CARBONDIOXIDE SEQUESTRATION AND RECLAMATION OF SALINE LAND USING ALGAE

By

Rabia Shaukat (2010-NUST-BE-ENV-28)

Noor ul Subah Riaz (2010-NUST-BE-ENV-26)

Iram Sifat (2010-NUST-BE-ENV-16)

Hifsah N Qaisrani (2010-NUST-BE-ENV-12)

Bachelors of Science in Environmental Engineering

Institute of Environmental Science and Engineering (IESE)

School of Civil and Environmental Engineering (SCEE)

National University of Science and Technology (NUST)

Islamabad – Pakistan

(June 2014)

Carbon dioxide Sequestration and Reclamation of Saline Land Using Algae

Thesis Submitted In Partial Fulfillment

Of The Requirement for the Degree Of

BACHELORS OF SCIENCE

IN

ENVIRONMENTAL ENGINEERING

By

Rabia Shaukat (2010-NUST-BE-ENV-28)

Noor ul Subah Riaz (2010-NUST-BE-ENV-26)

Iram Sifat (2010-NUST-BE-ENV-16)

Hifsah N Qaisrani (2010-NUST-BE-ENV-12)

Bachelors of Science in Environmental Engineering

Institute of Environmental Science and Engineering (IESE)

School of Civil and Environmental Engineering (SCEE)

National University of Science and Technology (NUST)

Islamabad – Pakistan

June 2014

CERTIFICATE

This is to certify that the thesis titled

Carbon dioxide Sequestration and Reclamation of Saline Land Using Algae

submitted by

Rabia Shaukat (2010-NUST-BE-ENV-28)

Noor ul Subah Riaz (2010-NUST-BE-ENV-26)

Iram Sifat (2010-NUST-BE-ENV-16)

Hifsah N Qaisrani (2010-NUST-BE-ENV-12)

towards partial fulfillment for the award of degree of

BACHELORS OF SCIENCE

IN

ENVIRONMENTAL ENGINEERING

Embodies original work carried out by the students under my supervision.

Project Advisor:

Dr. Zahiruddin Khan
HoD – Environmental Engineering
IESE (SCEE)
NUST

Project Co- advisor:

Dr. Ehsan Ali
Associate Professor
Centre for Energy Systems (CES)
NUST

APPROVAL SHEET

This is to certify that the thesis titled

Carbon dioxide Sequestration and Reclamation of Saline Land Using Algae

submitted by

Rabia Shaukat (2010-NUST-BE-ENV-28)

Noor ul Subah Riaz (2010-NUST-BE-ENV-26)

Iram Sifat (2010-NUST-BE-ENV-16)

Hifsah N Qaisrani (2010-NUST-BE-ENV-12)

has been accepted towards partial fulfillment for the award of degree of

BACHELORS OF SCIENCE

IN

ENVIRONMENTAL ENGINEERING

Approved by:

Dr. Zahiruddin Khan Project Advisor	**Dr. Ehsan Ali** Project Co-advisor
Dr. Zahiruddin Khan HoD- Environmental Engineering	**Dr. Ishtiaq A. Qazi** Associate Dean (IESE)

SPECIAL DEDICATIONS

We grant this effort

to

the forgotten and unrecognized legacy of Muslim scientists and researchers;

whose contributions laid the foundations of modern-day science & technology

The pioneers of scientific knowledge and wisdom;

Al-Biruni, Al-Zahrawi, al-Haitham, Jabar bin Haiyyan, Al-Khwarizmi

and many others ...

Unfortunately their names have been lost in history.

ACKNOWLEDGEMENTS

We would like to express our gratitude and appreciation to all those who gave us the possibility to complete this project. A special thanks to our supervisor Dr. Zahir ud din khan (HOD IESE) for his patient guidance, enthusiastic encouragement and useful critiques of this research work. We would also like to express our deep gratitude to our co-supervisor Dr. Ehsan Ali (Associate Professor CES) for his valuable and constructive suggestions, guidance and his support throughout the research work. His willingness to give his time so generously needs appreciation.

Our grateful thanks to Dr. Usman Shah for his advice and assistance.

Special thanks to Environmental chemistry lab, Wastewater lab , Biotech lab and Microbiology lab at IESE(NUST), Biofuel lab at CES (NUST)and lab at SCME (NUST) for providing us facilities of equipment and chemicals as required by the project. A special thanks to the entire lab staff especially Sir Basharat at Environmental Chemistry lab (IESE), and all the other staff at our department (IESE) for helping us out.

We would also like to thank Mr.Tariq from Sargodha Remount Depot.

We would also like to pay our great appreciation to our seniors and Master Students Syeda Asma, Liaqat Kareem, Azra Nawar ,Muneeb Qayyum, Asif Khoja , and Abeera Ansari for their kind assistance to carry out the research work.

Very special thanks go to our School of Civil and Environmental Engineering, our department Institute of Environmental Science and Engineering and above all NUST for funding our project. Deepest thanks to our parents, families and friends for their support.

This page is intentionally left blank

TABLE OF CONTENT

LIST OF FIGURES

LIST OF EXHIBITS

LIST OF TABLES

ABSTRACT

The rapidly rising world population is shrinking the cultivable land and demands for more efforts to increase food production. Recovery of saline land would be of key significance to avoid the consequences of food and environmental crisis in near future. Pakistan has 14-20 million acres of saline lands including coastal belt. To deal with this problem, we introduced algae as a solution. Some algal species uptake the salts as a nutrients, hence reduce the salinity of the land. Algae have been predicted as most promising feedstock, they need salts, carbon dioxide which leading to CO2 sequestration and sunlight to grow and produce oil. Culturing of selected algal strains was performed on laboratory scale. Algal samples were collected from Sargodha Remount Depot. The algal samples were enriched in Modified Johnson medium with a pH of 6.8-7.2 range, incubated and illuminated under controlled conditions. Dilutions, micro-pipetting, heat exposure, spreading and streaking techniques were used for isolating strains. Algae were scaled up from culture tubes to beakers then to large flasks and finally to artificial saline land. The algal strains transferred to artificial saline land were *KKL-5 and* Chlorella Vulgaris. Salinity tests of ASL were carried out on daily basis and readings were recorded. While preparing algal inoculum and cultures, some other comparative methods i.e., leaching, bacterial activity and chemical treatment by gypsum were also studied to check the efficiency of salinity removal. This study concluded that Chlorella Vulgaris and KKL5 specie of algae can help removing 80% salinity from salt affected soil and make it re-useable.

Chapter 1

INTRODUCTION

"Some experts look at global warming, increased world temperature, as the critical tipping point that is causing a crash in coral reef health around the world. And there's no question that it is a factor, but it's preceded by the loss of resilience and degradation." (Sylvia Earle) Due to the increase in population and rapid economic development, human activities such as land exploration, mismanagement of irrigation, industrial pollution and the exceedingly use of fertilizers lead to increase in salination. 70 percent of the world's rural population earns its bread and butter from agriculture. Agriculture requires 1/3 of the world land area and takes more than 2/3 of the world's water. Pakistan's economy largely depends on the, agriculture. Pakistan has 79.61 million hectares of land out of which 22.17 million hectors are used as cultivated area and salt-affected land is about 6.30million hectares out of which saline is 1.89 hectare (Corbishley, J. and Pearce, D, 2007). According to one estimate salinity affected irrigated land in world at present is 20 percent and the loss of land by salinity is likely to increase in the coming 20 years, which would of course affect the world food supply. Table 1 provides some details about the saline soils in all provinces of Pakistan.

Table 1 : Land Area According to Soil Chemistry (Ha)

Province	Saline	Sodic	Saline Sodic	
			Non Gypsum	Gypsum
KPK	480	-	240	720
Punjab	55330	69160	96820	82990
Sindh/Balochistan	210070	26260	78780	577690
Total	265880	95420	175840	661400

Source: WAPDA (1990), Water Sector Investment Planning Study, Volume 1, Main Report, Federal Planning Cell, Lahore.

Soil salinity is the amount of salt content in soil. It is expressed as Parts Per Thousand (PPT) or Partical Salinity Unit (PSU).

Salt is a natural element in water and soils. Soil salinity can be caused by any natural (i.e. weathering) or artificial (i.e. irrigation) processes. Rise in the salt level in soil can be caused by capillary transport of salt laden water which is then accumulated by evaporation. It can also be by human activities such as use of fertilizers. Climate is also a factor in accumulation of salts in soil. Salinity of a soil is indicated by whitish crust on the row peaks. Sodic soils are the ones that refer specifically to the amount of excess sodium ions present. Saline soils contain excess concentration of soluble chloride, sulfate and carbonate salts that cause electrical conductivity to rise.

Salinity not only affects the crops directly by decreasing their water uptake but it also indirectly affects the soil structure and interferes with uptake of essential nutrients for plants. Highly saline

soil quality can cause problems for irrigation, depending on the type and amount of salts present, the specific plant species and growth stage, and the amount of water able to pass through the root zone. Salinity affects the plant growth directly, when soil becomes saline, it becomes unable for the plants to draw water. This happens because plant roots already have some concentration of salts, which creates flow of water from soil to plant roots through osmosis. Sodic soils also affect the metabolism and nutrition of plant as they contain sodium carbonate, bicarbonate and some other anions. These soils limit the emergence of seedlings as sodium attaches with clay particles and settle into impenetrable dense layers (Ann McCauley and Clain Jones,2005). Table 2 shows the effect of soil salinity on crop yield.

