HIGH DENSITY CULTURE OF MARINE MICROALGAE USING SEMI-CONTINUOUS AND CONTINUOUS SYSTEMS

Dissertation submitted by

LAMBADE SUNIL BHARGURAM

in partial fulfilment for the Degree of

MASTER OF FISHERIES SCIENCE (MARICULTURE)

of the

Central Institute of Fisheries Education

(Deemed University)

July 1999

POST GRADUATE PROGRAMME IN MARICULTURE
Central Marine Fisheries Research Institute
Cochin - 682 014

Dedicated to

Late Dr. D.R. Jalihal

My Beloved Professor

Contents

CERTIFICATE

Certified that the dissertation entitled "***HIGH DENSITY CULTURE OF MARINE MICRO-ALGAE USING SEMI-CONTINUOUS AND CONTINUOUS SYSTEMS.***", is a bonafide record of work done by **Mr. Lambade Sunil Bharguram** under our guidance at Central Marine Fisheries Research Institute during the tenure of his M.F.Sc. (Mariculture) programme (1997-99) and that it has not previously formed the basis for the award of any other degree ,diploma or other similar titles or for any publication.

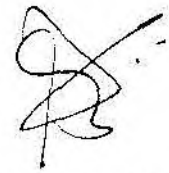
Dr. K. Sunilkumar Mohamed,
Scientist (Sr. Scale),
C.M.F.R.I., Cochin,
Major Advisor, Advisory Committee.

Shri. P. Vijayagopal
Scientist,
C.M.F.R.I., Cochin,
Member, Advisory Committee.

Dr. Mrs. Manpal Sridhar,
Scientist (Sr. Scale),
C.M.F.R.I., Cochin,
Member, Advisory Committee.

DECLARATION

I hereby declare that this thesis entitled *"HIGH DENSITY CULTURE OF MARINE MICRO-ALGAE USING SEMI-CONTINUOUS AND CONTINUOUS SYSTEMS."* is based on my own research work and has not previously formed the basis for the award of any degree,diploma,associateship, fellowship or other similar titles or recognition.



Cochin-682014

Lambade Sunil Bharguram

ACKNOWLEDGEMENTS

I wish to express my deep sense of gratitude to **Dr.K. Sunilkumar Mohamed**, ***Scientist, Sr. Scale*** and my Supervising Guide for his unstinted guidance, encouragement, constructive criticism and affectionate treatment, throughout the period of study.

I am greatly indebted to my co-guides, (Members, Advisory Committee) **Shri P. Vijayagopal** ***(Scientist)***, **Dr.(Mrs) Manpal Sridhar**, ***Scientist (Sr. Scale)*** for their guidance and supervision throughout my work.

I am grateful to **Dr. M. Devaraj**, ***Formar Director*** as well as **Dr. V.N. Pillai**, ***Director, CMFRI*** for permitting me to avail the facilities at the Institute during the course of this study.

I take this chance to express my gratitude to **Dr. C. Suseelan**, ***Senior Scientist*** and ***Officer-in-Charge, PGPM, CMFRI*** *for the patience and tolerance shown by him in* understanding and helping to overcome the problems faced during the period of study.

I express my gratitude to **Shri P.E. Sampson Manickam**, ***Senior Scientist and Officer-in-Charge, FHL of CMFRI*** in providing facilities for my work and valuable suggestions.

I wish to express my thanks to **Dr. V. Chandrika**, ***Senior Scientist*** for her help during the course of my study.

My sincere thanks are also due to **Shri. M.M. Shirdhankar**, *Ass.* ***Professor,FRESE, College of Fisheries, Ratnagiri*** for his suggestions and encouragement.

I would like to express my profound gratitude to **Shri. Mathew Joseph** ***(Technical Assistant)*** and **Dr. Prema** ***(Scientist, Sr.Scale)*** for their valuable suggestions and encouragement. I also express my sincere thanks to **Venu Gopal** (***Technical Officer***)**,Ms. Geetha antony** (***Technical Officer***)**,Thomas V.J.**(***Technical Officer***)**, Sreekumar K.P., Vinod T.V.,Sabu M.D., Murugesan K.S.,Vasudevan P.A., Gopi V.C.,Ms. Moly Augustin** for their help, co-operation and encouragement without which it was impossible to complete *my experiment.*

I acknowledge with thanks the help rendered by **Shri L.R. Khambadkar, Technical Assistant** in doing nutrient analysis and spectrophotometer work.

I acknowledge with thanks the help rendered by **Shri S. Nandakumar Rao,** ***Technical Assistant.***

I wish to express my thanks to the ***Propritor*****, IDEAL GAS APPLIANCES**, Thevara, Cochin-20 for making gas valve as per my experiment need without which my work would not be complete.

I wish to express my thanks to my classmate **Shri.Unnikrishnan** for the help given to me in my work.

My thanks are also due to **Mr. Aboobaker,** ***Technical Officer, PGPM*** for providing me with the necessary facilities.

I express my gratitude to **Ms. Rosalie Shaeffer,** ***Technical Information Specialist, U.S.A.*** for her promptness in providing me with the literature needed for my study.

I wish to express my deep sense of gratitude to my friends **Shri. Shardul Gangan and Shri. Abhijeet Shinde** for their help in collection of references for my research work.

The services rendered by the PGPM Office staff are also sincerely gratified. Thanks are due to the Library staff, **Mrs. Suvarna Mahesh, Mr.V. Mohan, Mrs.S. Girijakumari.**

My special thanks to **Shri. John Josephraj** for his encouragement, wholeheartd support , help and co-operation without which it would have been impossible for me to complete my work successfully.

I am filled with the utmost gratitude to my classmates and friends, **Sandeep, Unnikrishnan, Ashok, Prabhakar, Binu, Babu, Madhavi, Radhika and my juniors especially Paul Pandi, Sushant, Uday, Vishwamitra, Shankar, Hari** whose constant and whole-hearted support, help, advice and encouragement made it possible for me to complete my work without any hassles, successfully.

I acknowledge with thanks the help rendered by **Mrs. A.K.Omana** for typing my dissertation work.

Finally, I wish to express my thanks to **ICAR**, *for having awarded me Junior Research* Fellowship, during the tenure of which the present study was carried out.

साराँश

प्रयोगशाला स्थितियों मे प्रयोग करने लायक निम्न व्यय के लघुमानु प्रणलियों के आविष्कार के उद्देश्य से "अशं संतत और संतता प्रणलियों से सूक्ष्मशैवालों का उच्च घनत्व संवर्धन" नामक अद्ययन चलाया। यह अद्ययन सूक्ष्मशैवालके दो सामान्य जातियों यानी फ्लाजेल्लेट आइसोक्राइसिस गलबाना और डयाटम कीटोसिरोस जाति का उपयोग करके चलाया था।

इसकेलिए बनाये गये विदा जिस में तीन कतार के हस्तेन नियंत्रको साहित और स्वचालन रहित गुरुत्वीय प्रवाहके एक संशोधित टराबिडोस्टाट संवर्धन था। अंश संतत संवर्धन बढाने के लिए एक 60 लि. धारिते के आंतरी प्रदीप्त प्लास्टिक ग्लास टैंक संवर्धन रीती का प्रयोग किया।

मामूली पोषक सघनता के 2.5 गुना देकर, CO_2 के सीमित पूर्ति करके और निर्जर्म अवस्थाओं में सक्रियत काष्ठ कोयला निस्यंदित वायू के प्रेयोग करने पर मामूली बैच संवर्धन की तुलना मे 5 गुना वृध्दी पा सका। यह भी नही 2 से 5 हफताओं तक की विविध आवाधियों में किसी पात के बिना संवर्धन प्रणाली से उच्च घनत्व संवर्धन (2 लि धारिता) में दैनिक संग्रहण 500 मिलि था और अंश संतत (4 लि धारता) प्रणाली में 3 दिनों में एक बार संग्रहण 2-3 लि मे विविध था। 60 लि घारिताके एक अंश संतत प्रणालि में जो 36 दिनों की अवधि की थी, संग्रहण औसत 3.2 मिलियन कोश / मिली संवर्धन के 530 लि था।

समूद्री संवर्धन के संबन्ध में, इस अध्ययन से प्राप्त निष्कर्षों का प्रयोग निम्न व्यय में समूद्री शैवालों के वर्धित उत्पादन के लिए दक्ष साबित होगा।

ABSTRACT

This study entitled "High density culture of marine micro-algae using semicontinuous and continuous systems" was carried out with the objectives of devising small-scale low cost systems for use in laboratory conditions. The study was carried out using two commonly used species of microalgae viz., the flagellate *Isochrysis galbana* and the diatom, *Chaetoceros* sp.

The system devised was a modified turbidostat (2 l to 5 l) having three tier gravitational flow with manual controllers and without automation. For scale-up semicontinuous culture, a 60 l capacity internally illuminated plexy glass culture system was devised.

Using 2.5 times the normal nutrient concentration, limited supply of CO_2, activated charcoal filtered air and under sterile conditions it was possible to obtain upto 5 fold increase in culture cell density when compared to the normal batch cultures. Besides it was possible to maintain the cultures for durations varying from 2 to 5 weeks without collapse. In the continuous culture system of 2 l capacity . The daily harvest of high density culture amounted to 500 ml, and in the semi-continuous system (4 l capacity). The once-in-3 day harvest varied between 2-3 litres. From a single 60 l capacity semicontinuous system running for 36 days the total harvest amounted to 530 litres of average 3.2 million cells/ml culture.

With respect to mariculture, the application of conclusions derived from present work would result in increased efficiency and reduced cost of production of microalgae.

INTRODUCTION

Unicellular marine algae are widely used as food in the hatchery production of commertially valuable fish and shellfish. Oysters and clams feed by filtering them from sea water. Rotifers and brine shrimps also ingest algae and are then themselves used as food for larval fish and prawns. In some systems algae are added to the water containing fish or prawns to improve quality.

Marine algae are single celled plants and like all plants, contain chlorophyll which traps the energy from light and uses it to convert nutrients and carbondioxide in the seawater into organic growth. In the laboratory, or hatchery, a collection of algal cells, which are growing and dividing, is known as culture (Laing, 1991).

For algal culture most important parameters which regulates the growth are

1) Nutrient quality and quantity
2) Light
3) pH
4) Turbulence
5) Salinity
6) Temperature

Table 1: Generalised set of environmental conditions for culturing micro-algae.

(After Coutteau,1996)

Parameters	Range	Optimal
Temperature °C	16-27	18-24
Salinity ppt	12-40	20-24
Light intensity (lux)	1000-10000 (Depending on volume & density)	2500-5000
Photoperiod (light : dark,*hours*)		16:8 (minimum) 24:0 (maximum)
pH	7-9	8.2-8.7

There are many ways of culturing algae. These range from closely controlled methods on laboratory bench top, with a few litres of algae, to less predictable methods in outdoor tanks, containing thousands of litres. Several methods have been developed for production of algae for use as food for various marine animals. The terminology used to describe the type of algal culture include:-

1) **Indoor/outdoor** - Indoor culture allows control over illumination, temperature, nutrient level, contamination with predators and competing algae, whereas outdoor algal systems make it very difficult to grow specific algal cultures for extended periods.

2) **Open/closed** - Open cultures such as uncovered ponds and tanks (indoors or outdoors) are more readily contaminated than closed culture vessels, such as flask, carboys, bags etc. which are always kept closed either plugging with cotton plugs or by any other means.

3) **Axenic (=sterile)/xenic** - Axenic cultures are free of any foreign organisms such as bacteria and require a strict sterilization of glass wares, culture media and vessels to avoid contamination. The latter makes it impractical for commercial operations.

4) **Batch, continuous and semi-continuous** - There are three basic types of phytoplankton culture systems which are described by Coutteau (1996).

a) Batch culture - Batch culture is a system where the total culture is harvested and used as food. If required another culture can be set up to replace it.

b) Semi-continuous culture - Semi-continuous culture is a system where part of the culture is harvested and used as food and the amount taken is replaced with fresh culture medium (clean seawater and nutrient salts). After allowing 2-3 days for the remaining cells to grow and divide, the process is repeated. Semi-continuous culture may be operated upto 7-8 weeks.

c) Continuous culture - This falls in two catagories.

i) Turbidostat culture - In which the number of algal cells in the culture is monitored and as the cells divide and grow an automatic system keeps the culture density at a pre-set level by *diluting the culture with fresh medium.*

ii) Chemostat culture - In which a flow of fresh medium is introduced into the culture at a steady, pre-determined rate with both types, the surplus culture overflows into a collections container, from which it can be taken and used as food..

With semi-continuous and continuous culture methods, the number of food cells produced (the yield) varies with the density of the culture. For each type of algae the greatest yield is obtained by maintaining the culture at an optimum density. This optimum density can be determined experimentally.

Table 2: Advantages and disadvantages of algal culture techniques (After Coutteau,1996).

Culture types	Advantages	Disadvantages
Batch	Easiest, most reliable	Least efficient, quality may be inconsistent
Semi-continuous	Easier, somewhat efficient	Sporadic quality, less reliable
Continuous	Efficient, provides a consistent supply of high quality cells, automation, highest rates of production over extended periods.	Difficult, usually only possible to culture small quantities, complex equipment expenses may be high.

A number of culturing devices for different catagories of organisms including algae were described by Persoone et al. (1975). Continuous culture of algae that is culture that are partially harvested while new medium is added were first attempted in Zechoslovakia during the early twentieth century, but it was not untill 1940 that practical continuous culturing was developed by J.Monad, B.H. Ketchum and A.C. Redfield (Landau, 1991).

Considering the advantages of continuous and semi-continuous culture systems over the traditional batch culture systems, a number of workers have reported on several designs for continuous production of algae., among them are Persoone et al., 1975, Trotta, 1981; semi-continuous turbidostat algal culture using polyethylene bags was described by Trotta, 1981, Laing and Hepper, 1983, Laing and Jones 1983. Boussiba in 1988 grew *Isochrysis galbana* in open reactor first by batch and then by semi-continuous mode.

For continuous culture 200 litre translucent vertical air-lift tubular photo-bioreactor was designed by James & Abu-Rezeq in 1989 and a reactor and an automatic harvesting system was used by Gronados *et al.*in 1990. The use of carbon dioxide for a very short time is helpful for faster growth and not allowing the pH of culture to exceed above 9.0 (Laing, 1991).

It was found that optimal temperature for phytoplankton culture is generally between 20 and 24^{o}C, salinity range between 25 and 30 ppt for flagellate cultures and between 20 and 25 ppt for culture of diatoms (Laing, 1991). Ciliate-free aeration will provide thorough mixing of culture and making light and nutrient available to all of the cells.

SCOPE OF STUDY

In batch culture technique adopted presently at Fisheries Harboour Laboratory, CMFRI, production of microalgae is highly inconsistent, with frequent collapse of cultures due to ciliate infestation and consistent high production could never be achieved.

Through out the world semi-continuous and continuous systems of algal culture are used (Trotta, 1981, Spectorova, 1982, Hepper,1983, De paw *et al.*, 1984 etc.) to produce microalgae in high concentrations over a sustained period of time. Therefore, the objective of the present study was to devise a small scale low cost semicontinuous and continuous system for use in laboratory conditions.

The objectives of the study are -

1) To produce microalgae in high concentrations by
    a) Semi-continuous system and
    b) Continuous system
2) To assess the effect of CO_2 on the production of microalgae in semicontinuous and continuous systems.
3) Scale-up culture of microalgae in semi-continuous system, and
4) To compare the economics of production of microalgae in batch, semicontinuous and continuous systems.

The following two species were used for the culture experiments

Isochrysis galbana and *Chaetoceros sp.* while the former is a flagellate, the latter is a diatom. *Isochrysis* is used as the main food for the larvae of oysters in molluscan hatchery. In shrimp hatchery *Chaetoceros* is mainly used for feeding the developing stages of shrimp larvae.

