress or inhibit the growth of microorganisms. This class of compounds includes the antibiotics naturally occurring substances produced by yeast, molds and other microorganisms and the chemotherapeutics are substances that are chemically synthesized. In additional, the mineral element copper has anti-bacterial properties when present in high concentration. Antimicrobial agent have been used widely as feed additives for swine since the early 1950s. They are used at low level in feed for growth promoting, improvement of food utilization reduction of mortality and improvement of reproduction efficiency. Antibacterial agents also are used to moderate to high levels for the prevention of disease in exposed animals and at highly therapeutic(G L Cromwell., 1991).

Antimicrobial agents are used in textiles, including Quaternary ammonium compounds, N – halamines , Chistosan , polybiguanides ,triclosan, nanoparticles of noble metals and metal

oxides and bioactivity plant-based products (Barbara simoncic and Brigita Tonic 2010). The antimicrobial activity of plant extracts found in folk medicine, essential oils or isolated compounds such as alkaloids, flavonoids, sesqui terpenlactonse, diterpenes, triterpenes or naphthoquinones' among other. By previously detecting the antimicrobial activity of plant part some compound were isolated or obtained by Bio-guided isolation (Jose-Luis kios and Maria Carmen Recia.,2005).

Diffusion and dilution methods have been employed to study the antimicrobial activity of medicinal plants. Bioacutography is another method for studying antimicrobial activity. Since some factors can change LA results. It is difficult using these methods to standardize a procedure for the study of antimicrobial plants (Jose-Luis Rips., *et al.*, 1998). Rapid method used for assessing antimicrobial activity is by bacteriostatic and bactericidal assay. Dynamics of antibacterial action ,Quantitative assessment of growth and death of microbial populations three factors affecting antimicrobial activity, nine test for determining bactericidal activity, four test for determining bacteriostatic activity(Sally F Bloomfield .,1991).

The development of chemical synthesis has helped to produce the synthetic components which act as an antimicrobial agent against the pathogenic bacteria. These synthetic components are also called as antibiotics. Pathogenic bacteria can be killed by synthetic compounds at low concentration example, ampicillin and amoxcillin(C De Boer .,*et al.*, 1 1970).The structural requirements of peptides for antiviral and antibacterial activities are evaluated in light of the diverse set of primary and secondary structures described for host defence peptides (HavardJensen.,et al,2006).

8.2. MATERIALS AND METHODS:

8.2.1 Media used: Muller Hinton Agar (MHA)

8.2.2. Bacterial strains: *Staphylococcus aureus, E. coli*

The molten and cooled media was poured in sterilized petri dishes(20 ml/dish). The plates were kept overnight at room temperature to ensure contamination. Briefly, wells of 10 mm diameter were prepared in the agar plates with the help of sterilized stainless steel cork borer. Lawns were prepared on agar plates by the spreader employing 100microliter NB culture of each organism. The leaf and root extracts (100 lg/ml) were prepared in DMSO and from that 100 microliter was used for activity. The wells on each plate were loaded with samples and

the same procedure was carried out for standard antibiotics. The plates were aerobically incubated at 37 C for 24 h for bacteria. The diameters of inhibition zones were used as a measure of antimicrobial activity.

8.3. RESULT AND DISCUSSIONS:

The antibacterial activity of the acetone extraction of Palmyra palm leaf and root sample against human pathogens was determine by measuring the diameter of zone of inhibition expressed in millimetre(mm) (Wilkinson ,2007 and Joys Albert, 2015). *Borassus flabllifer* leaf and root extracts showed varied in the exploitation of antimicrobial activity of zone of inhibition from 5-9 mm against *E.coli* and *Staphylococcus aureus.*Root (acetone) extract gives the zone of inhibition against *E.coli* and *Staphylococcus aureus* is 8 mm and 9 mm.Leaf (acetone) extract gives the zone of inhibition against *E.coli* and *Staphylococcus aureus* is 7mm and 8mm. The *Borassus flabllifer* acetone extraction of leaf and root sample gives the minimum zone of inhibition against gram negative *E.coli*and gram positive *Staphylococcus aureus.*This can be further observed the antibacterial effects of the *Borassus flabllifer*root and leaf (acetone) extracts studied against both gram negative and gram positive bacteria in the present study may due to differences in permeability barriers .From the results , it was obvious that the extracts of Borassusflabllifer showed significant potency against the test organism(Saravanan C .,*et al.*,2012).

Table.8.1.Effects of antimicrobial activity Palmyra palm (*Borassusflabellifer)* leaf and root sample against bacterial strains

HUMAN AND SEWEAGE BACTERIAL PATHOGEN	ZONE OF INHIBITION(mm)			
	Positive Control	Negative control	Leaf (acetone) extract	Root(acetone) Extract
E.coli	5mm	0	7mm	8mm
Staphylococcus aureus	5mm	0	8mm	9mm