Table 2 : Effects of Soil Salinity on Crop Yield

Crop	Traditional Varieties				Imported Varieties			
	Non Saline	Slightly Saline	Moderately Saline	Strongly Saline	Non Saline	Slightly Saline	Moderately Saline	Strongly Saline
Wheat	17.5 (100)	15.8 (90.3)	9.5 (54.3)	6 (34.3)	23.6 (100)	20.2 (85.6)	13.4 (56.8)	4.2 (17.8)
Rice	23 (100)	22.5 (97.8)	17.5 (76.1)	-	32.3 (100)	28.7 (88.9)	24.4 (75.5)	12.4 (38.4)
Sugarcane	602 (100)	318 (52)	-	-	534 (100)	349 (65.4)	-	-

NOTE: Figures in parentheses indicate the average yield proportion of respective Saline land to yield attained at good farm land.(Source : Pakistan Economic and Social Review.2002)

1.1 Objectives of Study:

1. Reclamation of saline land by using algal activity (Algal Cultivation/ Farming)

1.2 Salinity Control:

There has been many several management strategies for reducing salinity of soil which include leaching of salts by supplying excess water, for which drainage is pre requisite and fertilizer management which includes gypsum treatment (Kijne, Jacob W., 1996).

In order to increase the fertility of saline soil, biological activity can also be used that includes bacteria and algae . The bacteria present in the saline soil can be used for this matter as these bacteria are salt tolerant and are growing in the saline soil. Aziz and Hashem (2003) reported that Cyanobacteria increases the soil fertility as well as organic matter in the soil.

Algae are the best CO_2 sequestration agents when compared to terrestrial photosynthetic plants. Algae require sunlight, salts and carbon dioxide for its growth. Depending upon the saline soil some algae can be grown. Algae will takeup the salts from saline soil for its growth and hence reduce salt concentration of soil. In order to irrigate algae saline water from ground can also be used. Algae can survive moderate temperature such as 20-30C and does not thrive under extreme temperatures as faced in Pakistan. In order to explore reduction of salinity by algae as an option for warmer climates, locally isolated algal strains will be investigated for their potential for reclamation of salt affected land in Pakistan.

According to Ali and Sandhu (1972) more than 15 algal species belonging to 6 genera grow well in highly saline soils. Algal growth results in significant reduction of pH, EC, salts, aggregation status of soil and increases the nitrogen content in soil.

1.3 Advantages of saline land recovery through algae (Schenk et al., 2008, Demirbas & Demirbas, 2010)

1. Algae can consume salts from saline soil.
2. Pakistan's weather is extreme and in summers it is very hot so algae can grow easily as it requires sunlight and salts.
3. Saline water can also be used to grow algae.
4. Algae can also be used as food for animals.
5. Algae are great for producing oil and biofuels.
6. On the recovered saline land the plants can be grown. Agriculture activity on this land would increase the economy and the utilization of land which was going to waste prior to the recovery.

Chapter 2

LITERATURE REVIEW

To manage the soil salinity, the first step is the determination of salt concentration in the soil which requires detailed salinity analysis including pH, electrical conductivity (EC) and water soluble levels of the soil (Provin and Pitt, 2008-07). EC is the assessment of amount of dissolved salts in the soil and water mixture.

2.1 Existing Common Methods for Salinity Reduction Of Soil

Salt affected soils can be treated by various methods some of which have been discussed in the following sections:

2.1.1 Leaching

This method involves application of an adequate amount of low salt water (1500 – 2000 ppm) to dissolve the soluble salts and move them beneath the root zone. It is suitable for the saline soils with fine configuration and internal drainage (Provin and Pitt, 2008-07). Leaching of highly saline soils may necessitate as much as 48 acres inches of water per acre of saline soils.

However to find out the exact amount of water required leach testing is necessary. Therefore the main problem with this application is that it requires the proper drainage and large quantity of good quality water which adds extra cost to this method.

2.1.2 Chemical Treatment

Addition of calcium in soluble form in the soil such as gypsum (Calcium Sulfate) , to remove or exchange sodium from the saline soil is another common treatment approach. Provin (2008) suggested chemical treatment as the most frequent modification used to correct saline soils with no calcium source such as gypsum or free carbonates. If free carbonates already exists in the soil then addition of acid is required for the formation of gypsum which further removes exchangeable sodium.

Acid + Calcium carbonate $\longrightarrow$ **Gypsum + Water + Carbon Dioxide**

Chemical treatment of saline soil is only applicable for soils having low pH as soils with high pH do not allow the calcium sources to dissolve where the required quantity of calcium is determined by lab study (Provin and Pitt, 2008). This application must be followed by the leaching method to drain out the exchanged sodium which makes this method ineffective and extravagant.

2.2 Biological Reduction of Salt By Biological Activity:

Salt affected soil can result in either the declination of crop productivity or abandonment of many agricultural areas (Maas, 1990). Plant uses ACC (1- amino cyclopropane 1- carboxylic acid) as a precursor to biosynthesize ethylene called salt stress induced ethylene under high salinity stress conditions. Several reports have documented that ethylene could inhibit the elongation of plant root and shoot (Jusaitis, 1986; Penrose and Glick, 2003) suppress leaf expansion (Peterson etal., 1991) and promote epinasty (Abeles etal., 1992). Therefore to increase agricultural production in saline soil one of the major challenges is to reduce the production of salt stress induced ethylene.

In the last decade, the concept of plant growth promoting bacteria (PGPB) containing ACC deaminase for promotion of plant growth under environmental stress conditions has gained importance (Berg, 2009) These bacteria are capable of metabolizing ACC in the root of developing plants thereby reducing the adverse effects of ethylene on plant growth (Ghosh etal., 2003; Glick, 2005; Hontzeas etal, 2006). Although the achievements of ACC deaminase containing bacteria in promoting plant growth under various environmental stresses were reported immensely (Grichko and Glick, 2001; Dey etal., 2004, Mayak etal., 2004, Zahir etal., 2008) little information is available on effectiveness of salt tolerant bacteria containing ACC deaminase under high salinity (Nadeem etal.,2006). Therefore research study carried out to screen and select efficient salt tolerant bacteria containing ACC deaminase that could use ACC as a sole nitrogen source under highly salt stressed conditions. A study was conducted in which eighty four bacterial strains isolated from saline soil were used. Screening procedure comprised three consecutive screening steps. In the first step, the ACC deaminase of eighty four salt tolerant bacterial strains, were analyzed for their abilities to use ACC as a sole nitrogen source (Chookietwattana et.al.,2012). The first five bacterial strains with the highest OD were selected in this step. In the second step the effects of temperature and salinity on the growth of five selected bacterial strains and their ability to produce enzyme ACC deaminase was analyzed. The first three bacterial strains with the highest growth in a wide range of temperature and salinity were selected. Finally in the last step the ACC deaminase activity of the three selected bacterial strains were determined according to method of Penrose and Glick (2003). Bacterial strains with the highest ACC deaminase activity was selected as efficient salt tolerant bacteria.

One of the major factors affecting the plant growth is the salt stress depending on the plant species. The results showed that the selected salt tolerant bacteria containing ACC deaminase increase the germination percentage, germination index, and root length and seedling dry weight especially at very high salinity. These observed results match the studies of Mayak et al. (2004); Kausar and Shahzad (2006) in which the effectiveness of Rhizobacteria containing ACC to improve the plant growth under high salinity was recorded.

Salinity is the major cause of soil degradation with deleterious effects on crop production (Sumner, 2000) which concerns large areas of cultivated land in the world. Salt stress possess problems for microbial diversity and metabolic activities (Rothschild and Mancinelli, 2001; Oren, 2002; Ritez and Haynes, 2003; Jiang etal. , 2007). Microbial Fe (111) reduction significantly contributes to iron and organic matter cycling in different environments (Lovley, 1995; Slobodkin etal. , 1999). Microbial Fe reduction in soils which can results in the release of heavy metal (Mark-Wiese and Colberg, 2000; Islam etal. ,2004; Li etal. 2011) can play a key role in soil quality by releasing major and trace elements and modifying the pH and the redox potential (Eh).

In the saline environments Fe (111) reduction has not been observed beyond a pH 9 (Booone etal. 1995; Kanso etal. 2002). However, alkaliphilic organisms such as Bacilllus Spp. And alkaliphilus metalliredigens, capable of Fe (111) reduction have been isolated under the saline environment (Gorlenko etal. 2004; Ye etal. 2004; Pollock etal. 2007). According to Pollock etal. (2007), microbial Fe (111) reduction in natural saline environment could not only significantly contribute to mineralization of organic matter but also to the formation of reduced iron minerals. Iron reducing bacteria can control and enhance the quality of water and the soil as well as crop production by changing the environmental parameters (pH, Eh

and release of trace and major elements) especially when the crops are subjected to salinity stresses.

Saline environments are considered as extreme habitats for microbial life (Rothschild and Mancinelli, 2001; Oren , 2002). Jiang etal.(2007) had shown that the abundance composition diversity and metabolic functions of microbial communities were lower in the saline and hypersaline environments. This type of environment favored the development of halo-tolerant bacteria which do not require NaCl for growth but can tolerate high salt concentration and also the development of halo-phiilic bacteria which require NaCl for growth. Ol-livier etal. (1994) and Kerkar (2004) classified three groups of halophiles based on their response to NaCl. Table 3 provides a glimpse of salt tolerance by bacteria.

Table 3: Salt tolerance percentage of bacteria

Bacteria Type	% NaCl
Slightly Halophilic	2 – 5
Moderately Halophilic	5 – 20
Extremely Halophilic	20 – 30

Source: (Ol-livier etal. (1994) and Kerkar (2004)

2.3 Algal Cultivation – Potential of algae for saline land reclamation

Growth of algal biomass caused significant reductions in pH, electrical conductivity, salinity of sodic soils (exchangeable sodium) and aggregation condition of the soil. Significant increase in the total nitrogen content of the soil was observed during experimentation due to the algal growth (Subhashini and Kaushik, 1981). In addition, the comparative study was done by

combining gypsum and algal cultivation and more appreciable results indicated algae as a preferable mean of bioremediation of sodic soils.