REVIEW OF LITERATURE

Microalgae are indeed the biological starting point for enegry flow through most aquatic ecosystems and as such are the basis of food chain in many aquacultural operations (Bardach, et al., 1972).Hence, maintaining algal cultures is an integral part of aquaculture.

A *number of culturing devices* for different catagories of organisms including algae were described by Persoone *et al.*,(1975). Continuous culture of algae that is culture that are partially harvested while new medium is added were first attempted in Czechoslovakia during the early twentieth century, but it was not until the 1940 that practical continuous culturing was developed by J.Monad, B.H. Ketchum and A.C. Red Field.

Considering the advantage of continuous and semicontinuous culture systems over the traditional batch culture systems, a number of workers have reported on several designs for continuous production of algae. A multistage, continuous culture apparatus was designed by Palmer et al., 1975 and tested for the production of algae. Trotta in 1981 developed continuous monoxenic culture system for marine phytoplankton utilizing low cost and easily available material.

High density culture of flagellate with reference to composition of nutrient media, regime of continuous cultivation and equipments in laboratory condition was discussed by Spectorova (1982). Microalgae could also be an important resource for special chemicals and other byproducts (Litchfield, 1979; Aaronson et al.,1980; Oswald, 1980; Soeder 1980; Batton *et al.*, 1981; Depauw *et al.*, 1984) described the demerits of outdoor batch culture. The usual technique for microalgal production in hatcheries has been a multistep back up batch culture system whereby small cultures are grown and used to innoculate larger scale cultures a procedure which is a trouble-some and costly in terms of space and manpower utilization. Also outdoor algal cultures which are conventionally used in hatcheries, rapidly lead to

collapse of the culture or are taken over by other species better adapted to the prevailing outdoor conditions. As a result, outdoor culture of several cubic meter usually last for periods of time that rarely exceed a few weeks.

In recent years semi-continuous, turbidostat algal cultures have been demonstrated using polythene bags for the production of marine phytoflagellates by Trotto (1981), Liang and Hepper (1983), Liang and Jones (1983).

Spektorova et al., in 1986 grew *Monchrysis lutheri* in a 1.5 litre culture vessels of the bubbling type and fitted with thermostat to maintain constant temperature., further they carried out experiments for determining the optimal light regime for cultivating Marine chlorella, where they used two types of cultivator, a 2 litre bubbling cultivator with a 5 mm. thick suspension layer and a circulation cultivator with a 2 mm thick suspension layer. The batch and continuous cultures were grown in a mineral nutrient medium with the content of nutrients maintained at a stable level of 300 mg Nitrate/L, 90 mg Phosphate/L, 30 mg Magnesium/L and 30 mg Silicate/L, at the temperature of 33 degree C and pH 7.0-7.5. Light sources used were RHL-1000 W and RNaH-400 W lamps as well as sunlight. The effect of light intensity on yield was studied with light intensities of 75, 150, 250 and 300 W/m^2 PAR (Photosynthetically Active Radiation). 250 W/m^2 PAR proved to be optimal at dry biomass concentration of no less than 0.5 gm/lit. At this light intensity the maximal daily yield obtained per $1m^2$ of the illuminated surface was 50 gm of dry biomass (15 mm-thick layer), the maximal growth rate was 0.1/h and the efficiency of radient enegry utilization was 4%.

A number of closed photobioreactors such as horizontal serpentine tubular reactors (Pirt *et al.*, 1983; Gudin and Therpenier, 1986; Lee, 1986) and small scale vertical tubular reactors (Behemann *et al.*, 1978, Miyamoto *et al.*, 1988) have been tried with varying degree of success.

Techniques for culture of selected marine phytoplankton in continuous flow through culture serving as a year round food source was described by Walsh *et al.*,(1987). Yield optimi-zation of the algae was achieved in these nutrient saturated, light limited cultures by the proper selection of depth and dilution rate empirically determined for each month of the year. The cost of producing cultured algae was also computed. Taub (1970) discussed about ther batch of marine algae, protein content of algae, their production reliability and effect of several types of additives.

Laboratory and mass production of the marine microalgae *Isochrysis galbana* in an open reactor was described by Boussiba *et al.*, (1988). James *et al.* (1988) showed algal productivity was considerably higher in the continuous culture system than any of the other algal culture systems available. James and Abu-Rezeq (1989) James and Al-Khors (1990), reported on the design of an automated intensive continuous culture system based on the principals of chemostat. Further, a 200 litre capacity translucent vertical air-lift tubular photobioreactors was also designed.

A continuous culture system which is a turbidostat composed of a reactor and an automatic hatvesting system was described by Reynoso Granadon *et al.*(1990), in which the reactor was made with 280 litre polyethylene bag, included in cylindrical metal structure with four 40 W Fluorescent lamps for illumination. An air line was inserted at the bottom of the bag providing, vigorous stirring to the cultures. The algal concentration was kept steady by means of an inexpensive automatic socket with a photocell, which pumps fresh medium into the reactor, when the critical point of the photocell is reached. The product was harvested by overflow.

Liang (1991) gave the detailed procedure for culture of marine, unicellular algae through batch culture method, continuous culture method and semicontinuous culture method in a laboratory leaflet published by MAFF'S Fisheries Laboratory at Conway; North Wales, UK.

Spektorova et al. (1991) showed the changes in the chemical composition of algal biomass in relation to season and nitrogen nutrition. A photobioreactor for high density

culture of marine photosynthetic microorganisms which are capable of using solar energy as the light source was constructed by Matsunaga (1991). Excellent light distribution throughout the culture was achieved by use of a bundle of optical fibres which diffuse light energy out through their surface perpendicular to the axis of the fibre. The fibre bundle passed through the cell culture supplied light to it. This biosolar reactor was used and optimised for both the production of marine cyanobacterial biomass and for enhanced carbondioxide removal from the input gas. Enrichment culture of microalgae with carbondioxide at a level similar to industrial exhaust was carried out by Kodama et al.(1993).

Economics of microalgae (Chaetoceros calcitrans) production using multistep method was described by Samonte et al. (1993). The algae were grown in batches using successively larger containers. A cell density of 2.65 x 10^6 cells/ml was obtained from the final 4-day culture of *C. calcitrans* The production cost using this method was P 715.50/ton (US$ 1.00=25 Philippine pesas) = 28.68$ = 1087.56 Rs (US$ 1.00= 38 Rs Indian).

Veen (1994) developed a culture system for the investigation of UV-B effect on microalgae. Also the effect of vitamin B12 on a culture growing continuous culture chaemostat system was studied by Wilhelm *et al.*(1995). Semicontinuous cultures of *Phaeodactylum tricornatum* for optimization of nutrient concentrations and renewal rates was carried out by Fabregas *et al.*(1996). Coutteau (1996) gave the complete description of various methods used for the culture of microalgae in mariculture practices.

Semicontinuous culture of the marine microalgae Tetraselmis suecica with two nutrient concentration was carried out by Oteno and Fabregas (1997). Fukami *et al.* (1997) developed a continuous culture system for benthic food diatom *Nitzschia* sp by using properties of high nutrients in clean of deep seawater (DSW) collected from 320 m depth in *Muroto city, Japan.*

Flavobacterium species which was found to promote growth of a marine diatom *Chaetoceros gracilis* in axenic culture condition was examined by Suminto *et al.* (1997).

INDIAN WORK ON MICROALGAL CULTURE

Published reports on microalgal culture in India is sparse. Gopinathan (1982) has described the methods for culturing marine phytoplankton. He described the methods for isolation and culture of phytoplankton. The cultures were maintained by occasional replacement with nutrients or by regular subculturing. Also differential growth rates of microalgae in various culture media was studied by Gopinathan (1986) and growth characteristics of some nanoplankters was given by Gopinathan (1987).

Based on practical experience various research agencies have brought out a few working mannuals, like

1) Methods of culturing phytoplankton as C.M.F.R.I. Special publications,8: 113-118.
2) Live feed culture by Marine Product Export Development Agency

There is no published research work available on continuous algal culture as well as semicontinuous algal culture from India. It was after the scanning of all these peripheral and primary literature the present study was undertaken.

MATERIALS AND METHODS

Place of Experiment

All experiments were conducted at the Fisheries Harbour Laboratory (FHL) of C.M.F.R.I. (Central Marine Fisheries Research Institute) situated at Thoppumpady, Cochin, Kerala. The experiments were conducted inside the airconditioned Algal Culture Laboratory of FHL as well as in covered outdoor conditions.

The following experiments were carried out -

- ⊗ *High density culture of marine unicellular algae using continuous culture system and* semi-continuous culture system.
- ⊗ To find out the effect of use of carbondioxide bubbled through culture system.
- ⊗ To find out economics of algal culture using continuous and semi-continuous systems and as compared with batch cultures.

The species of microalgae used in the experiments were the flagellate *Isochrysis galbana* (5 μ) and the diatom *Chaetoceros sp* (2.5 μ). The stock cultures were procured from the algal culture facility of FHL of CMFRI.

Seawater (Source & Treatment)

The seawater for culture of the algae must be clean otherwise unwanted species of algae and other contaminants which may feed on or compete with the cultured algae will grow in the medium.

Seawater used for experiment purpose was pumped from nearby harbour area having connection to sea. *The seawater was pumped during high tide to get seawater of salinity 30-37 ppt.*

Following treatments were followed before it was used for experiments.

1) Seawater was pumped into a 10 tonne capacity FRP tank and alum was added so as to settle the debris.
2) The clean sea water was pumped in another tank where it was chlorinated @ 30 ppm (i.e. 30ml/ ton seawater) and the same was kept overnight and then vigerously aerated to remove chlorine.
3) The **O-Toloudine** test was done for detecting the presence of residual chlorine in the dechlorinated seawater.

   Testing procedure was as follows:

   A. Take 50 ml of sample
   B. Add 2-3 drops of O-Toloudine solution with help of dropper in the sample
   C. If an yellowish pink colour residue appears, it indicates the presence of residual chlorine.
   D. In that case water was aerated again vigorously and after some hours the water was again tested for residual chlorine.
   E. If the water remains colourless on addition of O-Toloudine it shows that residual chlorine is absent and then only chlorine-free water is to be used for culture purposes.
   F. The salinity of rhe seawater was adjusted to have the range of 25-28 ppt. with the help the procedure stated earlier..

4) This chlorine-free sea water was filtered through 10μ filter bag and 2 litre and 4 litre were taken in culture flasks of capacity 3 litre and 4 litre respectively. The reservoir bottles also filled with the water and nutrient salts were added to them.
5) For Exp.No.**I** to **X**, Culture flask and reservoir flasks were plugged with cotton and autoclaved for 20 minutes.
6) For batch culture (Control) seawater was heated until it boiled.
7) For semicontinuous outdoor culture, the water was chlorinated twice and dechlorinated since it was not practical to auto-clave 60 litres of seawater.

A simple calculation was used to prepare seawater of the required salinity from the available seawater. The formula used was :

Desired salinity (ppt) X Volume of SW / Available salinity = Volume of SW to be added

..... **Eq.1**

Nutrient media

Concentration of cells in phytoplankton cultures are generally higher than those found in nature. Algal cultures must therefore be enriched with nutrients to make up for the deficiencies in the seawater. Macronutrients include nitrate, phosphate (in an approximate ratio of 6:1) and silicate. Silicate is specifically used for the growth of diatoms which utilize this compound for production of their outer shells. Micronutrients consist of various trace metals and the vitamins Thiamin (B_1), Cynocobalamin (B_{12}) and sometimes Biotin.

For culturing the micro-algae, the following culture media are commonly used :

1. Conway or Walne's media (Walne, 1974)
2. TMRL media (Tung kone Marine Res. Lab.)
3. Guillard's F/2 medium
4. PM Media (Gopinathan, 1982)
5. **Laing media** (Laing, 1991)
6. Erd-Schreiber's and Miquel media (Miquel,1992)

Media 1 to 6 were found to be effective. In the present instance experiments were carried out using the media described by Laing (1991) in the Laboratory Leaflet, MAFF. Direct. Fish. Res., Lowestoft (67): 31 pp. Composition of algal media used for culture experiments is given below.

Table 3: Constituents of the nutrient salt solution used in the experiment (after Laing, 1991)

CONSTITUENTS	Quantity available in Gms.	Rate Rs.	Quantity required in Gms.	Price Rs.
SOLUTION "A"				
Ferric chloride	500	75	0.8	0.12
Manganous chloride	500	95	0.4	0.076
Boric acid	500	95	33.6	6.384
EDTA,disodium salt	500	95	45	8.55
Sodium di-hydrogen orthophosphate	500	120	20	4.8
K-nitrate	500	57.5	100	11.5
Solution 'B"			1ml.	0.09314
Make up to 1L with fresh water				
SOLUTION "B"				
Zinc chloride	500	105	2.1	0.441
Cobaltus chloride	500	1400	2	5.6
Ammonium molybdate	100	180	0.9	1.62
Cupric sulphate	500	135	2	0.54
Concentrated HCl	500ml.	57	10ml.	1.14
Make up to 100ml. with fresh water				
SOLUTION "C"				
Vitamin B1	25	225	0.2	1.8
SOLUTION "E"				15
Make up to 200ml. with fresh water				
SOLUTION "D" *(for culture of diatoms used in addition to solutions "A" and "B")*				
Sodium metasilicate	500	120	40	9.6
Make up to 1L with fresh water				
SOLUTION "E"				
Vitamin B12	1	1500	0.1	150
Make up to 250ml. with fresh water				

*All chemicals used were sourced from HiMedia, Qualigens and Citra Chemical Co.

Table 4: *Concentration of solutions A, C and D used in preparation of nutrient enriched seawater.*

Solution	1x ml/l (Control)	Cost (Rs)	2.5x ml/l (Experiments)	Cost (Rs)
A	1.0	0.03152	2.5	0.0788
C	0.1	0.0084	0.25	0.021
D	2.0	0.0192	5.0	0.048

Determination of algal cell concentration

Among many available procedures, for determining the algal cell concentration, haemocytometric counting is the simplest method. Other means such as Coulter counters, Turbidometer, Spectrophotometer, Video endoscopy etc. have been used.

The apparatus used for counting the algal cells was a haemocytometer with an impproved Neubaeur ruling. The device is quite suitable for counting algal cells less than 3μ size. Before counting, both the cover slip and the chamber were rinsed with clean distilled water and dried with blotting paper. Occasional cleaning with alcohol ensures the Free Flow of algae over the counting area. The face of counting chamber is composed of two gridded surfaces separated by canals. The coverslip was placed on the support bars along the canals and a drop of homogenously mixed algal suspension was delivered from a pasteur pipette by touching the pippette tip to the edge of the coverslip where it hangs over the V-shaped loading part. Slight pressure will cause the algal suspension to flow evenly across the surface, but not into the canals or on the top of the coverslip.

Both sides of chamber must be loaded to seal the coverslip properly. Each half of the haemocytometer surface contains nine large grids. Only those algal cells which fall with the

four large corner grids were counted. Each large corner grid is further sub-divided into 16 small squares cells which fall on a border were counted if atleast half the cell is within the square, but only two borders were acknowledged (either top or bottom and either left or right) so that cells were not counted twice.

To determine the algal cell density (number of algal cells per millimeter) of the suspension, the number of algal cells counted is divided by the large corner grid area covered multiplied by 10^4. All the four corner grid areas were covered and the mean was taken. The mean value when multiplied by 10^4 gave the actual cell concentration per ml of the sample. If the algal suspension is very thick suitable dilution with seawater was made.

Similarly, three to four samples were taken from each algal suspension and the average value was taken as the final cell concentration.