Plate 8.1.Zone of inhibition of *E. coli*

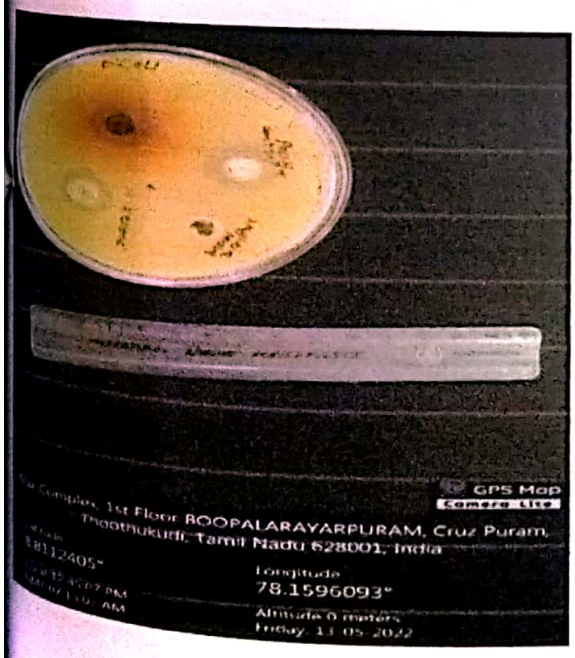
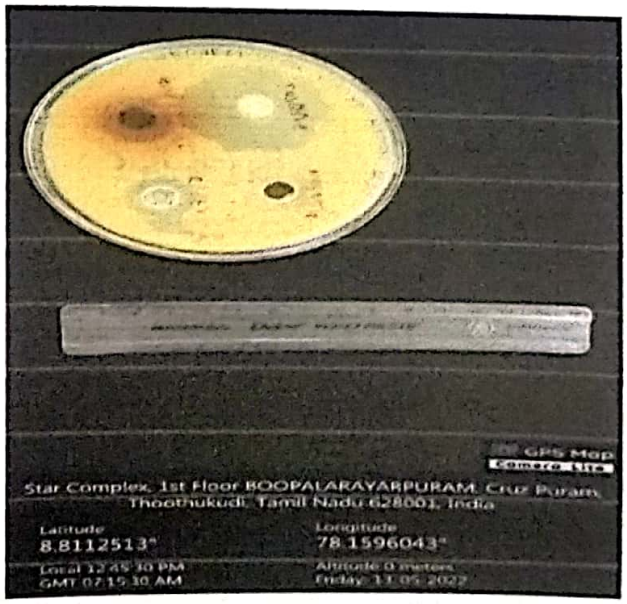


Plate 8.2. Zone of inhibition of *S.aureues*



CHAPTER 9

SUMMARY

Plant play an important role in health as medicine from the beginning of human era where Palmyra palm is a dioecious palm tree which are highly beneficial towards biological action in our body .In this study *Borassus flabellifer* was collected from Veerapandiyapattinam, Thiruchendur , Tamil Nadu. The leaf and root of Palmyra palm was fired and blend into powder. The powder was further proceeded for solvent extract by soxhlet apparatus where acetone was used as solvent.This was run for 8 hours, then the extract was collected by filtering through Whatman filter paper no.1.The solvent were evaporated and crude extract were stored for further use . The acetonic extract of *Borassus flabellifer*were analyzed to identify the presence of phytoconstituents. Test were carried out for saponins, terpenoids, flavonoids, phenol, tannins, steroids. The root extract showed the presence of steroids, saponins, phenolic and tannins compounds whereas the leaf extract revealed the presence of steroids and tannins.The wound sample was collected from Government Hospital in Thoothukudi.The collected sample was processed to isolate the microflora in the sample.The isolated organism was inoculated in Nutrient broth by determining the colonies on Mannitol salt agar. Similarly *E. Coli* was isolated from sewage sample from Pudukottai by determining the colonies on EMB agar. The pathogen from wound sample and sewage sample were *S. aureus* and *E. Coli* respectively. Muller-Hinton agar plates were used to demonstrate the antimicrobial properties of acetone extract of *Borassus flabellifer*. The extract of *Borassus flabellifer* showed moderate zone of inhition against *E. Coli* and *S. aureus*. Antiulcer assay was carried out by following the method of (Shay et al.1945 suzuki et al 1976)Albino rats (120-180 gm) were starved for 48 hours with access of water only. During this time, they were housed singly in cages with raised bottom of wide wire in order to avoid cannibalism and coprophagy. The root and leaf extract of *Borassus flabellifer* showed ulcer inhibition of 73.33% and 59.33% respectively in Albino rats. Anti-inflammatory activity was assessed by method suggested by (winter's ET al1962) using carrageenan as Phlogistic Agent where different dose of root and leaf sample was significant activity of antioxidant. This study determine us the spark about *Borassus flabellifer* nature towards the phytocompounds and Antiulcer, Antioxidant activity which would led to pathway of arise of new drug discovery.

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EP23

Phytochemical and Antimicrobial activity of Palmyra Palm (*Borassus flabellifer*) Leaf and Root

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The phytochemical and antimicrobial activity of palmyra palm (Borassus Flabellifer) root and leaf was identified by the activity of acetone as a solvent. The leaf and root sample was collected, dried and coarsely powdered and then the extract was collected by using acetone in the Soxhlet apparatus and by cold extraction using acetone as solvent respectively. A phytoconstituent analysis of alkaloids, glycosides, triterpenoids, steroids, flavonoids, tannins, and phenols, Saponins were performed on various solvent extracts. Antimicrobial activity was analyzed by Agar well diffusion method against two pathogenic microorganisms, *E. coli* and *S.aureus*. The maximum zone of inhibition was exhibited for *E. Coli* compared to *S.aureus*. The extract of the palm root has shown consistently significant inhibitory activity on different bacterial species tested.

Key words: Antimicrobial activity, Phytochemical Screening, Palm root, palm leaf

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