A biological approach to the problem of saline soils using cynobacteria (blue- green algae) were employed for reclamation of saline/alkaline 'usar' lands typical of certain north Indian states (Singh, 1950,1961). It absorbed and permanently immobilize Na+ intracellular, resulting in decreased soil salinity. Cynobacteria require sodium for growth, photosynthesis and nitrogen fixation (Apte and Thomas, 1980,1983,1984).The possibility of simultaneously augmenting the carbon and nitrogen status of saline soils were suggested to be an additional attraction of such a reclamation strategy (Singh, 1961).Such attempts have been made subsequently also reportedly with some success(Kaushik and Venkataraman, 1983). Research shows that blue green algae reduce soil salinity by entrapping Na+ in their extra cellular mucopolusaccharide sheaths.

Dunaliella salina, one of the main member of genus Dunalella, which is green, unicellular, motile and above all extremely halotolerant specie which can survive a very high concentration of NaCl in the range of 0.05 M to saturation which is 5.5 M while keeping low sodium intracellular concentrations in the cells (Fraser and Bramley, 2004)

Picochlorum Atomus is a green euryhaline (organisms able to adopt to wide range of salinities) microalgae which can tolerate wide salinity range , freshwater to marine salinities, without affecting the growth and the biomass production (Becker, 1994).

Nannochloris atomus which is a marine microalgae has a a very high biomass production and tolerance to broad variations of salinity (Cho and Park etal. , 2007; Witt and Nellen etal. 1981). In additional, these are the algal super species that can tolerate wide range of environmental conditions such as salinity, temperature and the nutrient loads (Gronlund etal. , 2004).

2.3.1 Nanochloropsis:

A study was conducted on two algal species Chlorella Vulgaris and Nanochloropsis Oculata and it was found that Chlorella V. grow better at a low temperature (15 – 20C) with low salinity (10 – 20 mg/l) while N. Oculata requires a high temperature (25 – 30 C) and high salinity (30 ppt) for better growth (Sung Hwoan, Yong Chae etal. , 2007)

Chapter 3

MATERIALS AND METHODS

3.1 Algae

3.1.1 Sample Collection

Samples of Chlorella Vulgaris, a freshwater foreign strain and KKL5 from Kallar Kahar Lake were provided by Centre For Energy Systems (CES) – NUST laboratory . Samples of local algae and saline soil were collected from Sarghodha Rem ont Depot, by students according to the standard algal and soil sampling procedures.

3.1.1.1 Soil Sampling

Saline soil used as the samples for performing laboratory analysis. Field visits were conducted for collection of soil samples at this place. Two types of saline soil were collected according to standard Sampling Procedures: Dry West Paddock Soil and Wet West Paddock Soil (Sub-Soil) collected from the depth of 2.5 ft. table 4 shows the soil sample collection details.

Table 4 Sampling of soil from project site

Date	Location	Soil Type	Collected By
December ,062013	Sargodha Remount Depot	Saline Soil (Dry Surface Soil, Wet Subsurface Soil)	Dr Ehsan Ali, Rabia Shaukat, Noor ul Subha, Hifsah Qaisrani, Iram Sifat

3.1.1.2 Water Sampling

Saline water analysis was also required by the project. Field visits to the Sargodha Remount Depot and Bhulwaal Fish Farm were conducted for water sample collection. So by following standard sampling procedures, water samples were collected as given in Table 5.

Table 5 : Water Sampling details

Date	Location	Water Type	Collected By
December ,062013	Sargodha Remount Depot	Saline water	Dr Ehsan Ali,Rabia Shaukat, Noor ul Subha, Hifsah Qaisrani, Iram Sifat
December ,062013	Bhulwaal Fish Farm	Saline water	

3.1.1.3Algae Sampling

Local Strains:

Following the standard sampling procedures, local Strains were collected from the selected sites. Local strains of algae were found both on the soil and water as given in Table 6 and exhibit 1 below:

Table 6: Sampling of local algal strains

Date	Location	Algae Type	Collected By
December ,062013	Sargodha Remount Depot	Unidentified local algal strain	Dr Ehsan Ali,Rabia Shaukat, Noor ul Subha, Hifsah Qaisrani, Iram Sifat

Exhibit 1: Sample collection from Sargodha remont depo

Besides these indigenous strains , some foreign strains (Marine algal strains) were also sought to carry out the comparative study under the local condition. Some of the algal strains were also provided by Centre for Energy Systems- NUST Lab. These strains were:

1. Chlorella Vulguris
2. KKL5
3. Nano Chloropsis Oculata

3.1.2 Enrichment

Chlorella vulgaris obtained as sample was enriched in Modified Johnson Medium (See Appendix A) . The isolated strain of Chlorella Vulgaris growth rate was observed fast in Modified Johnsons Medium (MJM) in all the different salt concentrations which shows that MJM is useful for culturing Chlorella Vulgaris. MJM is a universal medium used for culturing fresh water

strains. The salt concentration (NaCl) of MJM was adjusted according to the algae type and the pH of MJM lies in the range of 6.8-7.4.

For KKL5 algal strain, enrichment was carried out in Bold Basal Medium(BBM). Bold Basal .medium (See Appendix B) has low salinity and is used for culturing a variety of fresh water algal strains and also it is a highly enriched medium as well. BBM has a salinity of 0.6% and PH adjusted to 6.8.

The marine strains, i.e Nano Chloropsis Oculata were also enriched in Modified Jhonson Medium . Since all the local samples were collected from fish pond, they were enriched in MJM.

For growth of algae CO2 was also provided by aeration through air pumps that are normally used in aquariums. The enrichment was carried out at a temperature of 25 ± 1°C regulated by an incubator.

3.1.3 Isolation & Microscopy

The local algal strains were not pure but they contained multiple of different algal strains. A single algal cell or a whole colony was picked up from the slide using micro-pipettes. This was then carefully transferred to sterile culture tube containing a minute quantity (often 1-2 ml) of the growth medium. The colony was then diluted before being transferred to the culture tube. For samples of Kallar kahar temperature was increased beyond 38°C. Only this specie which was able to thrive at such a high temperature survived, while the rest died off. In this way, isolation of a single species was achieved. This was also confirmed by the microscopy of the sample. Isolated algal strains of Chlorella Vulgaris and KKL5 were observed under microscope. Exhibits 2-3 show microscopic reflection of Chlorella Vulgaris and KKL5 strains used in this study.

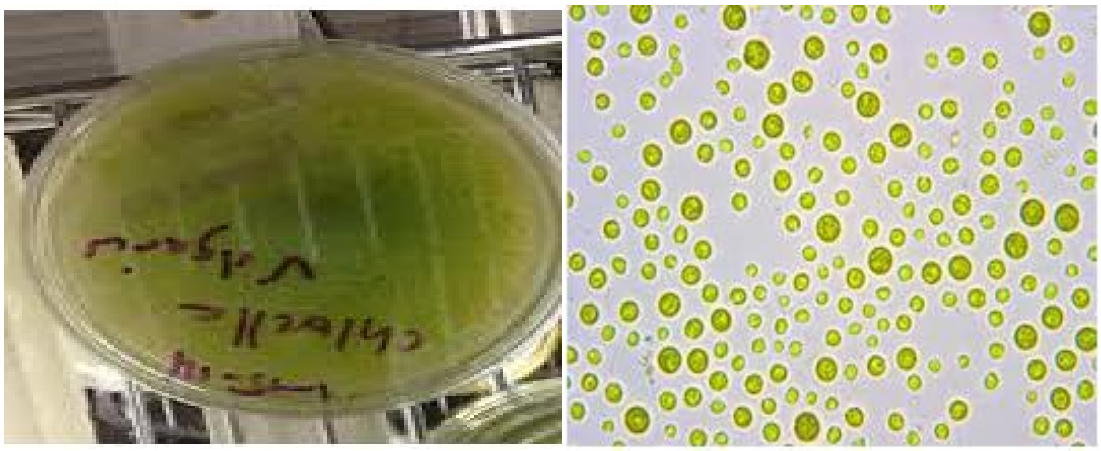

Exhibit 2 : Chlorella Vulgaris Microscopy

Exhibit 3: KKL5 Microscopy

3.1.4 Growth circumstances

Algae are photosynthetic organisms they require CO2 and a source of light to carry out the process of photosynthesis. For the growth of algal strains indoor in environmental chemistry lab-IESE and CES bio fuel lab, algae were scaled up from culture tubes to flasks and at last, to large containers provided with the following growth conditions in the lab scale set up:

1. Constant supply of CO2 to the culture was insured via filtered aeration, provided with diffusers.

2. Natural sunlight was provided to culture vessel via windows.
3. Nutrients were provided via autoclaved Modified Johnson's Medium and Bold Basal Medium

By maintaining these growth conditions, the volume of algal cultures was increased as shown in Exhibit 4.

Exhibit 4 : Experimental laboratory setup

3.1.5 Growth rates

In order to measure growth rates under local conditions, standard curves of all the strains were established by measuring their cell counts. Growth rates of both local and foreign strains are required to be measured in order to carry out a comparative study.

Chlorella Vulgaris strain showed a very high growth rate as compared to other algal strains being researched upon in this study. The *KKL5 strain* showed high growth rate on lab scale but not on the artificial saline land. However, identification of the genus and species of this particular strain requires further analyses.