The average number of cells in 1 ml was calculated as

Average count per chamber X 10^4 = *Total number of cells/ ml*

..... **Eq.2**

Harvesting of cells

Culture should reach densities suitable for harvesting. The harvested culture should be subsequently made up to total volume for maximum yield. The amount of harvest which achieves this yield can be calculated from the following equation (From Laing,1991)

Volume harvested in litres =

Vol of culture - (Vol of culture X Density to which culture need to be diluted) /
Actual culture density of algae harvested

..... **Eq.3**

Table 5: Experimental Treatments

Experiment No,	Particulars	Culture Capacity (litres)
I	Isochrysis batch culture (Control)	2 or 4
II	Chaetoceros batch culture (Control)	2 or 4
III	Isochrysis continuous culture	2
IV	Isochrysis continuous culture with CO_2	2
V	Isochrysis semicontinuous culture	4
VI	Isochrysis semicontinuous culture with CO_2	4
VII	Chaetoceros continuous culture	2
VIII	Chaetoceros continuous culture with CO_2	2
IX	Chaetoceros semicontinuous culture	4
X	Chaetoceros semicontinuous culture with CO_2	4
IX	Chaetoceros semicontinuous culture, outdoor	60

CONTROL - *Isochrysis galbana* and *Chaetoceros sp.* (Indoor) [Exp.No. I&II]

For this purpose pre-cleaned conical flasks of 3 litre/ 5 litre capacity were taken. Flasks were filled with de-chlorinated and filtered seawater of about 25-28 ppt salinity upto 1.8 litre for 2 litre culture and 3.6 litre for 4 litre culture. The flasks were plugged with cotton plug and heated to boil the water inside it completely and then allowed to cool. When flasks were properly cooled, nutrient were added at 1x ml/litre as per the concentrations given in table. 10% of innoculum from stock culture was added to flasks i.e 200 ml for 2 litre culture and 400 ml for 4 litre culture. The flask were kept on rack with light illumination (1 40W flourescent tube) provided. No aeration was provided to the flask. Following parameters were recorded daily.

1) Algal cell concentration
2) Temperature of air
3) Temperature of water
4) pH
5) Salinity

These parameters were recorded between 0800 and 1000 hours. When the culture concentration density starts declining, then both the cultures were harvested completely. All data were maintained in a record book.

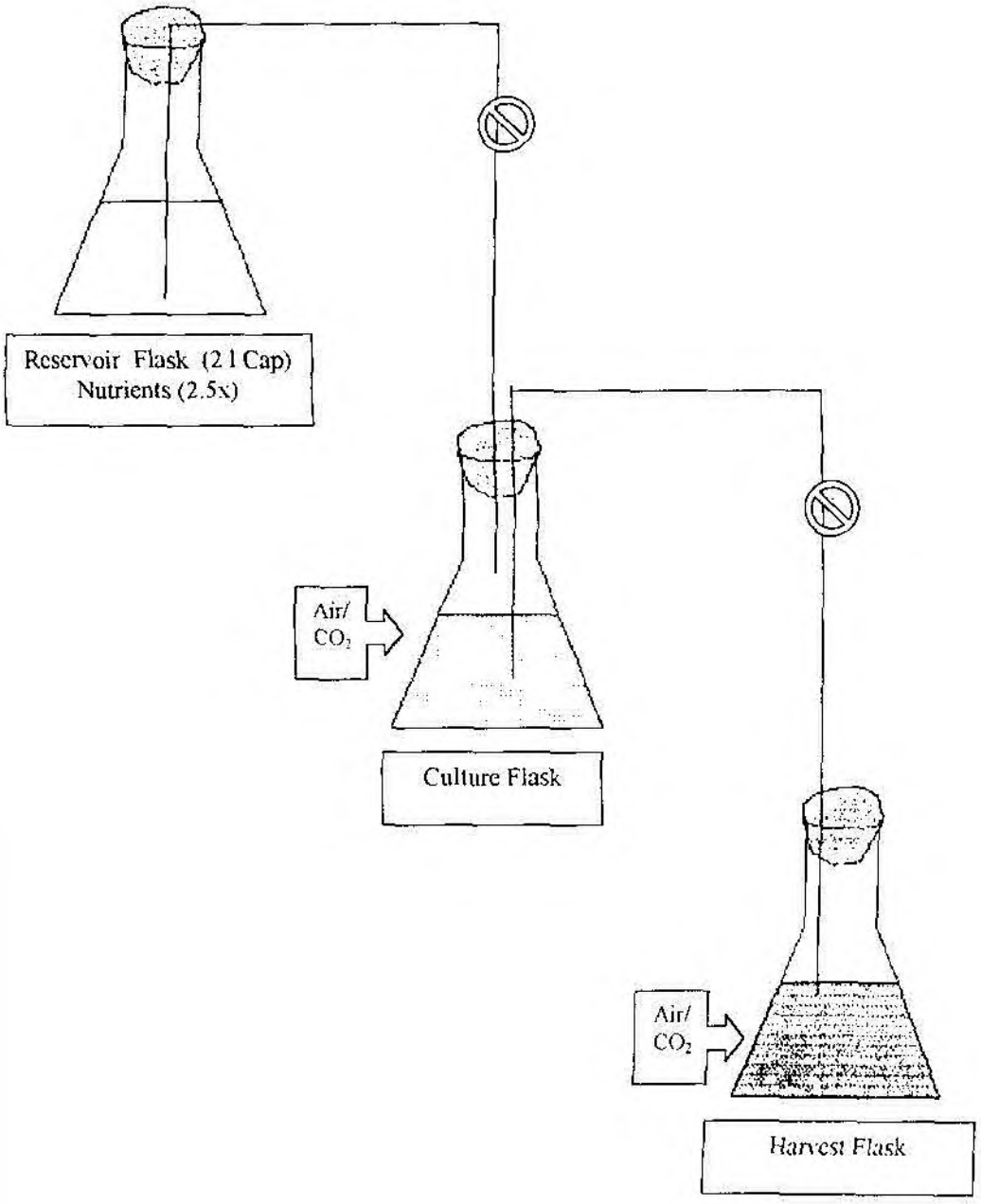
Continuous culture system of *Chaetoceros sp.* and *Isochrysis galbana* (Laboratory, Indoor) [EXP. III, IV, VII & VIII]

For this purpose cleaned 3 litre conical flask were taken. 1.8 litre dechlorinated and Filtered seawater of 25-28 ppt was taken in each flask. At the same time reservoir bottles were also filled with filtered seawater of 25-28 ppt. Nutrient salt solutions were added as per the concentrations (2.5x) given in Table 4. The flask and reservoir bottle mouths were plugged with cotton plug and then autoclaved at 115 psi for 20 minutes and then allowed to cool. The aeration system tubing was cleaned properly and then attached to a newly constructed air filter prepared as shown in Fig.1A. Gravity was used for transfering media from reservoir to culture flask. Reservoir flask was placed on top shelf in laboratory and on the immediate next shelf below, the Culture flask was kept and on the thirdshelf from the top the harvest flask was kept (Fig.1; Plate 1). The culture flask was connected to the reservoir flask with tubing having a controller to maintain the constant level of culture (2 litre) in culture flask by adding nutrients from reservoir bottles. Harvest flask was connected with tubing having a controller to culture flask through which everyday 500 ml of harvest was drained into harvest flask from culture flask. In this three tier system, air/CO_2 connections were provided to the culture flask and harvest flask. The continuously bubbling aeration helps to keep the culture and harvest in agitation and therefore allowing it to multiply fast. Flourescent lighting (4800-7400 lux) was provided on the back side of culture flask and harvest flask as shown in Plate No.1. In culture flask 200 ml innoculum from laboratory grown five day old stock culture was added. For experiment no IV and VIII CO_2 was provided by bubbling through culture flask and harvest, flask for less than min/day so that the pH of the medium would be reduced to 6.5-7.5. The following parameters were noted daily.

1) Temperature of air

2) Temperature of water

Fig.1 - CONTINUOUS CULTURE OF MICROALGAE
(Experimental Setup)



3) Salinity

4) pH

These readings were taken daily in between 0800 and 1000am. Within 3-4 days the culture reached above 3 million cells 1ml, then 500 ml. culture was harvested into the harvest flask. The volume harvested was replaced in the culture flask with nutrient mixed sea water from the nutrient reservoir bottle. The harvested volume in the harvest flask was allowed to multiply for one day with continuous aeration and supply of bubbling CO_2 (Exp. **IV&VIII**). During this period, the cultured cell concentration in the harvest flask would double and this was the final harvest, used for feeding the larvae. In this manner, daily 500 ml was harvested in harvest flask and nutrient mixed sea water was added to culture flask from reservoir bottle. New nutrient reservoir bottles were made ready . All parameters mentioned above were measured daily. When foam appears on the surface of the culture, culture colour starts fading this is an indicating that culture is going to collapse. Then the total harvest was done. All data were maintained in a record book.

***Semicontinuous culture system for Chaetoceros sp.* and *Isochrysis galbana* (Laboratory, Indoor) [Expt V, VI, IX & X]**

For this purpose cleaned 5L conical flask was taken. The flask was filled with dechlorinated and filtered seawater of 25-28 ppt upto the 4L mark.. The nutrient salt solutions were added as per the concentrations given in Table 4. The flask mouth was plugged with cotton plug. The flask was then autoclaved at 115 psi for 20 minutes. Cooled autoclaved flask along with nutrient media added seawater was used for the culture. The aeration system tubing was cleaned properly and then attached to a newly constructed air filter prepared as shown in Fig.1A . Other tubings were provided inside culture flask (as shown in Plate 2) for removing harvested culture and for adding carbondioxide to culture for experiment using CO_2. Light system was provided on the back side of the flask as shown in Plate No.2. Aeration (of continuous bubbling type) was started through a glass tube. The 400 ml culture of phytoplankton was innoculated to 3.6 litre of nutrient media added to seawater i.e. (10%

innoculum). The culture concentration was counted in million cells/ml. The following parameters were noted daily.

1) Temperature of air

2) Temperature of *water*

3) pH

4) Salinity

These readings were taken daily in between 0800 and 1000a.m. period.

Once the cell concentration reached 3.5 million cells/ml within 3-4 days, a part of the culture was harvested and the rest was diluted with newly autoclaved cooled nutrient added seawater of 25-28 ppt to make a cell concentration of approximately 1 million cells/ml in the 4 litre water in the flask. CO_2 provided from a CO_2 cylinder. The CO_2 was bubbled for less than a minute a day through the culture to bring down its pH to 6.5 - 7.5. The pH reading was taken after this. The harvest was repeated after every 2-3 days since within the 3rd day the culture regained the concentration of 3.5 million cells/ml. When even after 3-4 days the concentration of cells was not increasing and foaming at the surface was observed it indicated that the culture was going to collapse. Then, total harvesting was carried out and the experiment was closed. All data were maintained, in a record book.

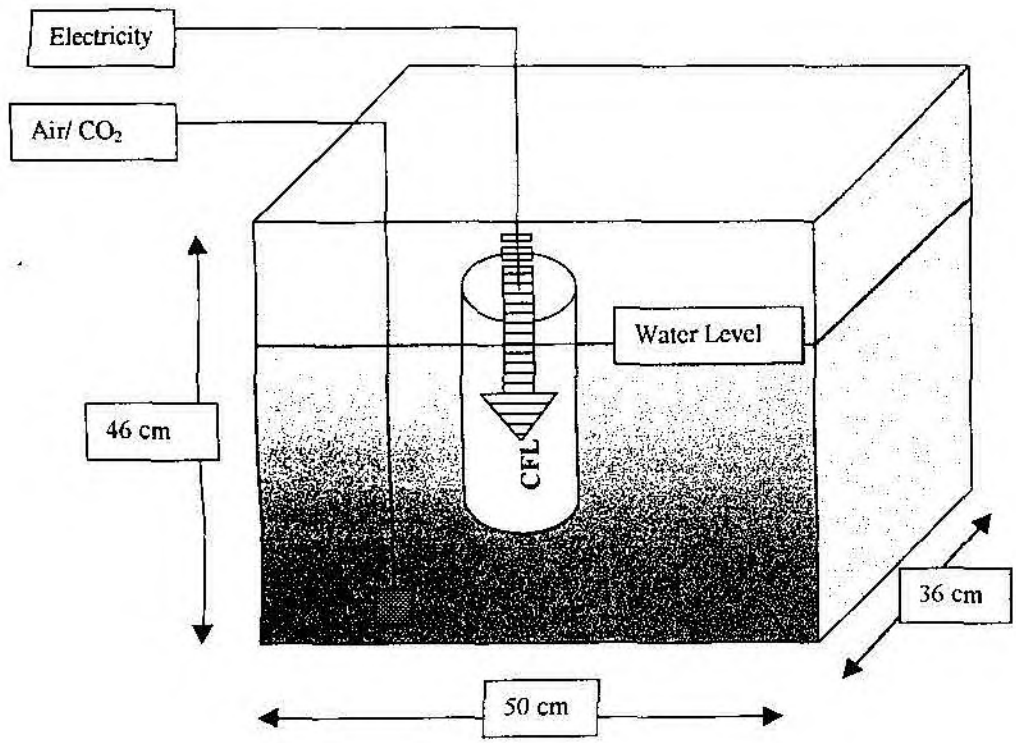
Scale-up *Chaetoceros* semicontinuous culture 60 litre capacity, Outdoor with use of CO_2 (Exp. XI)

The preparation of perspex tank as culture vessel -

A plexy tank of 80 litre capacity (Plate 3. & Fig..2) was cleaned thoroughly. The tank was placed outdoor in a covered gallery on a wooden table. The tank was filled with dechlorinated seawater of 25-28 ppt salinity upto 60L capacity and chlorinated again @ 30 ppm chlorine. A cleaned airfilter (constructed as shown in Fig 1A) was inserted between air line to avoid ciliate attack on culture and 2 air stones were provided for aeration. Reservoir tank was also cleaned and filled with dechlorinated sea water of 25-28 ppt salinity and chlorinated again with 30 ppm chlorine @ 30 ml/ton of sea water and after 12-14 hr aerated vigorously to dechlorinate it

Fig.2 - Semi-Continuous Microalgal Culture in 60 litre Capacity Internally Illuminated Outdoor Perspex Tank

(Experimental Setup)



60 litre capacity plexy glass outdoor semicontinuous culture system with internal illumination provided by a compact fluorescent lamp (CFL)

completely. The tank was covered completely with a white paper and plastic sheet so as to keep all the illumination reflected back in the tank. The water was used for culture only when all chlorine residues were removed this was checked with 0-Toloudine test.

For fitting an internal illumination system, a 5L capacity plexy jar was taken. To the lid a hole was made in such a way that the wire and the bulb holder can be inserted inside jar in a water tight manner. The jar was fixed at the centre of tank in such a way that the 3/4 of jar would be immersed in the water and no water would enter inside jar. A small hole was made to lid in order to ensure air circulation into the jar. The nutrients were added to the culture tank at 2.5x ml/litre. 600 ml culture from laboratory stock culture was added into the 60 litre seawater present in the tank. The bubbling aeration was started. The carbondioxide was provided through aeration system in bubbling made by stopping aeration for some time and attaching the aeration tube to CO_2 cylinder. The CO_2 was bubbled for less than a min./day and then again aeration was restarted.

The following parameters were recorded daily

1. Culture concentration
2. Temperature of air
3. Temperature of water
4. pH
5. Salinity

These parameters were recorded daily in between 0800 and 1000 am. When the culture concentration reached about 3.5 million cells/ml, harvesting of the culture was done as given in earlier section and volume again made upto 60 litre (1 million cells/ml) by adding water from reservoir tank and adding nutrients again. Harvesting was done mostly after 2-3 days as within 2-3 days culture grows from 1 million to 3-3.5 million cells/ml. This procedure was continued until the culture starts producing foam on the surface and culture colour starts fading. At this point complete harvesting was done. The data were recorded daily and maintained in record book.

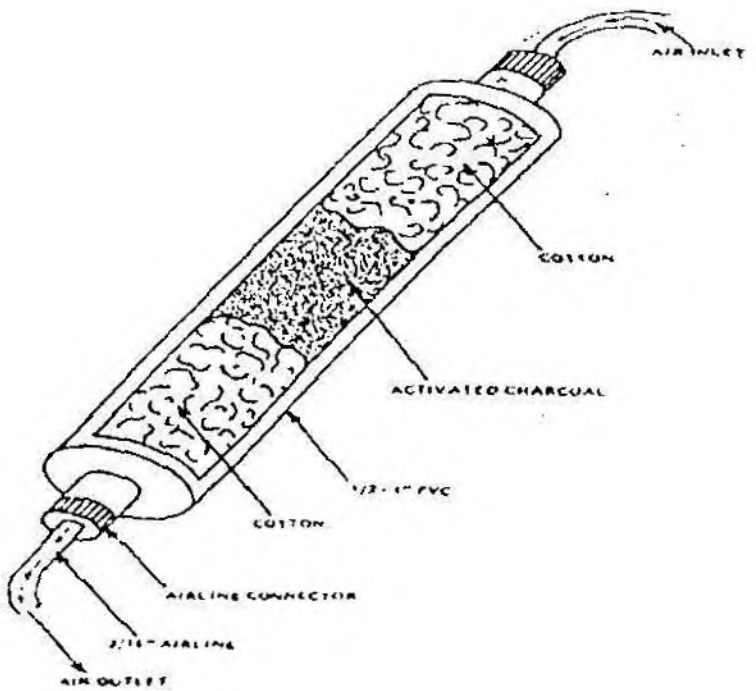


Fig. 1A: Schematic representation of the activated charcoal filled air filter used for providing air supply in the present experiment.

Inputs for Experiments

1. Air Supply

A common source of contamination is the condensation in the air lines which harbour ciliates. For this reason air lines should be kept dry and both air and carbondioxide should be filtered through an in-line filter of 0.3 or 0.5 m before entering the culture. For larger volumes of air small filter units can be constructed using PVC tubing, cotton, activated charcoal, bolting silk cloth as shown in Fig 1A (Modified from Fox, 1983).

Algal cultures are usually mixed by bubbling air through them, so that necessary light and nutrient become available to all the cells. During the experimental period, air was supplied from rotary blower.

2. CO_2

Providing the algae with extra carbon dioxide in the form of the gas carbon dioxide will give much faster growth. (Laing, 1991). CO_2 is usually supplied from compressed gas cylinder.

In this experiment small CO_2 cylinder of 270 gm capacity (M/s Mr. Butler Soda Maker Refill) which is already filtered as it is to be used for human consumption in bottled beverages was used.

CO_2 was provided once a day through tube supplying air to the culture. The CO_2 passed through culture will not allow the pH of culture above 9.

Filtering of air as well as CO_2 through air filter helped to prevent contaminating organisms from getting into the culture.

3. Light

Light was provided by 40W flourescent tubelights in indoor culture conditions. For outdoor culture a PL-S gw/86 [Cool Day light/6500^{O}K] compact flourescent lamp (CFL) was used. The intensity of light was measured with the help of a digital lux meter.

For indoor culture photoperiod was Light: Dark- 16:8 h and for outdoor culture the photoperiod was 24 h light period. The light intensities available to the different experiments is given in Table 6.

Table 6. Intensity of light during experimental period.

	Intensity lux	**Start of culture** lux	**Before harvest** lux
Control	2970-3000	1380	680
Semicontinuous culture	2970-3000	1380	680
Continuous culture Harvest flask	2970-3000	1380	680
Continuous culture Culture flask	4850-7420	2800	1430-1450
Outdoor culture	Near light Near edge Near bottom	1980-1400 760 200	1380-1400 170 22

Increasing light intensity usually mean, better growth and faster division of algal cells and therefore production of more microalgae.

4. Salinity

Salinities between 25 ppt and 30 ppt are generally best for the culture of flagellates and between 20 ppt and 25 ppt for culture of diatoms. These salinities can be obtained by diluting the seawater with fresh water as per the procedure given in Equation 1. Salinity was measured with the help of a pocket refractometer.

5. Temperature

Most types of algae grow well at temperatures from 17° to 27°C. Lower temperature will not usually kill the algae but will reduce the growth rate. Above 36°C temperature will be lethal for number of species. The optimal temperature for phytoplankton culture is generally between 20° and 24°C.

Above 27°C, most of the algae will not survive, *Chaetoceros* is an exception.. Even a sudden drop or increase in temperature will affect the growth of microalgae. If necessary cultures can be cooled by a flow of cold water over the surface of the culture vessel or by controlling the air temperature with refrigerated air conditioning units. For the present experiments (I to X) cultures were maintained in an air conditioned room, where, the

RESULTS

1. *Isochrysis* control culture Exp I

The results of control culture of *Isochrysis galbana* using 1x nutrient concentration is shown in Fig.3. The graphs shows at the time of innoculation (10%) the culture concentration was 0.86 million cells per ml. The culture started showing increased cell concentration as days progressed. It showed three peaks during its exponential and stationary phase. Highest was at 19th day following by 17th and 10th day. From 20th day onward declining phase was observed.

The culture lasted for 27 days with an average cell density of 2.15 million cells/ml. The pH of the culture ranged from 7.25 to 9.12. Temperature of air was in between 22.0 and 29.5^{o}C and that of water was in between 23.4 and 28.0^{o}C.

2. *Chaetoceros* control culture Exp II

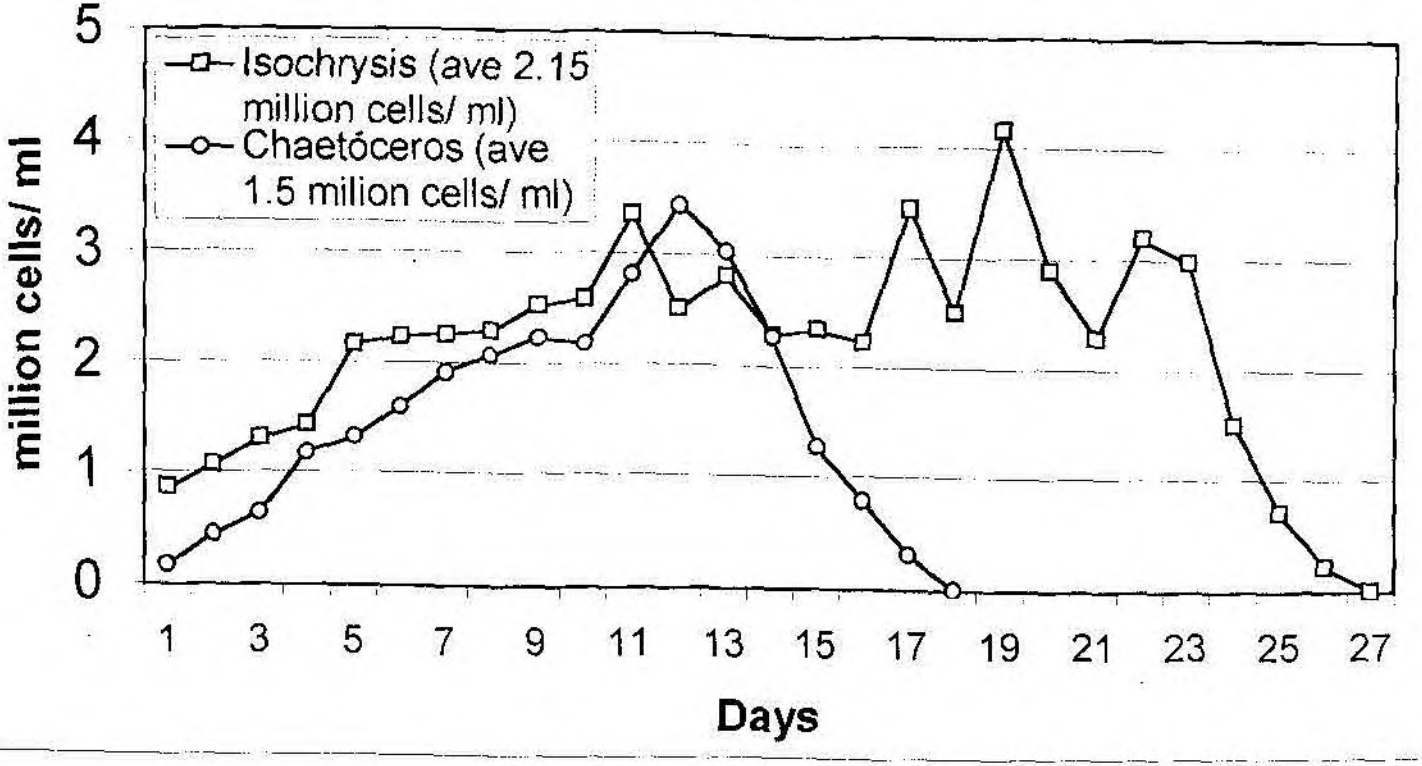
The growth curve of *Chaetoceros* using 1x nutrient concentration is shown in Fig.3. The initial concentration of culture was 0.18 million cells/ml. The exponential phase was observed upto day 7, when the cell concentration was 1.92 million cells/ml. After this the stationary phase was observed till the 12th day when the cell concentration was 3.44 million cells/ml. The culture collapsed on the 18th day and the cell density observed was 0.01 million cells/ml.

The culture gave on an average 1.5 million cells/ml. The pH of the culture during 18 days ranged from 6.54 to 9.18. The temperature of air was in between 21.2^{o}C to 27.3^{o}C and that of water was 23.4^{o}C to 28.0^{o}C. Salinity remained at 30 ppt. throughout the culture period.

3. *Isochrysis* continuous culture Exp III

Results of the continuous culture of *Isochrysis galbana* is shown in Fig.4. The culture was started with an average cell density of 0.39 million cells/ml. The culture density reached 4.07 million cells/ml in 6 days, then the first harvest of 500 ml was done. The harvest flask was kept under similar conditions for one more day and within this period the culture concentration became 7.68 million cells/ml. Then the culture was used for feeding oyster

Fig.3 - Algal Culture - Control



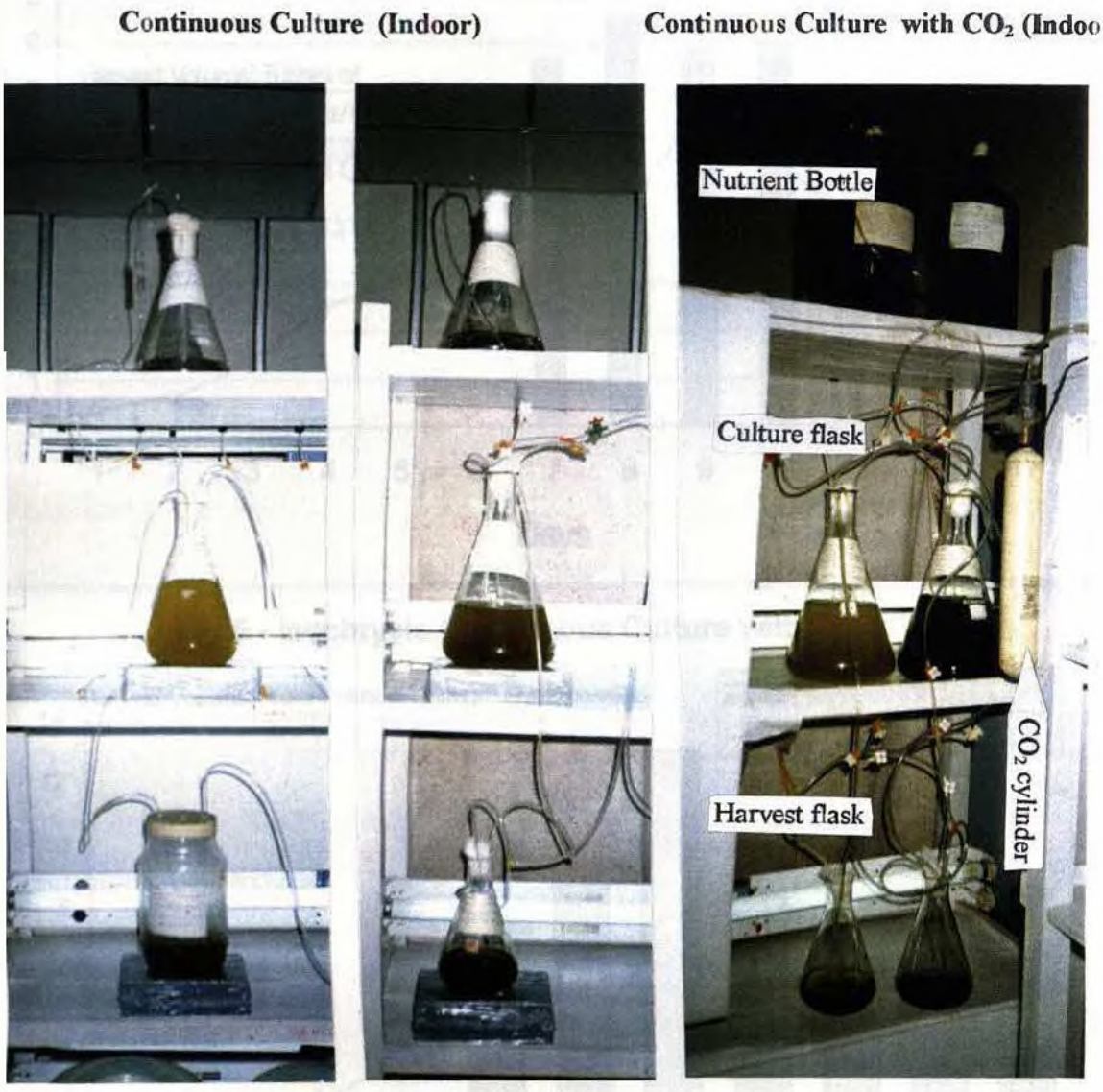


Plate 1. Photograph of continuous culture of *Isochrysis galbana* and *Chaetoceros* sp. with and without CO_2, showing nutrient bottles, culture and harvest flasks. Note the high density in harvest flasks.