3.1.5.1Salinity Measurement

Salinity test was performed by measuring conductivity in mS/cm or uS/cm by using Conductivity Meter equipment. As salinity is the function of temperature and conductivity therefore by keeping temperature constant conductivity can be converted into salinity (ppt)by using Salinity Calculators as shown in Exhibit 5. In addition rough estimate was done which shows that

Conductivity (mS/cm or uS/cm)/2000 = Salinity (ppt)

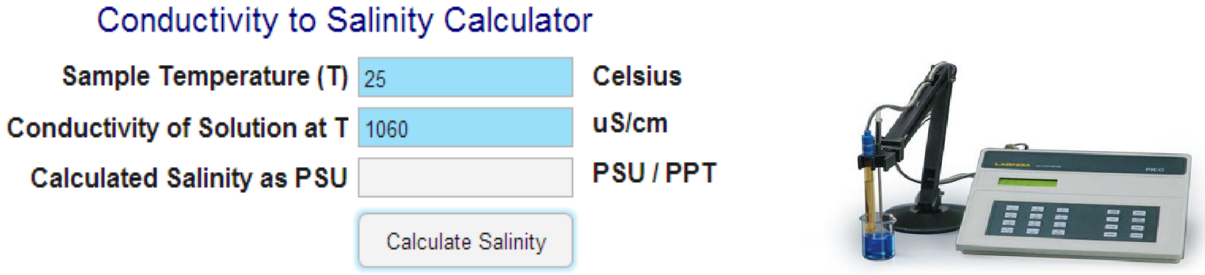

Exhibit 5 :Salinity Calculator and Conductivity Meter

3.1.6 Preservation

Stock cultures are kept in specialized maintenance media, which may be enriched nutrient-enriched agar plates or slants, under closely controlled conditions of temperature and illumination. A special temperature-controlled area or room adjacent to the algal culture room is usually allocated for this purpose.

Stock cultures containing sterile, autoclaved media are kept in small, transparent, autoclavable containers such as 25-mL test tubes or 250- to 500-mL borosilicate glass, flat-bottomed, conical flasks fitted with cotton wool plugs at the necks (or polyethylene beakers can serve as caps). To minimize potential contamination, an enclosed culture transfer hood outfitted with a Bunsen .

The following sterilizing procedures were followed:

1. Wipe all inner surfaces of the laminar hood and working surfaces with 70 percent ethanol.
2. Place all flasks that will be used in the hood, including flasks to be transferred from (the transfer flask) and flasks containing sterilized media that will be inoculated under the culture transfer hood.
3. Ignite a small Bunsen burner; remove caps from one transfer and one new flask; and flame the neck of each flask by slowly rotating the neck through the flame.
4. Tilt the neck of the transfer flask toward the new flask. In one motion, remove both stoppers and pour an inoculum into the new flask. Once the inoculum is added, replace the stopper in the transfer flask. Slowly flame the neck of the new flask before replacing its stopper.

5. Replace the cap over the neck of the new flask and use a waterproof marker pen to label the new flask with the algal species inoculated and the date of transfer.
6. After all inoculations are completed, turn off the burner and transfer all new flasks to an algal incubator or a well-lit area in the algal culture facility.
7. Empty test tubes, flasks, stoppers and/or caps should be removed, thoroughly washed, and sterilized or discarded.
8. Remove all materials from the working area and wipe the surface with 70 percent ethanol.

3.1.7 Selection

Depending on the growth rate and the salt reduction efficiency the strain was selected for further study. In this context, Chlorella Vulgeris and KKL5 were selected prior to the local strains which did not show up positive results due to the environmental factors .i.e. Temperature

3.1.9 Preparation of artificial Saline land (ASL)

In order to see the effect of algae on saline land we created a lab scale artificial saline land. This is created using the soil sample that were collected from Sargodha Remount Depot. This artificial saline land was created on 19th march, 2014 using 10.2kg of soil. Initially the measured soil salinity was 32 ppt. Two ASLs were prepared separately for two different algal strains as shown in Exhibit 6. In order to create saline water for algal growth, over the artificial saline land initially 6250ml of water was added and was allowed to settle over night.

Exhibit 6 : Artificial saline land

3.1.10 Transfer of algae to artificial saline land

The centrifuged wet mass of algae transferred to the artificially made saline land. The artificial saline land is provided the favorable conditions that is, proper sunlight and natural aeration. Salinity testing of both the tubs was carried out on daily basis and the readings were recorded.

3.1.10.1 ASL1- Chlorella Vulguris

Algae i.e. Chlorella Vulgurus was harvested using centrifugation and was spread over the artificial saline land in order to algal grow and reduce the salinity of soil. Amount of Chlorella Vulguris initially added was 37.2g. Algae settled on the soil surface the next day and it started growing on the artificial saline land. Meanwhile, the salinity of soil is recorded every day and the declination of soil salinity was noticed. Favorable conditions were provided to the tub i.e the tub was kept in open air for natural aeration and light. After duration of almost one and half month a very thick layer of algae was seen in the tub and a remarkable reduction of soil salinity was also observed.

3.1.10.2 ASL2- KKL-5

While the artificial land for chlorella was made, also a KKL5 setup was set, i.e 9.91 g of wet mass of KKL% along with 200 ml of culture medium was added in the solution form to another tub. As the KKL 5 was added along with the media (MJM) because after lab experimentations it was noticed that it cannot grow well without media. Therefore it was transferred with media and the KKL 5 tub was monitored the same way as the chlorella tub on daily basis as shown in Exhibit 7. With the passage of days, we noticed the growth of KKL 5 on the surface of the saline soil but it was a little bit slow. Anyhow, a thick layer of KKL 5 was seen after two and half month.

Exhibit 7 : Transfer of KKL5 to ASL

3.1.8 Algae Harvesting

The separation of algae from growth medium is known as harvesting. In this case, centrifugation was employed as a harvesting technique. Harvesting increases the biomass concentration. Algae are photosynthetic organisms they require CO2 and a source of light to carry out the process of

photosynthesis. By maintaining these growth conditions, the volume of algal cultures was increased. Dark green, thickened algal biomass was achieved after the process.

Several techniques have been introduced for algal harvesting, i.e. flocculation, centrifugation, filtration, ultra-filtration, air-flotation, auto-flotation, etc (Dragone & Fernandes et.al, 2010).However, following methodology was adopted for harvesting of the algal strains under study.

In this study, we used centrifugation as a harvesting technique. The aqueous algal solution was subjected to centrifugation at 5000 rpm for 15 minutes. Dark green, thickened algal biomass was achieved after the process as shown in Exhibit 8.

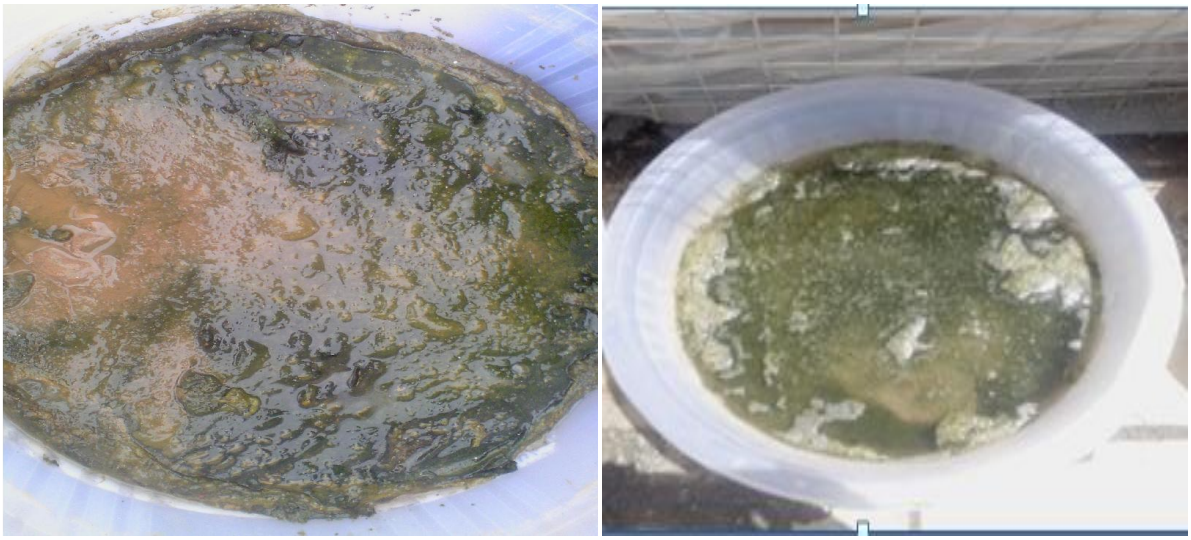

Exhibit 8: Chlorella V. and KKL5 on ASL

3.2 Bacteria

3.2.1 Bacterial Activity on Saline Soil

3.2.1.1 Sample collection

Bacteria were isolated from saline soil sample that was collected from Sargodha Remount Depot for conducting bacterial examination regarding its activity in salinity reduction of soil.

3.2.1.2 Enrichment

The bacterial strains isolated from soil were enriched in Luria Broth Media (LBM). LBM is enriched media having salt concentration of 1% and pH adjusted to 7.3-7.4..The soil sample was streaked in the agar plates and placed over night in incubator at 37 C for bacteria to grow .The growth of bacteria was observed and the pure colonies were collected for inoculum in test tubes.

3.2.1.3 Growth Conditions

Bacteria are microorganism that grows in sterilized conditions providing autoclaved equipment and incubator temperature of 38°C. These bacteria samples were cultured indoor in environmental chemistry lab- IESE as shown in Exhibit 9 below.

3.2.1.4 Growth Rates

In order to find bacterial growth the streaked petri plates were placed overnight in incubator to grow and the next day using colonies showed bacterial growth which was measured by Colony Counter. Then some of selected bacterial colonies were placed in the saline water to grow and the growth was measured using Spectrophotometer instrument by measuring optical density (OD).