Fig.4 - Isochrysis Continuous Culture

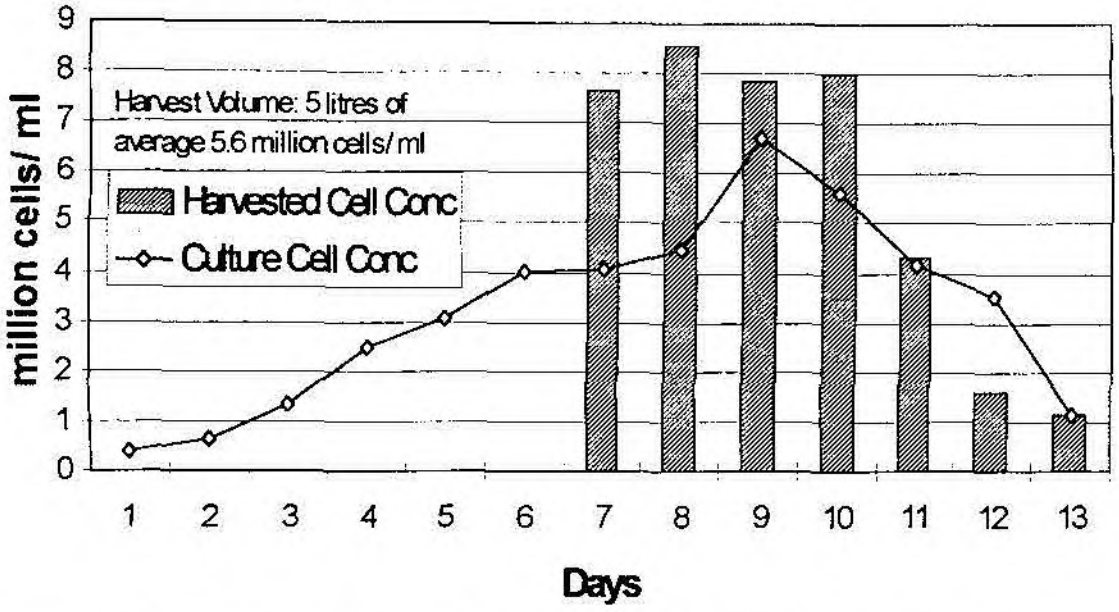
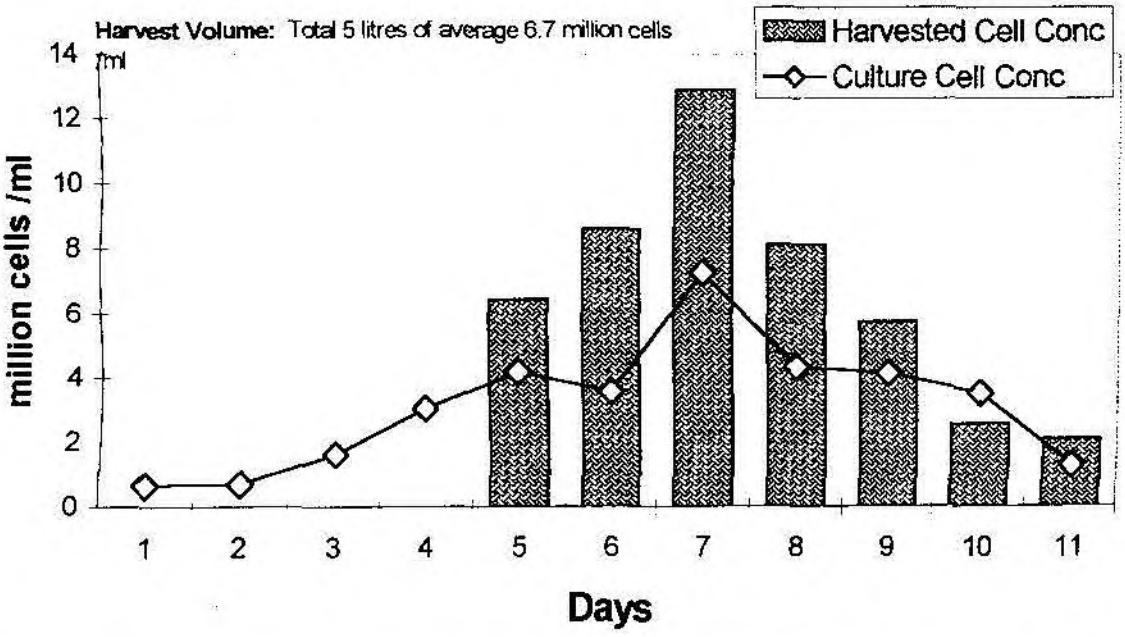


Fig.5 - Isochrysis Continuous Culture with CO2



larvae. Like this 500 ml was harvested daily. This process was continued upto the 6th harvest. On 11th day sudden fall in temperature and on 12th day sudden increase in temperature resulted in collapse of the culture, and so, on 12th day complete harvest was done. The temperature fluctuation on 11th and 12th day was the main reason for collapse of the culture. The pH of culture was within range of 7.62 to 8.78.

4. *Isochrysis* continuous culture with CO_2 Exp IV

The continuous culture of *Isochrysis galbana* is shown by Fig. 5. At the start of experiment culture cell density was 0.64 million cells/ml. The culture attained a cell concentration of 4.33 million cells /ml on 5 th day, when first harvest was carried out. The harvest attained cell concentration of an 6.48 million cells/ml on next day after which it was used for feeding oysters. From 5th day onward continuous harvesting was carried out . The 7th day harvest flask on 8th day showed 12.9 million cells/ml culture concentration. On 10^{th} day the air temperature was 19.5 °C and water temperature as 22.1 °C and that on 11^{th} day the temperature of air was 27.2 °C and water temperature was 28.9°C. This sudden fluctuation in temperature because of air-conditioning failure resulted in collapse of culture. So on 11^{th} day culture concentration was only 2.08 million cells/ml, hence complete harvesting was carried out. The pH of culture was within range of 5.83 to 8.29.

5. *Isochrysis* semicontinuous culture Exp V

Fig.6 shows results of semil-continuous culture of *Isochrysis galbana*. The culture was started with a cell concentration of 0.37 million cells per ml which went on increasing during the log phase and on 6th day culture attained a concentration of 6.32 million cells/ml. The first harvest was taken here, the culture was then made up to 4 l with newly autoclaved cooled nutrient solution so as to maintain a cell concentration of 0.57 million cells/ml. On 10th day, the cell concentration increased to 4.96 million cells/ml and the same harvest procedure was repeated. In this manner, the culture continued upto 29 days, when air conditioning failure resulted in collapse of the culture. Totally 8 harvests (total 26 lit) of

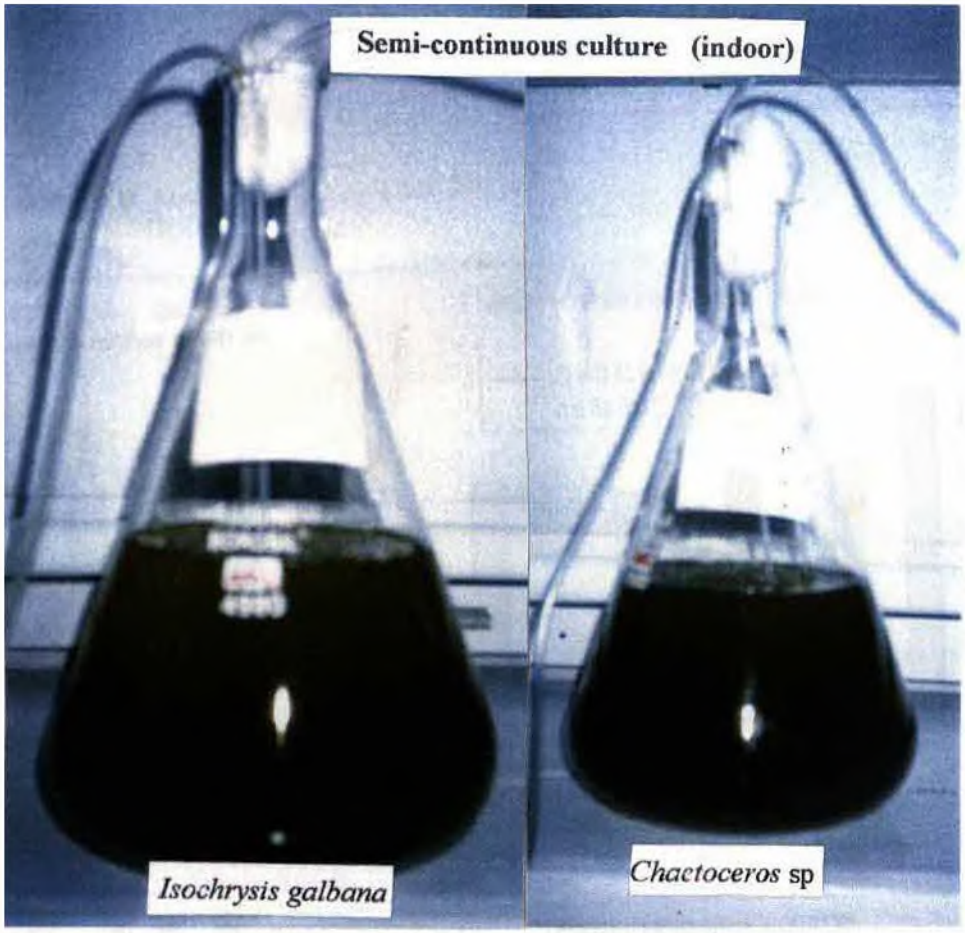


Plate 2. Photograph of semicontinuous culture of *Isochrysis galbana* and *Chaetoceros* sp. just before harvest with and without CO_2

Fig.6 - Isochrysis Semi-Continuous Culture

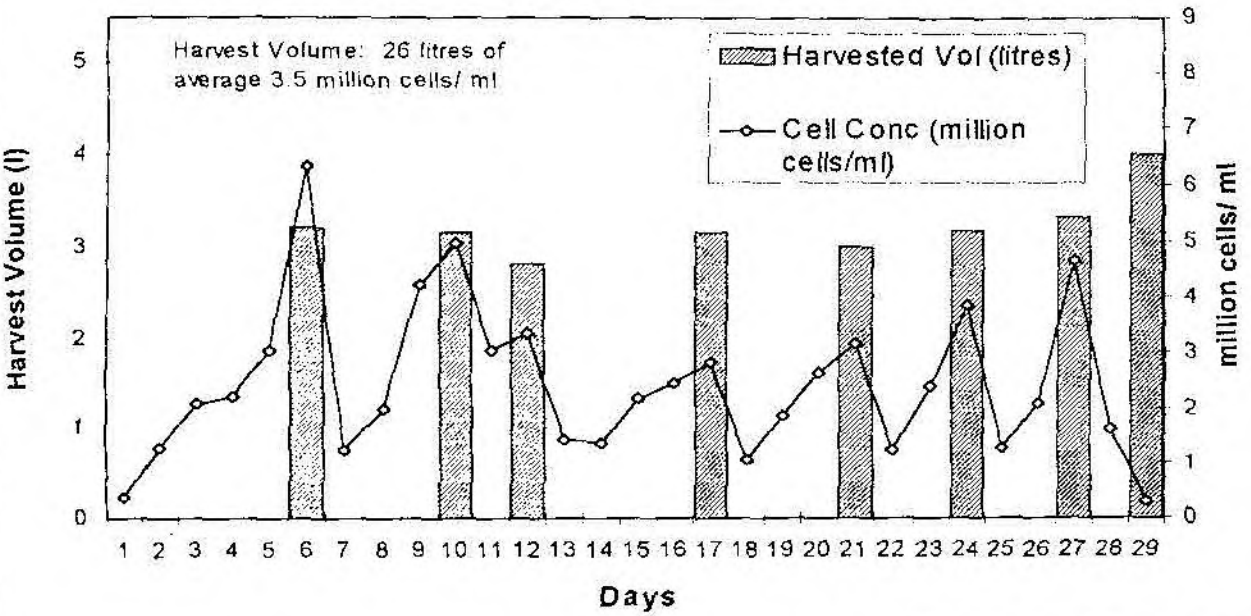


Fig.7 - Isochrysis Semi-continuous with CO2

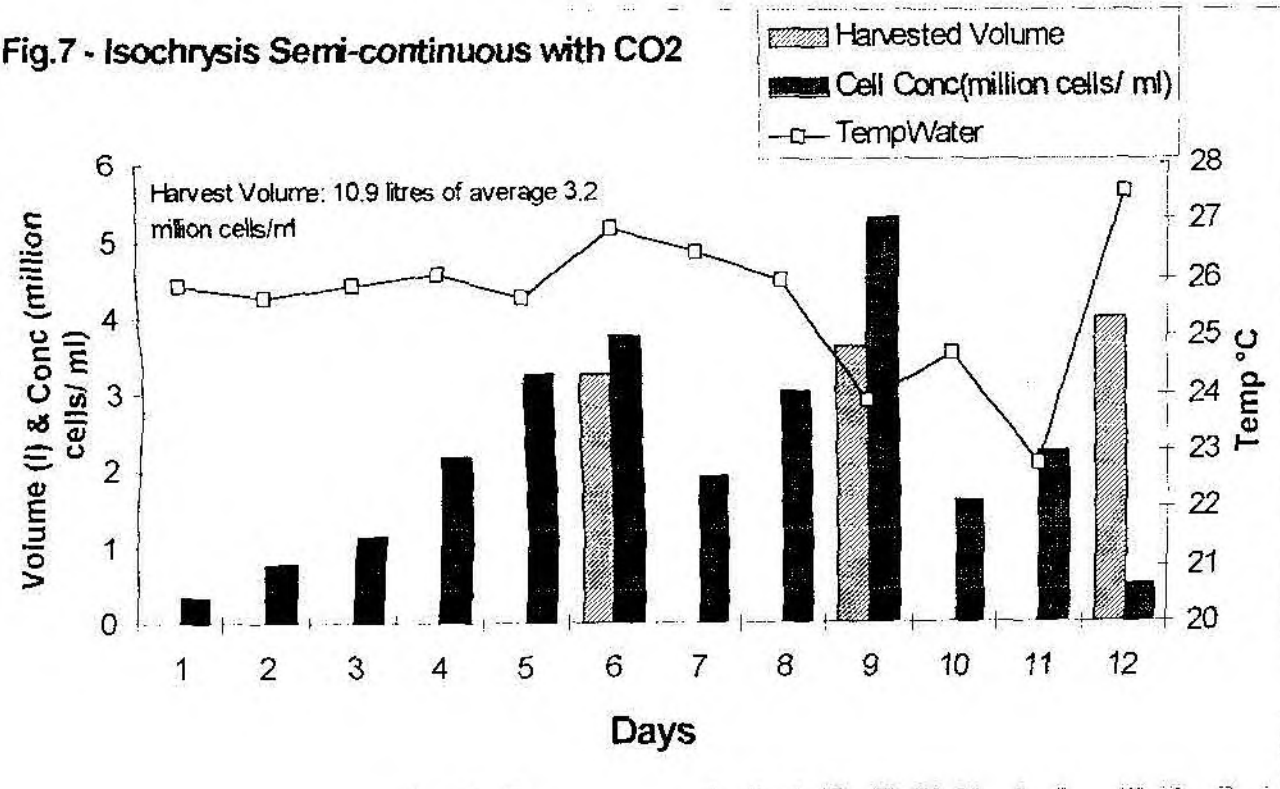


Fig.8 - Chaetoceros Continuous Culture

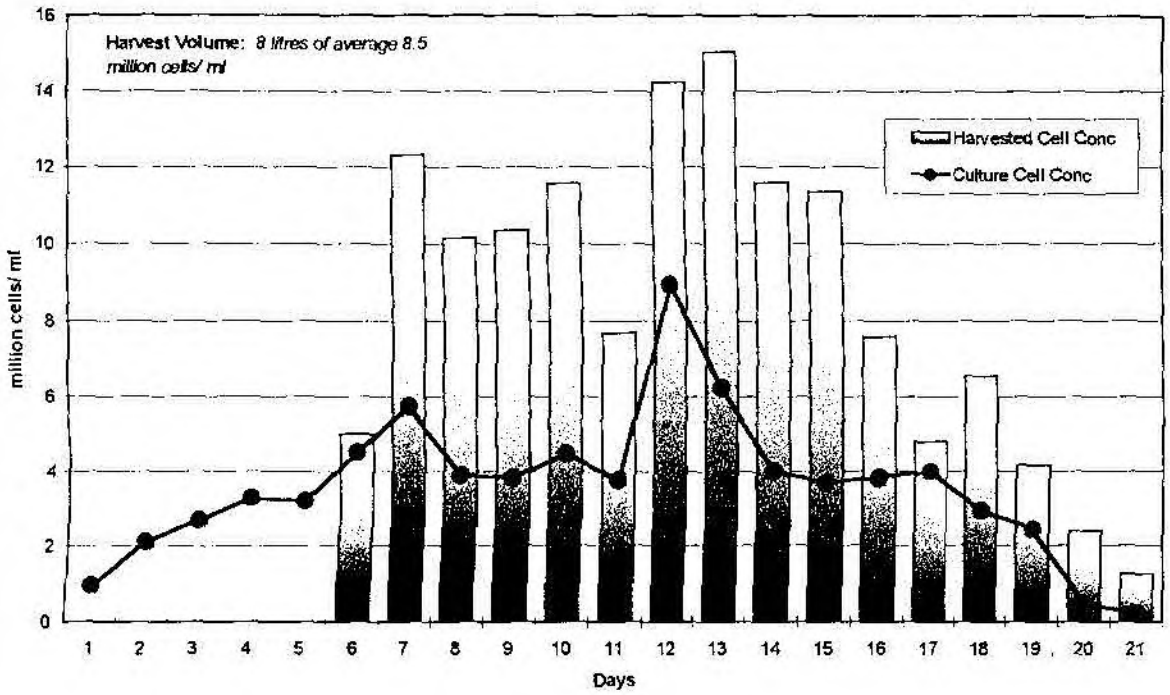
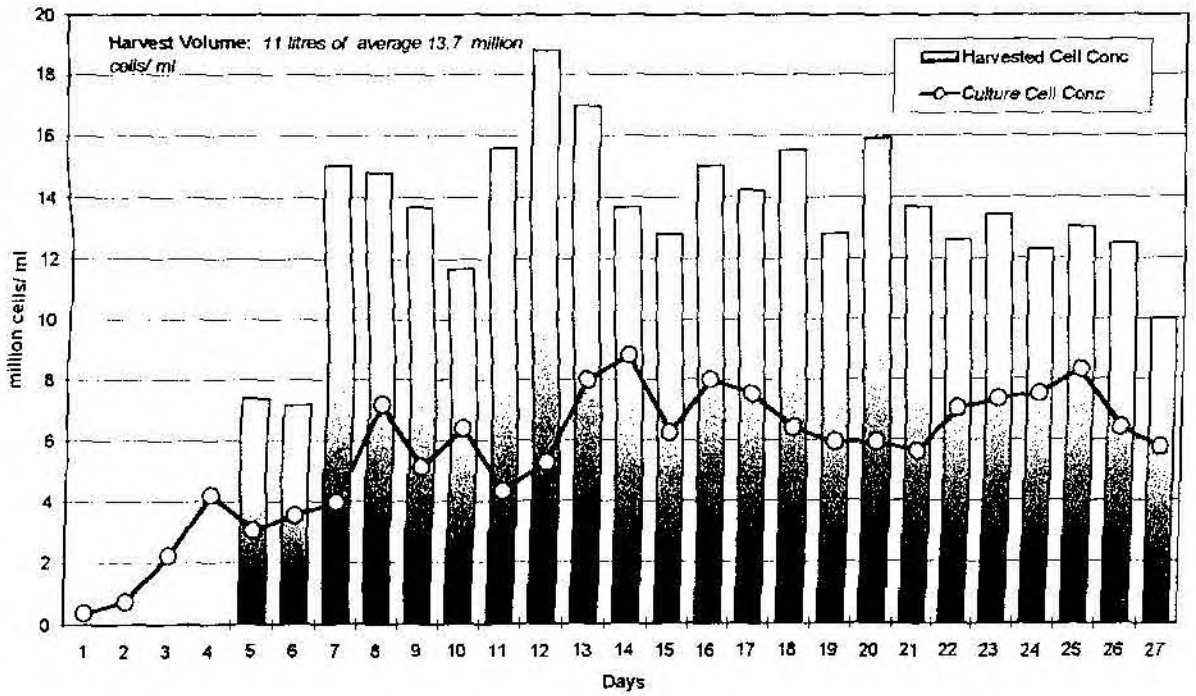


Fig.9 - Chaetoceros Continuous Culture with CO2



average 3.9 million cells/ml was obtained. The pH ranged from 6.56 to 8.75 and temperature of air fume 19.5^{O} ro 29.5^{O}C and that of water from 21.5 ^{O} to 28.0^{O}C.

6. *Isochrysis* semicontinuous culture with CO_2 Exp VI

The results of semi-continuous culture of *Isochrysis galbana* using CO_2 is shown in Fig. 7. On 6th day when culture reached a cell concentration of 3.73 million cells/ml, where as in control it took 11 days to reach 3.35 million cells/ml. The culture was harvested and diluted with fresh nutrient added seawater to achieve a cell concentraton of 1.66 million cells/ml the total volume harvested was 10.9 litres and it was achieved in 3 harvests with an average cell concentration of 3.2 million cells/ml. The culture collapsed on 12th day because of airconditioning failure and the subsequent sudden increase in the culture temperature from 19.5^{O}C to 27.2^{O}C of air and 22.8^{O}C to 27.5^{O}C of water. The pH of culture was in range of 5.69 to 7.96.

7. *Chaetoceros* continuous culture Exp VII

The culture was started with an initial cell concentration of 0.96 million cells/ml. On 5th day it reached a concentration of 5.01 million cells/ml, when the first harvesting was done (Fig. 8) and harvest was utilised only on the next day. The cell concentration in the harvest flask reached 7.2 million cells/ml after the 24h incubation. From 7th to 14th day the culture showed a cell concentration of more than 10 million cells/ml with the exception of 11th day when it showed 7.7 million cells/ml concentration. After the 14th day, declining phase was observed and on 21st day complete harvesting was done. The temperature difference between 15th and 16th day was about 7-8^{O}C of both air and water due to airconditioning failure which may be the main reason for the culture to collapse. From 5th to 20th day 500 ml was harvested daily and on 21st day complete harvesting (2 litres) of culture was done. The pH of culture was in range of 7.03 and8.69 . The temperature of water was in range of 21.3 and 28.7^{O}C and that of air was 19.5 and 28.7^{O}C. During culture period a total of 8 litre of harvest was obtained with an average cell concentration of 8.5 million cells/ml.

Fig.10 - Chaetoceros Semi-Continuous Culture

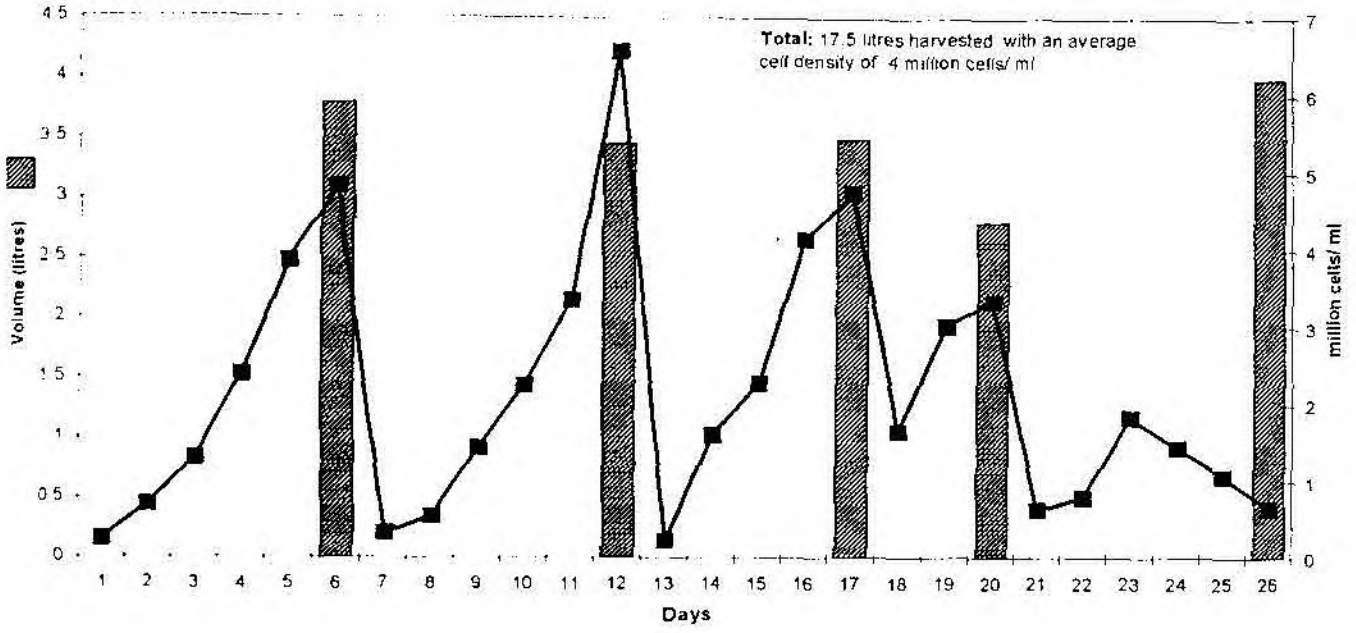
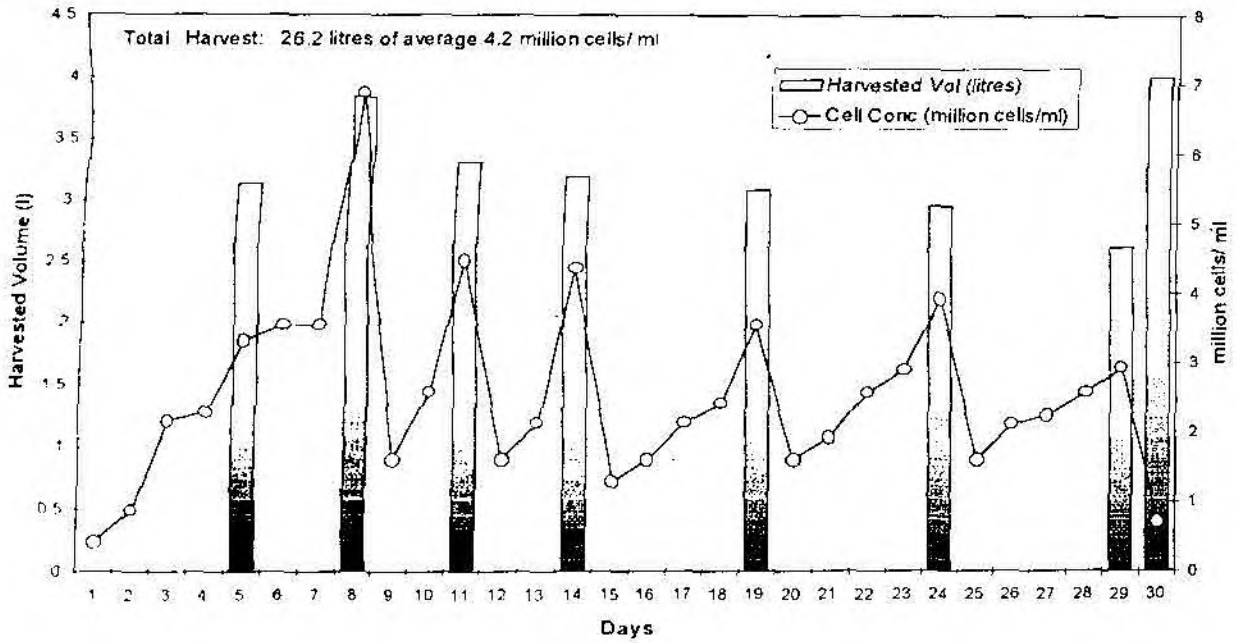


Fig.11 - Chaetoceros Semi-Continuous with CO2, 4l Cap



8. *Chaetoceros* continuous culture with Co_2 Exp VIII

Fig.9 shows the variations in algal cell density during continuous culture of *Chaetoceros* using CO_2. The daily harvest of 500 ml was initiated from day 4 when the cell concentration reached 7.4 million cells/ml. After incubation of the harvest for 24 hr, further increase in cell concentration was noted.. This daily harvesting was continued upto 27 days after which culture showed declining phase when complete harvesting of culture was carried out. Altogether 23 harvests were made resulting in harvest of total 11 litres with an average density of 13.7 million cells/ml.

The pH of the culture ranged from 5.54 to 8.50. The temperature of air was in range of 19.5^{O} to 27.2^{O}C and that of culture was 22.3^{O} to 28.7^{O}C.

9. *Chaetoceros* semi-continuous culture Exp IX

Fig.10 shows the results of semicontinuous culture of *Chaetoceros* cultivated in 5 litre flask. The initial culture density was 0.25 million cells/ml and the culture reached a cell concentration of 4.82 million cells/ml within 6 days. The first harvest was done on that day and culture was diluted to attaining a cell concentration of about 0.33 million cells/ml with fresh nutrient added sea water. After 6 days when culture reached cell concentration of 6.56 million cells/ml second harvesting carried out. In this manner a total of 4 harvests were made and in the 5th one complete harvest was done on the 26th day. From 20th day onward the culture was in the declining phase. Totally 17.5 litres of culture was harvested with an average cell density of 4.0 million cells/ml. The pH of culture was within range of 7.0 to 9.0. Temperature of air was in range of 21.3^{O} and29.5^{O}C and that of water was in range of 22.4^{O} and 28.0^{O}C.

10. *Chaetoceros* semicontinuous culture with CO_2 Exp X

Fig.11 shows the results of semi-continuous culture of *Chaetoceros* with CO_2. Initially, the culture concentration was 0.43 million cells/ml, and on the 5th day culture reached a concentration of 3.32 million cells/ml, when first harvest was carried out. The remaining culture was diluted with fresh autoclaved nutrient added sea water so as to obtain a

Chaetoceros Semi-continuous Culture (Outdoor)

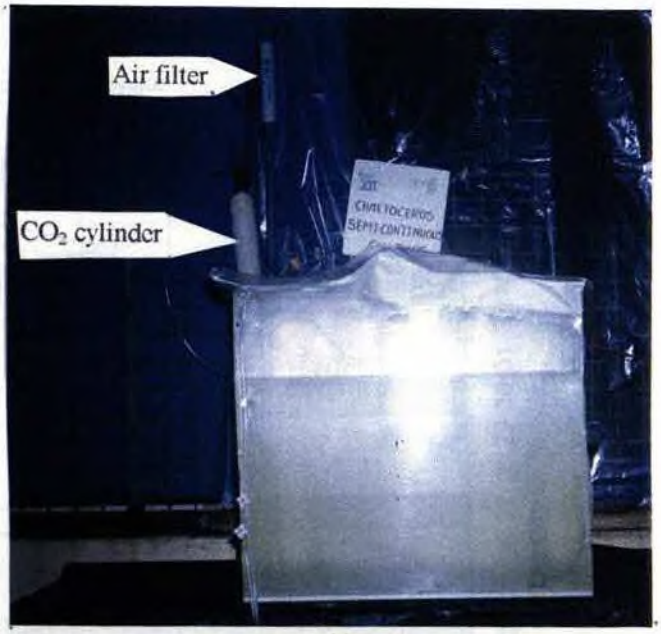


Plate 3. 60 litre capacity *Chaetoceros* sp. semicontinuous culture system with internal illumination. Note the high density of culture at the time of harvest.