Exhibit 9: Bacterial culturing setup (Laminar hood)

3.2.1.5 Bacterial Preservation

For transferring liquid bacterial cultures using glass Pasteur pipettes, following steps were followed:

1. Place the pipette bulb adjacent to the Bunsen burner and clean the inside with 70 percent ethanol.
2. Place the bulb on the pipette, pick up the cell culture vessel, and flame at an angle of at least 45 degrees.
3. Remove the vessel from the flame, insert the tip of the pipette into the liquid and collect the desired amount of inoculum by controlling the pressure of the bulb.
4. Slowly discharge the cell suspension, remove the pipette, flame the mouth of the vessel, and replace the cap.
5. Remove the pipette bulb and place the used pipette into a discard container to be discarded or reused. Clean the bulb with 70 percent ethanol for reuse.

6. Once all transfers are completed, turn off the Bunsen burner, remove all materials from the working area, and wipe the surface with 70 percent ethanol.

RESULTS AND DISCUSSION

4.1 Algal Readings

After transferring algae wet biomass in artificial saline land, salinity tests were carried out on daily basis. Salinity calculations were performed as follows:

Calculations:

Soil from artificial saline land = 250 g

Water = 1000 ml

Dilution factor = D.F = 1000/250 = 4

Salinity (ppt) = [(D.F x Salinity)/250] x 1000

4.1.1 Control Growth

A control artificial saline land was also made along with the other two ASLs of KKL 5 and Chlorella. Saline soil in a third ASL was taken and wheat seeds were sown in it. All the other parameters were kept same as given to the other two ASLs, the only difference was that the control growth ASL was not treated with algae. It was the same saline soil taken from the sample area. The purpose of control growth set up was to compare the growth of wheat in it with the growth of wheat in the treated soil with algae (Chlorella Vulgaris). Wheat growth was found resistant in untreated saline soil whereas remarkable growth of wheat crops was observed in the

algal treated artificial saline land which ensured the land reclamation for cultivation of crops. Exhibits 10 and 11 show the controlled and reclaimed ASLs.

Exhibit 10: Control Artificial Saline land (ASL)

Exhibit 11: Reclaimed Saline land

4.1.2 Results for Chlorella Vulgaris

37. 2 g of pellet of chlorella v. was separated and transferred to artificial saline land ASL 1 and was kept under normal field conditions. Salinity observations were performed from 2nd April, 2014 to 25th May, 2014. Results are shown in figure 4-1.

Readings showed that chlorella V. has a very good potential for salinity reduction and reclamation of land.

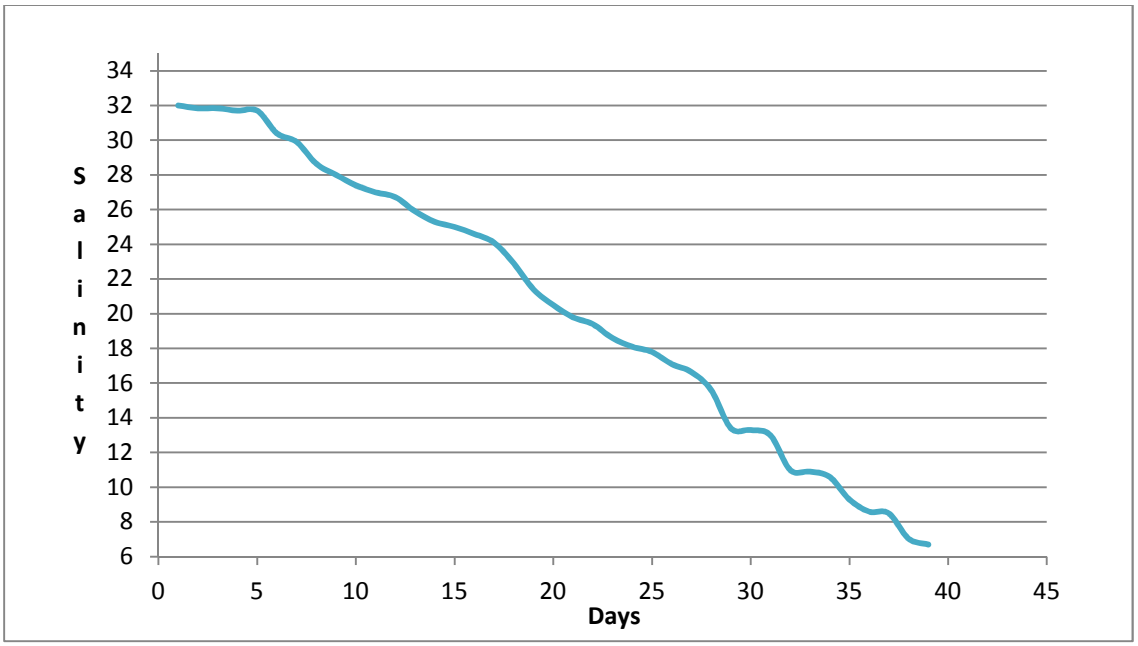

Figure 4-1 Salinity reduction curve of Chlorella V. vs Time

4.1.3 Results for KKL5

9.9 g of pellet of KKL5 provided with 200 ml medium was transferred to artificial saline land ASL 2 and was kept under normal field conditions. Salinity observations were performed form 25 march, 2014 to 6th June, 2014. Results are shown in figure 4-2.

Readings showed that KKL5 has a relatively slow growth rate demonstrating low potential for salinity reduction.

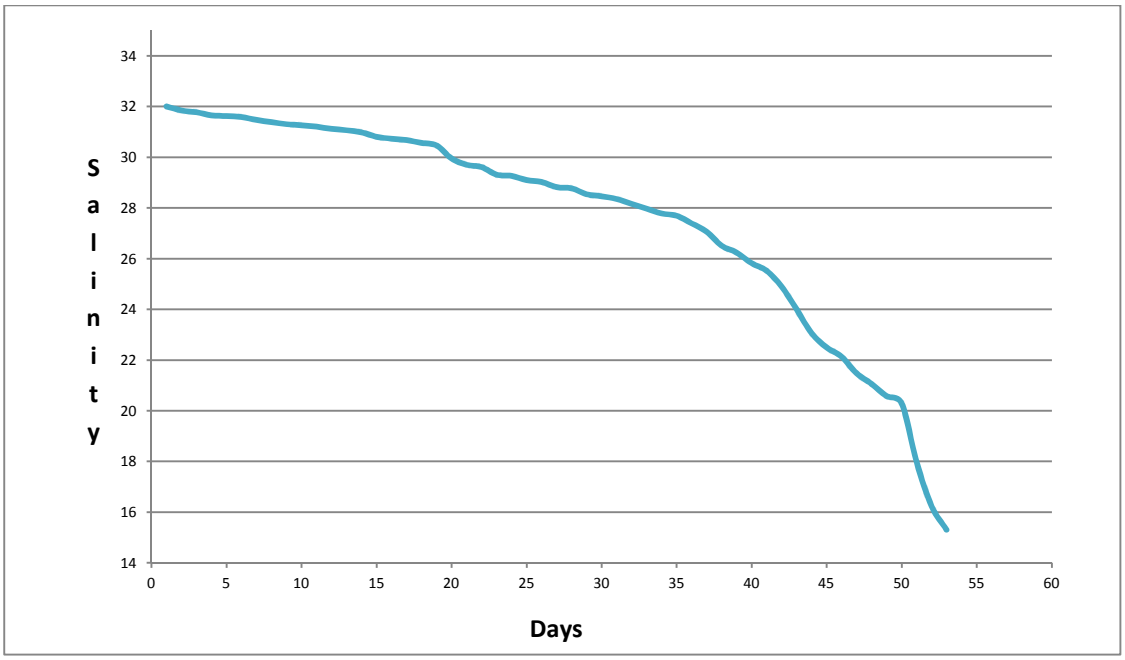

Figure 4-2 Salinity reduction curve of KKL5 vs. Time

4.1.4 Wheat Cultivation:

In order to check the salinity reduction of soil and to ensure land reclamation wheat seeds were sown in the ASL 1 provided with mixing algae (Chlorella V.) within a soil to use it as a fertilizer for the wheat seeds to grow. The whole set up was kept under observation and field conditions were provided till the desired results were achieved. Proper growth of wheat was observed after a period of one month. At this stage, to see whether the soil is now favorable for crops growth or not, we sow wheat seeds in the same tub containing chlorella. Chlorella was well mixed in the soil and it was expected to act as fertilizer for the wheat growth in the reclaimed soil. Hence, proved that algae can also be used as a fertilizer.

4.2 Bacterial Readings

In bacterial study we found out bacteria which we isolated as bacteria type A,B and C and used for salt reduction purpose. It howeve , did not come up with positive results and also showed no potential for any kind of salt resistivity. Bacterial growth was observed up to three days. Bacterial growth was measured as optical density by using Spectrophotometer. Three setups were established.

Setup 1: Saline Water + Bacterial Pellet

Two bacterial pellets of different colony, separated by centrifugation were transferred into saline water separately under sterilized conditions. Figure 4-3 shows the observed results. Bacteria type B resisted salt up to three days as compared to Bacteria C which was found dead within two days.