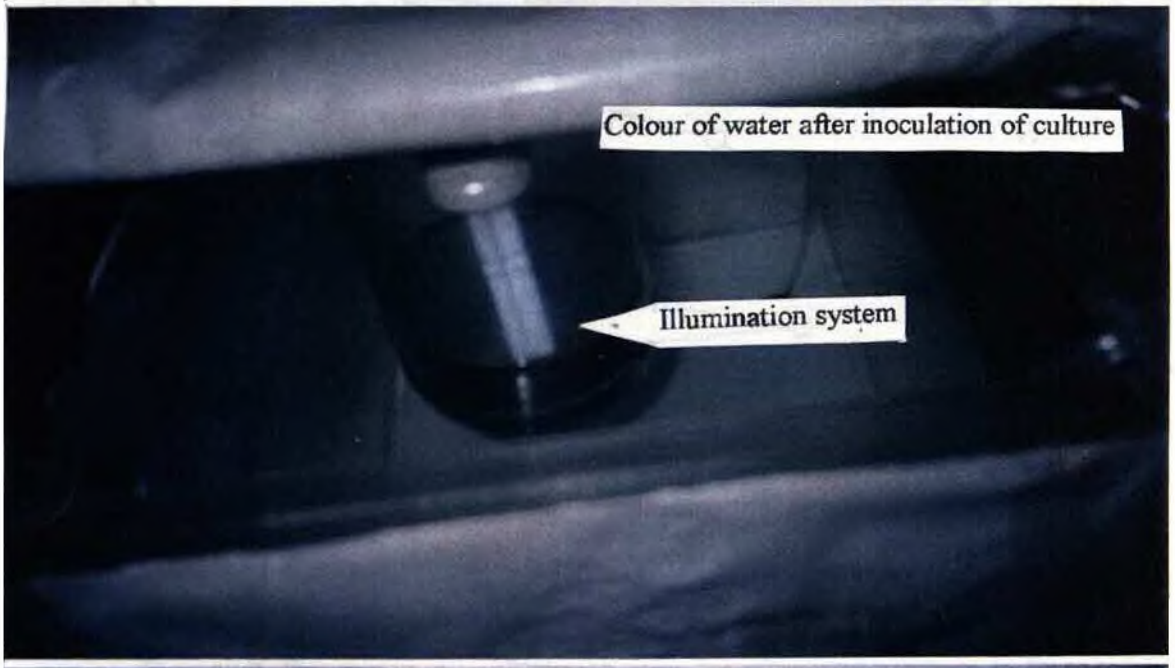
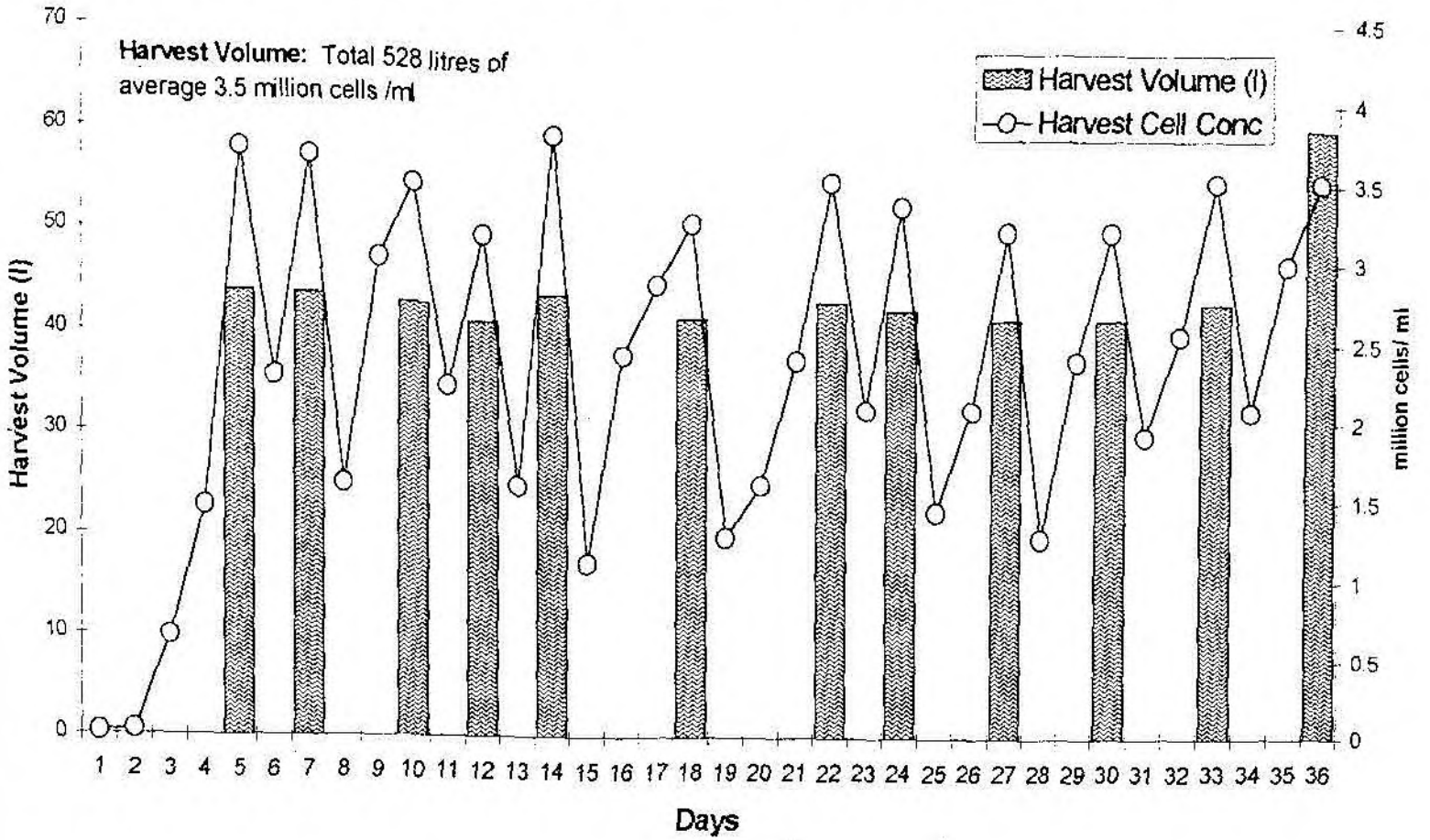


Fig.12 - Chaetoceros Semi-Continuous Culture 60 l Capacity - Outdoor



concentration of 1.62 million cells/ml. Again 4 days later, the culture attained a concentration of 6.88 million cells/ml when the 2nd harvest was done. Likewise, after a gap of 3-5 days, harvesting was carried out when culture attained a concentration above 3 million cells/ ml. From 25th day onward the culture started showing decrease in cell concentration and on 30th day complete harvesting was carried out. The pH of culture ranged from 6.22 to 8.12. The temperature of air was in range of 19.3 ^{O} and 27.2 ^{O}C and that of water was 20.9^{O} and 27.6 ^{O}C.

11. *Chaetoceros* semi-continuous culture with CO_2 EXP XI

The outdoor culture of *Chaetoceros* was started with an initial cell concentration of 0.02 million cells/ml which attained a cell concentration of 3.73 million cells/ml within 5 days, when 1st harvest was done. (Fig.12). The culture was made up to 60 litre with fresh dechlorinated seawater from reservoir tank and nutrients were added. This process was continued after every 3-4 days when the culture concentration reached to more than 3.0 million cells/ml. The culture was continued upto 36 days on 36th day complete harvesting was taken as the culture had entered the death phase. A total harvest of 528 litres with an average cell density of 3.2 million cells/ml was obtained from this experiment.

The pH of culture was in range of 5.77 and 7.86 the temperature of air was in range of 25.2^{O}C and 28.1^{O}C and that of water was in range of 25.7^{O}C and 31.9^{O}C.

12. Economics of high density microalgal culture

In order to ascertain the efficiency and advantages of high density culture a comparison of the economics of production of microalgal cells in different experimental treatments was comapred. The results are given in Table 7,8 and 9. For *Isochrysis* (Table 7) the cost of production per million cells was much lower than the control batch culture in all treatments except semicontinuous with CO_2. In the latter it was marginally more. Similarly for *Chaetoceros* also the cost of production in semicontinuous with CO_2 was marginally higher than control, while the continuous sytems with and without CO_2 were cheaper in production cost than the control batch cultures.

In the oudoor semicontinuous culture (Table 9), the cost of production per million cells was marginally cheaper as compared to the hypothetical control batch culture. However the advantages of higher production and increased volume of harvest over a sustained period of time are much superior.

Table 7. Details of production, input costs and cost of production per million cells of *Isochrysis galbana*. Assuming charges are same for facilities like container, treated seawater, aeration, illumination,etc.

Particulars	**Batch culture (Control)**	**Cont with CO2**	**Cont**	**Semi-cont with CO2**	**Semi-cont**
Duration (Days)	12 (2 batches)	9	12	13	29
Total volume harvested (litres)	8.0	5.0	5.0	10.9	26.0
Average cell density (million cells/ml)	2.15	7.416	5.6	3.2	3.9
Total cells harvested (Billion cells)	17.200	37.080	28.000	34.880	101.400
Cost (Rs.)of production					
1) Chemicals	0.3992	0.0998	0.499	1.08782	2.5948
2) Carbon dioxide	-	2.91	-	2.91	-
Total	**0.3992**	**3.145667**	**0.499**	**3.99782**	**2.5948**
Cost of production per million cells (Rs.)	**0.0000394**	**0.0000301**	**0.000017**	**0.0001146**	**0.0000255**

Table 8. Details of production, input costs and cost of production per million cells of *Chaetoceros*. Assuming charges are same for facilities like container, treated seawater, aeration, illumination,etc.

Particulars	**Batch culture (Control)**	**Cont.with CO2**	**Cont.**	**Semi-cont with CO2**	**Semi-cont.**
Duration (Days)	30 (5 batches)	27	21	30	26
Total volume harvested (litres)	20.0	11.0	8.0	26.2	17.5
Average cell density (million cells/ml)	1.5	13.7	8.5	4.2	4.0
Total cells harvested (Billion cells)	30.000	150.700	68.000	110.040	70.000
Cost (Rs.)of production 1) Chemicals 2) Carbon dioxide Total	 1.1824 - 1.1824	 1.6258 2.916 4.54246	 1.1824 - 1.1824	 3.87236 5.832 9.7056	 2.5865 - 2.5865
Cost (Rs.)of production per million cells	**0.0000394**	**0.0000301**	**0.000017**	**0.000082**	**0.00003695**

Table 9. Details of production, input costs and cost of production per million cells of *Chaetoceros* in outdoor culture. Assuming charges are same for facilities like container, treated seawater, aeration etc.

Particulars	**Semi-continuous culture Outdoor (60 litres)**	**Hypothetical batch culture Outdoor (60 litres)**
Duration (Days)	36	36 (6 batches)
Total volume harvested (litres)	530	360
Average cell density (million cells/ml	3.2	1.0
Total cells harvested (Billion cells)	1696	360
Cost of production (Rs.) 1) Chemicals 2) Carbon dioxide Total	 78.334 12.000 90.334	 21.78 - 21.78
Cost of production per million cells (Rs.)	**0.00005326**	**0.0000592**

DISCUSSION

Considering the advantages of continuous and semi-continuous culture systems over traditional batch culture system, the first attempt was made in Czechoslovakia during the early twentieth century, but it was not until the 1940 that practical continuous culturing was developed by the famous scientists J.Monod; B.H. Ketchum and A.C. Redfield.

Culture media used

Concentration of cells in phytoplankton culture are generally higher than those found in nature. Algal cultures must therefore be enriched with nutrients to make up for the deficiencies. The present study was carried out using nutrient salt solutions described by Laing (1991). For batch culture 1x ml/litre and for continuous and semi-continuous culture 2.5x ml/litre was used.

Results of the present study indicate that using 2.5 times the normal strength of media increased the culture cell density of both *Isochrysis* and *Chaetoceros* by many times. Besides the time taken by the culture to attain such high densities was also considerably reduced. Similarly Laing (1991) has described the culture system with the use of double strength nutrients with extremely high growth of microalgal culture.

Culture type

There are two main types of continuous culture being used all over world i.e. Turbidostat culture and Chemostat culture. Number of workers have reported on several designs for production of algae (Palmer *et al*, (1975); Trotta (1981), Gudin and Therpenier (1986), Lee (1986), also small scale vertical tubular reactors have been described by Behemann *et al* (1978) and Miyamoto *et al* (1988).

The present study is a sort of **Turbidostat culture** in which the number of algal cells in the culture was monitored. Depending on available facilities for culture the three tier gravitational flow system was preferred in which instead of automation, harvest of culture was

carried out using manual counting of cells and draining fixed amount of harvest into a harvest flask by use of gravitational flow through manually operated controllers.

The harvest was kept for one more day allowing for further multiplication of cells and ensuring total utilization of nutrients, after that it was used for feeding larvae.

Water temperature

For algal culture the ideal temperature range given by number of authors like Coutteau (1996), Gopinathan (1996) etc, is in between 16-27°C, the optimal being 18-24°C. The present experiments were carried out in the Airconditioned algal culture laboratory where temperature was maintained within range of 23-26°C. Only Exp XI was carried out in ourdoor conditions where the temperature of water ranged between 25.7 and 31.9 °C. *Chaetoceros* sp. can tolerate wide temperature range and it did not collapse (Exp. VII, VIII, IX, X and XI) where as in the case of *Isochrysis galbana* (Exp III, IV and VI.) a 8°C temperature difference between two successive days (because of airconditioning failure) resulted in collapse of the culture. Thus, maintaining an optimal temperature condition for each species is a most important factor.

pH

The ideal pH range for microalgal culture described by many authors lies between 7-9 from Table 1 we can see that pH range for control culture, continuous culture and semi-continuous culture without use of carbondioxide was within 7 to 9.5. However, the pH range was in between 6-8 for cultures using CO_2. This was achieved by use of CO_2 in the culture for a very short time through the aeration system.

Many authors (Liang, 1991; Gopinathan, 1996) have reported that lower pH of the medium will give extended period of culture as well as higher cell concentrations.

Light source

For indoor culture, light source used was flourescent tubelight. A single tubelight gives about 3000 lux light intensity when measured with help of a lux meter. The intensity of light available to the culture in the flask went on decreasing as culture concentration increases.

The provision of internal illuminations for outdoor tanks helped greatly in achieving higher cell densities in a short time period.

In outdoor culture where internal illumination was used, the algae starts adhering to the side of the tank and the sides of the illumination flask as the culture days prolong reducing the light penetration. It was found to be better to remove those adhering algae and clean the sides of the illumination flask for effective light penetration and to reduce the effect of self shading of culture.

Production trend

The control *Isochrysis* and *Chaetoceros* culture (Exp.I & II) took 11-12 days to reach a culture density of 3.35 million cells/ml whereas in the case of *Isochrysis galbana* culture using semicontinuous and continuous methods it took only 6 days to reach a cell concentration of 6.32 million cells/ml. Similarly *Chaetoceros* semicontinuous and continuous culture attained 3.32 million cells/ml cell concentration within 5 days.

Thus the advantage of using semicontinuous and continuous culture methods is clearly evident. Gopinathan (1987) reported that *Isochrysis galbana* reached its optimum growth during 9th to 12th day after which it showed declining phase.

In the present control culture of *Isochrysis galbana* showed maximum growth on 11th day and after which declining phase, again on 17th and 19th day it showed small elevation in cell count, there after it showed death phase. Whereas, in semi-continuous culture (ExpV) once reaching to 6.32 million cells/ml concentration within 6 days the harvesting was carried out and remaining culture allowed to grow in new nutrient enriched water in same culture flask. The culture period continued upto 29 days, and the death phase was observed only after 28 days

The *Chaetoceros* with CO_2 semicontinuous culture (Exp X) also showed prolonged culture period of about 30 days and *Chaetoceros* semicontinuous (Exp.IX) also lasted for about 26 days whereas *Chaetoceros* control remained only upto 18 days. This showed that the consistency of production and prolonged duration of algal supply can be achieved with help of semi-continuous and continuous culture system. Laing (1991) described the usual life of culture in continuous as well as semicontinuous culture system as significantly more than batch cultures.

He stated that continuous culture as only suitable for flagellates but the present study shows that with proper ciliate-free environment the diatoms can also be cultured successfully with continuous culture system.

Use of CO_2

The use of carbon dioxide in the form of gas in microalgae culture gives much faster growth and cell densities (Laing 1991).

However, Persoone and Sorgeloos (1975) stated that even without CO_2 enrichment, algal growth was very fast and not hampered by lack of CO_2 and in high density cultures photosynthesis seemed to be limited more by high optical density of culture than by insufficient supply of CO_2.

In the present experiment if we compare the production of *Chaetoceros* continuous without CO_2 and *Chaetoceros* continuous with CO_2 (Exp VII & VIII) we can see that the average cell density of culture is very much high (about twice) as compared to the cultures not using carbon dioxide . Also pH was maintained near to 7.5 on an average by the use of CO_2. This has also helped to increase the culture duration.

Economics of culture

The economics of microalgae (*Chaetoceros calcitrans*) production using multi-step methods in Philippines was given by Samonte *et al* (1998). According to their study production cost was P 715.50/ton (US$ 1.00 = 25 Philippines pesos). The present experiment was on a laboratory scale. Considering that charges are same for facilities like container, treated seawater, aeration, illumination etc., cost of production for batch culture is very less as

compared to continuous and semicontinuous culture, but due to high production of cells/ml the cost of production per million cells is lower especially in continuous sytems.

The average cell density in batch culture of *Chaetoceros* is only 1 to 1.5 million cells/ml, whereas in *Chaetoceros* continuous culture with carbon dioxide it is 13.7 million cells/ml and 8.5 million cells/ml in *Chaetoceros* continuous culture without CO_2 and *Chaetoceros* semicontinuous with and without carbondioxide. This itself shows that we can produce 9-10 times more cells/ml than batch culture.

A comparison of outdoor semicontinuous culture of *Chaetoceros* to a hypothetical *Chaetoceros* batch culture shows that though cost of production of cells with *Chaetoceros* semicontinuous outdoor is somewhat high, the average cell density obtained is more than double what is achieved through batch culture. Further, about 170 litre more harvest could be obtained from semicontinuous culture.

Moreover, in semicontinuous culture the labour charges and space requirements will be reduced as there is no need of arrangement of more culture containers. With one culture container it is possible to produce the required production over extended period of time. The labour cost will be further reduced as smaller volumes of high density cultures will be sufficient to meet the daily feed requirements.

SUMMARY AND CONCLUSIONS

- This study entitled "High density culture of marine microalgae using semi-continuous and continuous systems" was carried out at Fisheries Harbour Laboratory of CMFRI, Thoppumpady, Cochin during April-June, 1999.
- The objective of the present study was to devise a small scale low cost semicontinuous and continuous system for use in laboratory conditions.
- The study was carried out using two species *Isochrysis galbana*, a flagellate used as main food for the larvae of oysters in molluscan hatcheries, and *Chaetoceros* sp. a diatom used for feeding, developing stages of shrimp larvae.
- The study was carried out using following methods using above mentioned microalgal species.
    - Continuous culture system
    - Semi-continuous culture system

- The effect of carbondioxide on the production of microalgae in semi-continuous and continuous system was investigated.
- Semicontinuous culture of *Chaetoceros* sp. was scaled up from laboratory indoor condition to outdoor condition using 60 litre plexy tank and using internal illumination system.
- The economics of production of microalgae in batch, semi-continuous and continuous systems were compared.

The salient findings of the present study are:

- Semi-continuous and continuous culture are having advantages over batch culture like
    - High efficiency
    - Consistancy
    - Highest rate of production over extended periods and
    - Automation

- Use of 2.5 x ml/litre of nutrient media for semicontinuous and continuous culture with or without CO_2 gave excellent culture cell count than the cell count obtained using 1x ml/litre nutrient media in batch cultures.
- The present study was a sort of turbidostat culture without automation but instead of that a *three tier gravitational flow through system was preferred.*
- Results of the experiment revealed that maintaining optimal water temperature specific for each species is an important factor.
- Maintaining pH of the medium in between 6 and 8 will give higher cell conentration also extended period of culture.
- Use of internal illumination in outdoor cultures will enhance the production of *Chaetoceros* sp.
- Using semicontinuous and continuous culture system, the culture will take very less time to achieve highest cell concentration as compare to batch culture.
- Use of CO_2 in semicontinuous and continuous culture systems will help to extent the culture period and consistently higher production can be obtained
- Ciliate free aeration is most important for prolonging the culture period. Ciliate present in culture will make culture collapse within a short duration.
- By use of CO_2 it is possible to maintain pH near to 7.5 which is ideal pH for algal culture also it is *helpful for prolonged culture period.* The use of portable sodamaker CO_2 cylinder for microalgal cultures is a relatively easy and inexpensive method of increasing the culture cell concentration and maintenance of the culture for longer duration.
- Although the input costs for semicontinuous and continuous systems were found to be much higher than batch culture, the cost of production per million cells is less, if not equal.
- Use of semicontinuous and continuous systems will ensure supply of ciliate free good quality microalgal feed in hatcheries for larval rearing.

REFERENCES*

AARONSON, S., BERNER, T. AND DUBINSKY, Z., 1980. ***Microalgae as a source of chemicals and natural products.*** In: G.Shelef and C.J.Soeder (Editors), Algae Biomass, Production and Use. Elsevier, Amsterdam, pp. 575-601.

BARDACH, J.E., RYTHER, J.H. AND MCLARNEY, W.O., 1972. ***Aquaculture. The farming and husbandary of freshwater and marine organism.*** Wiley-Interscience, New York, 686 pp.

BATTER T.R., ANDERSON, D.B., HANSON, K.R., MCGEE, M.J. AND MOLTON, P.M., 1981. ***Microalgae as new and economical sources for industrial chemicals and drugs.*** Battele Pacific Northwest Laboratories, Richland, WA.

BENEMANN, J.R., PERSOFF, P. AND OSWALD, W.J. 1978. Cost analysis of microalgae biomass systems. ***CSO International Inc. concord, CA,*** pp. 1-99.

BOUSSIBA, S., SANDBANK, E., SHELEF, G.,COHEN, Z., VONSHAK, A., BEN-AMOTZ, A., ARAD, S. AND RICHMOND, A.,1988. Outdoor cultivation of the marine microalga *Isochrysis galbana* in open reactors. ***Aquaculture***, 72 : 247-253.

CHEN, J-F AND PAN, Y-Y,1987. Study on the growth of *Isochrysis galbana* Parke and it's nutrient component. ***Oceanologia et limnologia sinica*** 18(1) : 55-63.

COUTTEAU, P.,1991. Manual on production of live food for aquaculture. ***FAO Fisheries Technical Paper***,361 : 7-47.

DE PAUW, N.,MORALES, J., PERSOONE, G.,1984. Mass culture of microalgae in aquaculture systems:Progress and constraints. ***Hydrobiologia***,116/117:121-134.

FABERGAS , J., PATINO, M., MORALES, E.D., CORDERO, B. AND OTERO, A.,1996. Optimal renewal rate and nutrient concentration for the production of the marine microalga *Phaeodactylum tricornutum* in semi-continuous cultures. ***Applied and environmental microbiology***, 62(1) : 266-268.

FABERGAS, J., HERERRO, C., ABALDE, J., LIANO, R. AND CABEZAS, B.,1986. Biomass production and biochemical variability of the marine microalgae *Dunaliella tertiolecta* (Butcher) with high nutrient concentration. ***Aquaculture***, 53 : 187-199.

FABERGAS, J., HERRERO, C., ABALD,J. AND CABEZAS, B., 1986. The marine microalgae *Chlorella stigmatophora* as a potential source of single cell protein . Enhancement of protein content in response to nutrient enrichment . ***J.Int. Microbiol.***,1(4) : 251-257.

FABERGAS, J., HERRERO, J., CABEZAS, B. AND ABALDE, J.,1986.Biomass production and biochemical composition in mass culture of the marine microalga *Isochrysis galbana* Parke at varying nutrient concentrations. ***Aquaculture***,53:101-113.

FUKAMI, K., NISHIMURA, S., OGUSA, M., ASADA, M.AND NISHIJIMA, T.,1997. Continuous culture with deep seawater of benthic food diatom *Nitzschia sp.* ***Hydrobiologia***, 358(1-3) : 245-249.

GOPINATHAN, C.P., 1996. Life-food culture-microalgae . ***Bull. Cent. Mar. Fish.Res. Inst.***,1996,48 : 110-116.

GOPINATHAN, C.P.,1982. Methods of culturing phytoplankton : Manual of research mathods for fish and shellfish nutirtion. ***CMFRI Spl. Publ.***, 8 : 113-118.

GOPINATHAN, C.P.,1986. Differential growth rates of microalgae in various culture media. ***Indian J.Fish***,33(4) : 450-456.

GOPINATHAN, C.P.,1987. Growth characteristic of some nanoplankters. ***J. Mar.Biol. Ass. India***, 26(1&2) : 89-94.

GUDIN, C. AND THEPENEIER, C., 1986. Bioconversion of Solar energy into organic chemicals by microalgae. In: A.Mizrabi and A.L.Van Wezel (Editors), ***Advances in Biotechnological Processes***, Vol.6. Alan R.Liss, New York, NY, pp. 73-110.

HERRERO, C., CID, A., FABERGAS, J. AND ABALDE, J., 1991. Yields in biomass and chemical constituents of four commercially important marine microalgae with different culture media. ***Aquacultural Engineering***,10(2) : 99-110.

JAMES, C.M. AND AL-KHARS, A.M. ,1990. Intensive continuous culture system using tubular photo-bioreactor for producing microalgae. ***Aquaculture***,87(3-4) : 381-393.

JAMES, C.M. AND ABU-RAZEQ, T.S.,1989. An intensive chemostat culture system for the production of rotifers for aquaculture. ***Aquaculture***, 81 : 291-301.

JAMES, C.M. AND AL-KHARS, A.M.,1990. An intensive continuous culture system using tubular photo-bioreactor for producing microalgae. ***Aquaculture***, 87 : 381-393.

JAMES, C.M., AL-KHARS, A.M. AND CHORBANI, P., 1988. pH dependent growth of *Chlorella* in continuous culture system. ***J. World. Aquaculture. Soc.***, vol. 19(2) : 27-35.

KODAMA, M., IKEMOTO, H. AND MIYACHI, S.,1993. A new species of highly CO_2 tolerant fast growing marine microalgae suitable for high density culture. ***J. Mar. Biotech***.vol.1(1) : 21-25.

LAING, I. 1991. Cultivation of marine unicellular algae. ***Lab.Leafl.MAFF Direct.Fish. Res.,Lowestoft***, (67) : 31 p.

LAING, I. AND HEPPER, B.T., 1983. A simple method for the production of marine algae in polyethylene bags. ***Fisheries Notices*** (MAFF Directorate of Fisheries Research, Lowestoft), 73: 1-11.

LAING, I. AND JONES, E., 1983. Large scale turbidostat culture of marine microalgae. ***Aquacult. Eng.***, 2: 203-212.

LANDAU, M.,1991. ***Introduction to aquaculture.*** Published by John Wiley and Sons Inc. 431pp.

LAWS, E.A., POPP, B.N., BIDIGARE, R.R., KENNICUTT, M.C. AND MACKO, S.A., 1995. Dependence of phytoplankton carbon isotopic composition on growth rate and CO_2 (aq) : Theoretical considerations and experimental results. ***Geochemica et co-mochimica acta,*** 59(6) : 1131-1138.

LEE, Y.K., 1986. Enclosed bioreactors for the mass culti-vation of photosynthetic microorganisms. The future trend. ***Trends Biotechnol.***, 5: 186-189.

LITCHFIELD, J.H., 1979. ***Production of single cell protein for use in food or feed.*** In: M.J. Peppler and D.Periman (editors), Microbial Technology. Academic Press, New York, NY. pp. 93-155.

MATSUNAGA, T.,1991. Production of useful chemicals from CO_2 by marine microalgae using a biosolar reactor employing light diffusing optical fibres. ***Second International Marine Biotechnology Conference. (IMBC'91)*** : Abstract only.

MIQUEL, P., 1892. De la culture artificielle des diatomees. ***C.R.Acad. Sci. Paris,***94 : 1-780.
MIYACHI , S. ,1991. Studies towards decreasing atmospheric CO_2 with marine microalgae.***2. Int. Marine Biotechnology Conf. (IMBC'91)*** : Abstract only.

MIYAMOTO, K. WABLE, O. AND BENEMANN, J.R., 1988. Vertical tubular reactor for microalgae cultivation, ***Biotechnol. Lett.,*** 10: 703-708.

OHGAI, M., MATSUI, T., UNO, K., TSUJINAKA, K. AND IDANAKA, H.,1993. The effects of carboxylic acids and their carboxylates on the growth of four species of marine microalgae. ***Nippon Suisan Gakkaishi/Bull.Jap. Soc. Sci. Fish.*** 59(4) : 647-652.

✓OSWOLD, W.J., 1980. ***Algal production problems, achievements and potential.*** In: G. Shelef and C.J. Soeder (Editors), Algal Biomass, Production and Use. Elsevier, Amsterdam. *pp.1-8.*

OTERO, A. AND FABERGAS, J., 1997. Changes in the nutrient composition of *tetraselmis suecica* cultured semicontinuously with different nutrient concentrations and renewal rates. ***Aquaculture,***129 :111-123.

PALMER, F.E., BALLARD, K.A. AND TAUB, F.B.,1975. A continuous culture apparatus for the mass production of algae. ***Aquaculture,*** 6 : 319-331.

PERSOONE, G. AND SORGELOS, P.,1975.Technological improvements for the cultivation of invertebrates as food for fishes and crustaceans.I : Devices and methods. ***Aquaculture,***6 : 275-289.

PRIT, J.S., LEE, Y.K., WALACH, M.R., WATTS.PRIT, M., BALYUZI, H.H. AND BAZIN, M.J., 1983. A tubular bioreactor for photosynthetic production of biomass from carbondioxide. Design and performance. ***J.Chem. Tech. Biotechnol.***,33: 35-58

REYNOSO GRANADOS, T., MAEDA MARTINEZ, A., ROMAN ECHAVARRI, C. AND MONSALVO SPENCER, P., 1990. Continuous culture of phytoplanklton by means of a turbidostat. ***Investigaciones marinas CICIMAR*** ,(5,1) : 12-18.

SAMONTE, G. PB., ESPEGADERA, C.C. AND CATURAO, R.D.,1983. Economics of microalgae (***Chaetoceros calcitrans***) production using the multistep method in the Philippines. ***Aquaculture***,112:39-45.

SOEDER, C.J., 1980. ***The scope of microalgae for Food and Feed***. In: G.Shelef and C.J. Soeder (Editors). Algal Biomass, Production and Use. Elsevier, Amsterdam. pp 9-20.

SPECTOROVA, L., CRESSWELL, R.L. AND VAUGHAN, D.,1997. Closed tubular cultivators : An innovative system for commercial culture of microalgae. ***World Aquaculture***, 28(2) : 39-43.

SPECTOROVA, L.V., GERADZE, K.N., PAN'KOVA, S.A. AND PAN'KOVA, S.L.,1991. Mass cultivation of marine microalgae as food for larvae of oyster and marine fish. ***Sp. Pub. European Aquaculture Society***, 15 : 91-94.

SPECTOROVA, L.V., GORONKOVA, O.I., NOSOVA, L.P. AND ALBITSKAYA, O.N.,1982. High density culture of marine microalgae - Promising items for mariculture. I : Mineral feeding regime and installations for cultureing *Dunaliella tertiolecta* Butch. ***Aquaculture***, 26 : 289-302.

SPECTOROVA, L.V., NOSOVA, L.P. , GORONKOVA, O.I., ALBITSKAYA, O.N. AND FILIPPOVSKIJ, YU.N.,1986. High density culture of marine microalgae-Promising items for mariculture .II : Determination of optical light regime for *Chlorella sp. f. marina* under high density culture conditions. ***Aquaculture***, 55 : 221-229.

SPECTROVA, L.V., GORONKOVA, O.I., NOSOVA, L.P., ALBITSKAYA, O.N. AND DANILOVA, G.YU.,1986. High density culture of microalgae-promising items for mariculture,III. Mass culture of *Monochrysis lutheri* Droop. ***Aquaculture***,55 :231-240.

SUMINTO AND HIRAYAMA, K., 1997. Application of growth promoting bacteria for stable mass culture of three marine microalgae. ***Hydrobiologia***, 358 : 223-230.

TROTTA, P.,1981. A simple and inexpensive system for continuous monoxenic mass culture of marine microalgae. ***Aquaculture***,22 : 383-387.

VEEN, A.,1996. A continuous system for the study of UV-B effect on microalgae. ***Scientia Marina***, 60(suppl.no.1) : 89-93.

WALNE, P.R.,1974. Culture of bivalve mollusc. ***Fishing News(Books) Ltd. Surrey***, pp1-173.

WALSH, D.T., WITHSTANDLEY, C.A., KRAUS, R.A. AND PETROVITS, E.J.,1987. Mass culture of selected marine microalgae for the nursary production of bivalve seed. ***Journal of Shellfish Research*** ,6(2) : 71-77.

WILHELM, S.W. AND TRICK, C.G.,1995. Effects of vitamin B-12 on chemostat cultured *Synechococcus* sp strain PCC 7002. ***Canadian J. Microbiol***. 41(2): 145-151.

* Not all referred in original