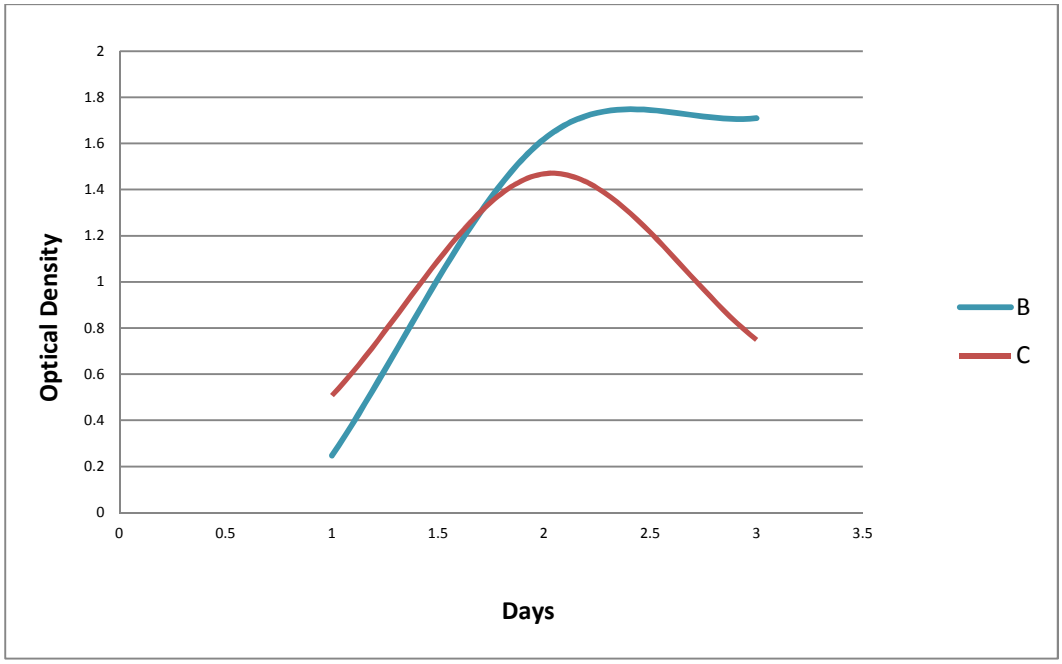

Figure 4- 3 Optical Density of saline water & bacterial pellet with bacterial type B & C

Setup 2: Saline Water + Bacterial Culture

Three different bacterial cultures were transferred into saline water separately under sterilized conditions. The purpose of culture transfer was to provide the nutrients for the bacterial growth. Figure 4-4 shows the observed results. Bacteria type A and B could resist salt up to three days as compared to Bacteria C which was found dead within two days. Bacteria A and B showed almost similar results.

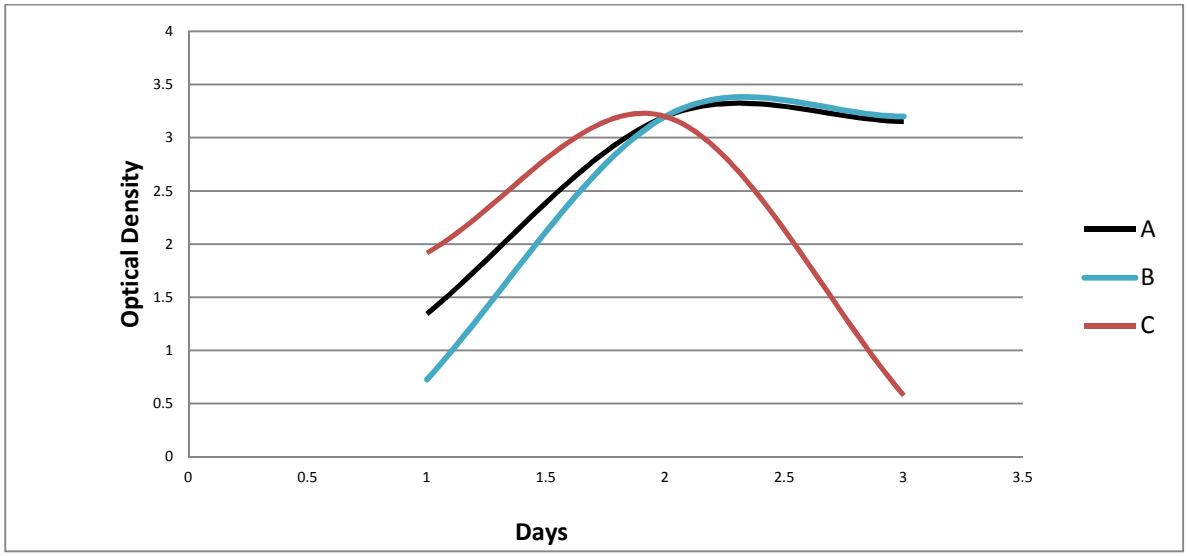

Figure 4-4: Optical Density Of saline water & bacterial culture with bacterial type A,B & C

Setup 3: Test Tubes with 1- 10 % Salt Concentration :

As three bacterial colonies was isolated from the soil, three test tubes setups for every bacterial category, were set up ranging from different salt concentration (varying from 1% to 10%). Results of this work are shown in Figure 4-5, 4-6 and 4-7 below:

Bacteria Type A:

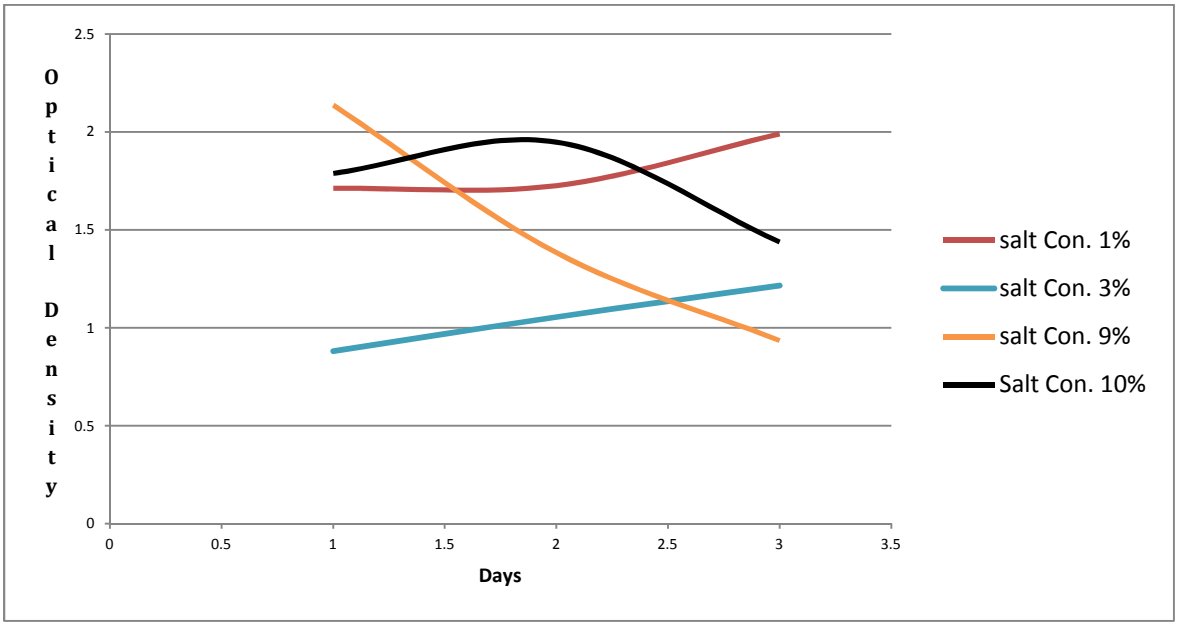

Figure 4-5 Optical Density Of saline water with bacterial type A at salt concentration (1 to 10 %)

Figure 4-5 shows that bacteria A can resist salt upto 3 % only above that it showed negative results.

Bacteria Type B:

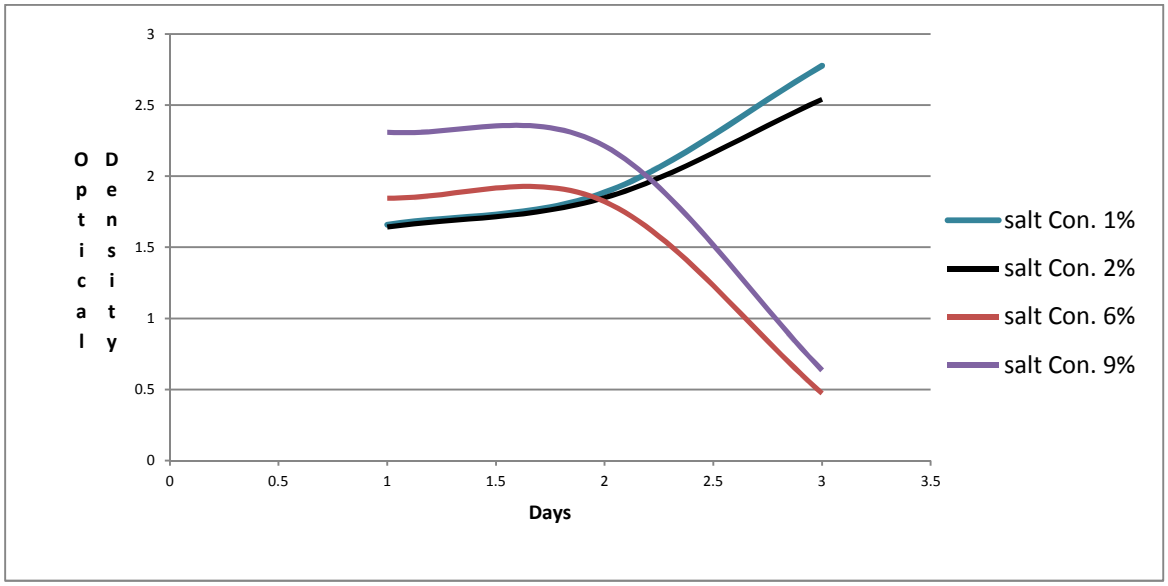

Figure 4-6 Optical Density Of saline water with bacterial type B at salt concentration (1 to 10 %)

Figure 4-6 shows that Bacteria A can resist salt upto 3 % only above that it showed negative results.

Bacteria Type C:

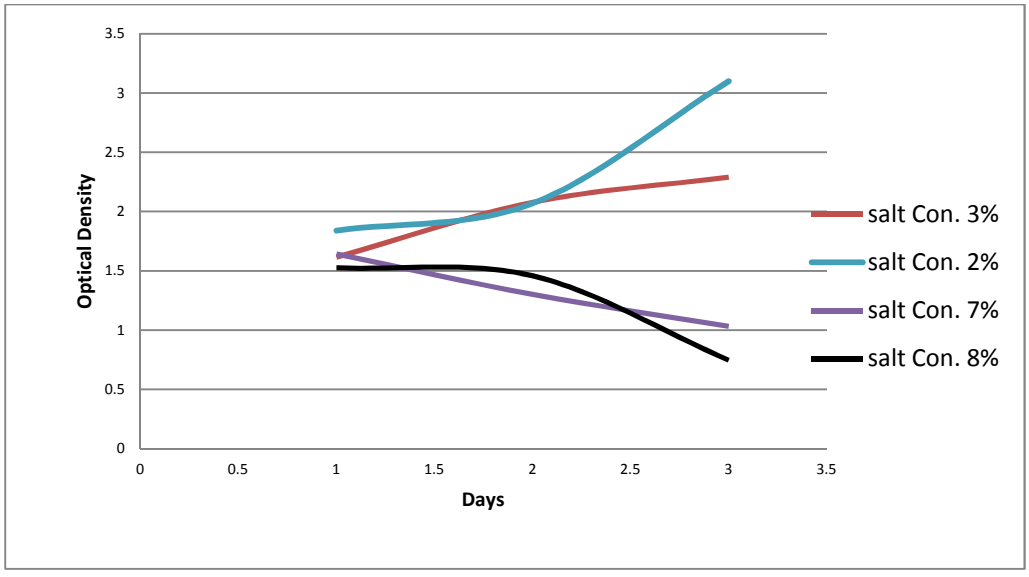

Figure 4-7 Optical Density Of saline water with bacterial type C at salt concentration (1 to 10 %).

NOTE: Figure 4-7 shows that Bacteria A can resist salt upto 3 % only above that it showed negative results.

4.3 Gypsum

Mixture of soil and gypsum with different ratios were setup. Table 9 below shows the types and ratios of gypsum and soil in samples A through E. Wheat seeds were sown in all sample soils. Same quantity of fertillzer and water were added to all but no growth was observed.This shows that gypsum is not a solution to all kinds of saline soils. Control setup with NUST normal soil without gypsum was established and normal growth was observed.

Table 7 Different ratios of gypsum and soil mixtures

Gypsum + Soil Mixture		
Sample Name	**Soil Ratio**	**Gypsum Ratio**
A	50	50
B	60	40
C	70	30
D	80	20
E	90	10

Fertilizer water provided daily to samples for growth of wheat seed, it contained urea 1g and 0.5g of Dipottasium Ammonium Phosphate in 500 ml of water. The growth was measured of these samples and results were observed. No positive results were found in wheat growth.

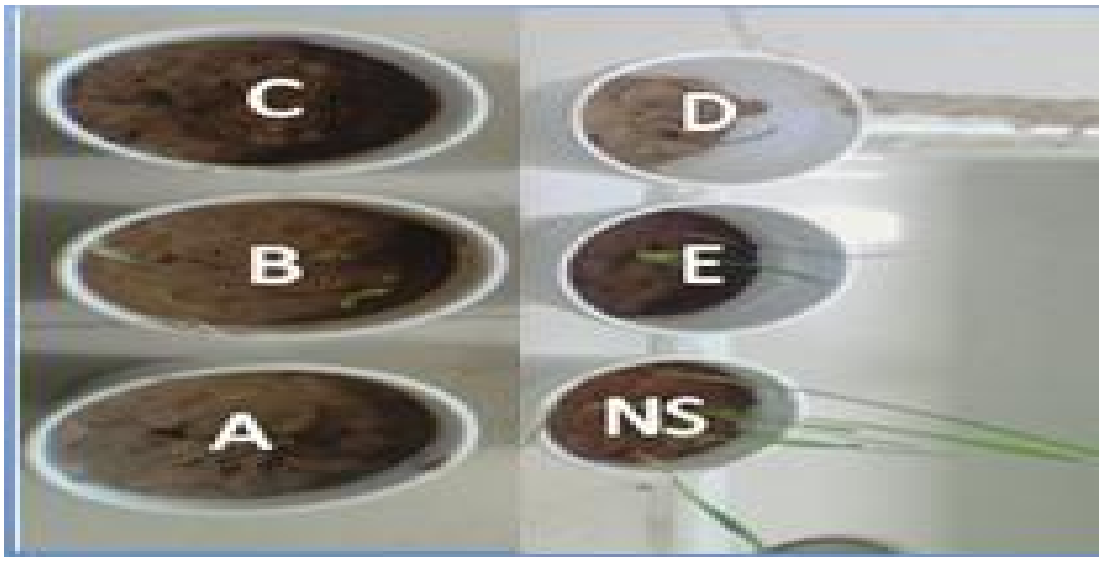

Exhibit 12 : Soil + Gypsum mixture setup

Chapter 5

CONCLUSIONS

The significance of algae is that it grows on saline land utilizing salts and reclaiming the land from salinity therefore it is an efficient and economic way to overcome the salinity issues. Several land areas in Pakistan that are exhibiting low productivity and are barren due to accumulation of salts can be treated by algal activity because of its fast productivity cycle .This would help making the wasted land useful for agriculture purposes leading to national prosperity through added crops.

The uniqueness of the this project is that the algae farming gives multiple outcomes i.e. Biodiesel production, animal fodder , suppose to be cash crop, CO_2 sequestration, raw material for paper industry and as a fertilizer.

Algae have a high growth rate and it yields more oil than conventional terrestrial crops like soya bean, Jatropha, corn, coconut etc. Algae are predicted as most promising feedstock, they need salts, carbon dioxide which results in CO2 sequestration and sunlight to grow by resisting local environmental conditions. On average, algae contains 40% of oil and 60% residual bio-matter. After extracting oil which can be used for biodiesel production, the left over 60% bio-matter can be used for animal feed, bioethanol production, protein and vitamin supplements, papermaking and some other purposes.

Hence, utilization of solar energy and carbon dioxide sequestration by algae makes our project more cost effective and environmentally sustainable.

RECOMANDATIONS

Our resources on the Earth are getting depleted day by day. The resources are greatly needed to be allocated properly and utilized carefully. If the resources are not used sustainably, then the future generation will face drastic problems regarding food security, land depletion, ,climatic changes and many other environmental problems. As the growing world population demands more efforts to get a considerable increase in food production, in order to fight against the threats and to get self-sufficiency, recovery of saline land would be of key importance to avoid the consequences of food and environmental crisis in coming future. It has been observed that some algal strains can reduce the salinity of land while some can be used as a cash crop on saline land. As there are thousands of species of algae on the Earth.. A series of research work is required to be conducted to isolate and identify other species of algae as well which are able to reduce the salt content of the soil or which can be used as cash crop on saline lands. The algal species have the potential to produce biofuel, antioxidants, vitamins and a number of bioactive compounds. In addition to this, algae are designated as the best sequestering agents for carbon dioxide to remedy the greenhouse effect. There is also a view that if it had been possible to capture carbon dioxide from flue gases from power stations and industries to grow algae for biofuel/biomolecule production, it might have caused value addition to the products cost-effectively.

Carbon credits is another incentive to be obtained by growing algae, however the permissible carbon credits arise from the flue gas that is captured to replace fossil fuels. In other words, the carbon credits arise as a result of the displacement of the fossil fuel that would have been used if the biofuel had not become available.

More research can be carried out in order to determine the efficiency of the local strain *KKL-5*, in producing biodiesel as compared to other algal strains. The topics which need further consideration include:

- Identification of the algal species *KKL-5*
- Isolation of other species of algae which have salinity reduction potential
- Optimization of growth conditions (temperature, light, CO2)
- Optimization of the type of growth medium and salinity & pH
- Potential for growth on wastewater and saline water

REFERENCES

1. Corbishley , J. and Pearce, D., (2007), "*Growing trees on salt-affected land"*, ACIAR Impact Assessment Series, Report No. 51
2. McCauley, A. and Jones, C., (2005)," *SALINITY & SODICITY MANAGEMENT",* Soil and Water management, module 2.
3. Kijne, J.W, (1996), "*Water and salinity balances for irrigated agriculture in Pakistan."* ,Research Report 6
4. M.A. Aziz and M.A. Hashem, (2003), "*Role of Cyanobacteria in Improving Fertility of Saline Soil*", Pakistan Journal of Biological Sciences, Volume 6 , 20, 1751-1752.
5. Sikander ali and G.R. Sandhu,(1972), "*blue geen algae of the saline soil of the Punjab"*
6. Andersen, Robert A., and Masanobu Kawachi , J. , (2005). "*Traditional Microalgae Isolation Techniques."* Algal Culturing Techniques, 83-100.
7. Tony Provin and Pitt J.L ,(2008) *, "Managing Soil Salinity".* Vol 60, 3-12.
8. Walworth, J. ,(2012*). "Using Gypsum & other calcium amendments in Southwestern soils".*
9. Patel, A., Pandey, Amar Nath. et.al,(2010). *"Seedling emergence of Jatropha in response to soil salinity".*
10. Mark S. Wigmosta, André M. Coleman, Richard J. Skaggs, Michael H. Huesemann, Leonard J. Lane ,(2011), *"National microalgae biofuel production potential and resource demand"* ,Water Resources Research, Vol 47, Issue 3.
11. J.Letey., (2012) *"Soil salinity posses challenges for agriculture and wildlife".*

12. Ramaraj Sathasivam and Niran Juntawong (2013). ,*"Modified medium for enhanced growth of Dunaliella strains"*.

13. Kannika Chookietwattana &Kedsukon Maneewan ,(2012) *.,"Selection of efficient salt-tolerant bacteria containing ACC deaminase for promotion of tomato growth under salinity stress"*.

14. Michael Hannon, Stephen Mayfield et al.,(2010) *"Biofuels from algae: challenges and potential"*.

15. DOE/Pacific Northwest National Laboratory (2011). *"Algae could replace 17 percent of US oil imports, study finds."* ScienceDaily. <www.sciencedaily.com/releases/2011/04/110413133028.htm>.

16. Bellinger, Edward G, and David C Sigee, (2010). ,*"Freshwater Algae-Identification and Use as Bioindicator"*.

17. Borowitzka, Michael A, (2013), *"THE MASS CULTURE OF DUNALIELLA SALINA."* Food and Agriculture Organization of the United Nations. Fisheries and Aquaculture Department.

18. I. Rajpar, A.A.Maitlo et.al., (2012) *, "Sodicity tolerence of Jatropha seedlings"*.

19. Chinnasamy, Senthil, Balasubramanian Ramakrishnan, Ashish Bhatnagar, and Keshav C Das, (2009), *"Biomass Production Potential of a Wastewater Alga Chlorella vulgaris ARC 1 under Elevated Levels of CO2 and Temperature."* International Journal of Molecular Sciences, 518-532.

20. Edberg, Ann ,(2010),*"Growth of Chlorella vulgaris at high carbon dioxide levels in Swedish light conditions".* Umea Institute of Technology.

21. Gopinathan, C. P, (1986*), "Differential Growth Rates of Microalgae in Various Culture Media".* Tuticorin: Central Marine Fisheries Research Institute.

22. Moheimani, Navid Reza, Michael A Borowitzka, Andreas Isdepsky, and Sophie Fon Sing, (2013). *,"Standard Methods for Measuring Growth of Algae and their Composition."* , Algae for Biofuels and Energy, edited by Navid Reza Moheimani and Michael A Borowitzka, 265-284.

23. Anonymous, 2008, *Irrigation Association: Advanced Head Layout & Zoning*, Uniformity and Efficiency, Chapter 1.

24. Chookietwattana, Maneewan ,(2012). *"Selection of eefficient salt-tolerant bacteria containing ACC deaminase for promotion of tomato growth under salinity stress".* Department of Biotechnology, Faculty of technology, Mahasarakham University, Thailand, 31-35.

25. Maas, E.V. and K.K. Tanji ,(1990), *Agricultural Salinity Assessment and Management,* American Society of Civil Engineers, 262-304.

26. Kausar, R. and S.M. Shahzad, (2006). "*Effect of ACC-deaminase containing rhizobacteria on growth promotion of maize under salinity stress*", Journal of Agriculture and Social Sciences , Volume 2,(4), 216-218

27. Grichko, V.P. and B.R. Glick. (2006). "*Reaction mechanisms of the bacterial enzyme 1-aminocyclipropane-1-carboxylate deaminase." ,* Biotechnology Advances . volume 24 , (4), 420-426.

28. Sathasivam and Juntawong, (2013). *"Modified medium for enhanced growth of Dunaliella strains".* Department of Botany, Faculty of science, Kasetsart University, Bangkok, Thailand,volume (5), 67-73

29. Dragone, Fernandes, et.al. A. Vicente,(2010). *"Third generation biofuels from microalgae".*IBB - Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Campus de Gualtar.

30. Oren, A., Heldal, M., Norland, S. & Galinski, E. A. (2002). "*Intracellular ion and organic solute concentrations of the extremely halophilic bacterium Salinibacter ruber. Extremophiles*", 6, 491–498.

31. Mancinelli R. L.,(2001), " *Life in extreme environments".* Nature 409, 1092-1101.

32. Rothschild L. J., Mancinelli R. L., (2001) ,*Life in extreme environments*. , Nature 409, 1092-1101.

33. Ollivier, B.,P. Caumette, J.L. Garcia and R.A. Mah. 1994. "*Anaerobic bacteria from hypersaline environments*." Microbial .Rev. 58, 27-38.

34. Kerkar, S., (2004*), "Ecology of Hypersaline Microorganisms. In Marine Microbiology: Facets and Opportunities",* National Institute of Oceanography, pp. 37–47.

35. . Kaushik BD and Krishna Murti GSR. (1981). "*Effect of blue green algae and gypsum application on physico-chemical properties of alkali soils"*. Phykos, 20, 91-94.

36. Subhashini D and Kaushik BD. (1981), *Amelioration of saline sodic soils with blue-green algae,. Australian J Soil Res*. 19, 361-366.
37. Kaushik BD and Venkataraman GS. (1983) "*Response of cyanobacterial nitrogen fixation to insecticides"*. Curr Sci. 52, 321-323.
38. Apte, S. K. and Thomas, J. (1980) Curr. Microbiol., 3, 291.
39. Apte, S. K. and Thomas, J. (1982) "*Biological Nitrogen Fixation"* , Bombay, Department of Atomic Energy, Govt. of India, p. 150.
40. Apte, S. K. and Thomas, J. (1983) J. Biosci., 5, 225.
41. Apte, S. K. and Thomas, J (1984) J. Gen. Microbiol., 130, 116.
42. Singh, P. ,(1989), "*Waste lands - their problems and potentials for fuel and fodder production in India. Report of regional workshop on wasteland development for fuelwood and other rural needs"*, Vadodora, India 1-8 November, GCP/RAS/III/NET Fd. Document 19, 102-113.
43. Singh, K., Chauhan, H. S., Rajput, D. K. & Singh, D. V. (1989) "*Report of a 60 month study on litter production, changes in soil chemical properties and productivity under Poplar (P. deltoides) and Eucalyptus (E. hybrid) interplanted with aromatic grasses*." Agroforestry Systems , 9,37-45.
44. Fraser, P.D. and Bramley, P.M., (2004) ,*The biosynthesis and nutritional uses of Carotenoids*. Lipid Res. 43 ,228-265.
45. Becker, E. W., (1994), "*Microalgae: Biotechnology and microbiology",*. Cambridge University press,Cambridge. pp293.

46. Cho SH, Ji SC, Hur SB, Bae J, Park IS, et al. ,(2007), "*Optimum temperature and salinity conditions for growth of green algae Chlorella ellipsoidea and Nannochloris oculata*." Fisheries Science 73,1050–1056.
47. Witt U, Koske PH, Kuhlmann D, Lenz J, Nellen W, (1981) , "*Production of Nannochloris sp. (Chlorophyceae) in large-scale outdoor tanks and its use as a food organism in marine aquaculture"*. Aquaculture 23, 171–181.
48. Gronlund, S. D. , (2004)., "*Sequential lineups: Shift in criterion or decision strategy?*" Journal of Applied Psychology, 89(2), 362-368
49. Sung Hwoan, Yong Chae etal. , (2007). , "*Optimal temperature and salinity conditions for growth of green algae chlorella ellipsoidea and Nannochloris oculata*". FISHERIES SCIENCE 73, 10500 – 1056.

APPENDIX A: Modified Johnson's Medium – Recipe

Chemicals	Modified Johnsons Medium A	Modified Johnsons Medium B
1. Macronutrients (mg/L)		
H_3BO_3	0.61	0.61
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.38	0.38
$CuSO_4.5H_2O$	0.06	0.06
$CoCl_2.6\ H_2O$	0.05	0.05
$ZnCl_2$	0.04	0.04
$MnCl_2.4\ H_2O$	0.04	0.04
Na_2MoO_4	-	-
$NaVO_3$ [1]	-	-
$CuCl_2.6\ H_2O$	-	-
2. MACRONUTRIENTS (g/L)		
$MgCl_2.6\ H_2O$	1.5	1.5
$MgSO_4.7\ H_2O$	0.5	0.5
KCl	0.2	0.2
$CaCl_2.2H_2O$	0.2	0.2
KNO_3	1.0	1.0
KH_2PO_4	0.04	0.04
$FeCl_3.6H_2O$ [2]	0.0024	0.0024
Na_2EDTA	0.0018	0.0018
$NaHCO_3$ [3]	0.04	0.04
3. NaCl (g/L)	**87.7**	**87.7**
PH	7.3	7.5*

APPENDIX B: Bold Basal Medium – Recipe

Fresh water algae

Stocks	Per 400 ml
NaNO3	10.0g
$MgSO_4.7\ H_2O$	3.0g
NaCl	1.0g
K_2HPO_4	3.0g
KH_2PO_4	7.0g
$CaCL_2.2H_2O$	1.0g
Trace elements solution (autoclave to dissolve): $ZnSO_4.7H_2O$ $MnCl_2.4H_2O$ MoO_3 $CuSO_4.5H_2O$ $Co(NO_3)_2.6H_2O$	Per litre 8.82g 1.44g 0.71g 1.57g 0.49g
H_3BO_3	11.42g
EDTA KOH	50.0g 31.0g
$FeSO_4.7H_2O$ H_2SO_2 (conc)	4.98g 1.0ml
medium	Per litre
Stock solution 1 – 6	10.0 ml each
Stock solution 7 – 10	1.0 ml each
Make up to 1 litre with glass distilled or deionized water.	