"EVALUATION OF *Nannochloropsis* EXTRACT AS A FOETAL BOVINE SERUM ALTERNATIVE FOR ANIMAL CELL CULTURE"

Project Work By

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CERTIFICATE

This is to certify that the project entitled "**EVALUATION OF *Nannochloropsis* EXTRACT AS A FOETAL BOVINE SERUM ALTERNATIVE FOR ANIMAL CELL CULTURE**" submitted by Ms. **NEHA ANN JOE**, Reg no-**AB21ZOO011** in partial fulfillment of the requirement of Bachelor of Science Degree of Mahatma Gandhi University, Kottayam, is a bonafide work under my guidance and supervision and to the best of my knowledge, this is her original effort.

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CERTIFICATE

This is to certify that the project entitled **"Evaluation Of *Nannochloropsis* Extract As A Foetal Bovine Serum Alternative For Animal Cell Culture"** is an authentic record of work carried out by **Ms. Neha Ann Joe (Register number:AB21ZOO011) at Primordia Lifesciences Pvt.Ltd, Kochi** under my supervision and guidance in the partial fulfillment of the requirement for the degree of Bachelor's in Zoology, St.Teresa's College (Autonomous) ,Ernakulam. Affiliated to Mahatma Gandhi University,Kottayam.The work presented in this dissertation has not been submitted for any degree or diploma earlier.

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DECLARATION

I hereby declare that project work titled "**EVALUATION OF *Nannochloropsis* EXTRACT AS A FOETAL BOVINE SERUM ALTERNATIVE FOR ANIMAL CELL CULTURE**" submitted to St. Teresa's College (Autonomous), Ernakulam affiliated to Mahatma Gandhi University, Kottayam in the partial fulfillment of the requirements of Bachelor of Science degree in Zoology, is a record of original project work done by me under the guidance and supervision of Dr. Soja Louis, HOD, Department of Zoology, St. Teresa's College (Autonomous), Ernakulam.

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ABSTRACT

Animal cell culture is an essential tool used in research, biomedical, and pharmaceutical industries for various applications. One of the practices in cell culture is culturing of cells in a medium with serum (Foetal bovine serum), which is an animal product. Although it is used widely, it has several drawbacks, especially for pharmaceutical applications. This study aims to find an alternative for FBS that can reduce or replace it. The microalgae *Nannochloropsis* was selected due to its high protein content. Three cocktails (CC1,CC2,CC3) containing the algal extract (AE) were formulated by Primordia Lifesciences Kochi, Kerala, and evaluated for its cell proliferative and attachment properties. CC2 and CC3 showed promising results with CC3 showing comparable results to that of FBS (Foetal Bovine Serum). The results of this study show that the formulated cocktails with algal extract is a promising alternative to reduce or replace the use of FBS in cell culture.

1. INTRODUCTION

Animal/ mammalian cell culture is an essential technique in the research and development of recombinant protein production and for other pharmaceutical and biomedical applications. Animal cell culture is the culturing of animal cells in vitro under specialized conditions. Cells can be grown from an original tissue, cell line, cell strain, or from a primary culture. This process requires a sterile environment as animal cells are highly prone to contamination and subsequent death. Cell culture is broadly divided into primary and secondary cell culture. In primary culture, the cells are obtained directly from the host tissue or organ via chemical or enzymatic disintegration (1). The cells are then cultured in vessels containing the appropriate growth media and incubated to promote their growth. Once the cells reach confluency, they are sub-cultured to new vessels with fresh media. Subculturing provides more area for cell growth, maintains cell density, and thus prevents cell death. Secondary cell culture refers to the cells that have been sub-cultured from the primary culture. They are easier to maintain than primary cultures due to their longer life span. It also creates genetic modifications making them more resistant to contamination. Depending on the type, cells can be grown as monolayer cultures or suspension cultures. In monolayer cultures, the cells are anchorage-dependent and require a substrate for their growth and propagation. However, in suspension cultures, they are non-adhesive and remain suspended in the growth media. The growth cycle of the cells includes three phases; lag phase, log phase, and plateau phase. The cell count is taken during the log and plateau phase to characterize cell growth and determine the optimal conditions required for cell culture. Quantification of cells is performed using a hemocytometer or via electronic counting. Furthermore, cell viability is tested to study the health of the cells. These techniques allow us to understand the response of the cells to different chemicals, drugs, or other external factors (5).

Cell culture is fundamental for developing vaccines, testing drugs, and studying various molecular pathways in cells. It can also be used to study the physiology and biochemistry of cells. Cancer research is another field that relies on cell culture to study the differences between normal and cancerous cells, the mechanisms of cancerous cells, and to test anticancer drugs. Cell culture is used for toxicity testing to study the toxicity of various products like drugs and cosmetics, in virology it is used to study the growth and cycles of viruses. Studying such mechanisms aids in development of vaccines. Another application of

cell culture is tissue/organ replacement where organ culture techniques are applied on adult embryonic cells and stem cells to produce organs like kidney, liver, skin etc (5). According to the National Library of Medicine, cell culture has played a major role in studying the function, formation, and pathology of tissues and organs. In the agricultural field, animal cell culture has been used to develop biopesticides. Apart from its uses in research, cell culture has various other advantages. The in vitro culture allows to culture the cells in a controlled physiochemical environment where factors such as pH, osmolarity, temperature, nutrients, hormones, etc can be regulated. From this we can determine the optimal conditions required for cell growth (7). Clonal cells provide homogeneity, which increases the reliability of the study. Studies using these cells have shown consistency and reproducibility in its results. Microenvironments, cell-cell interactions, and regulation of the matrix can also be controlled. The cells when not in use, can be stored for long periods via cryopreservation. The cells can be quantified and characterized to study its growth and viability. In vitro cell culture uses reduced quantities of chemicals and reagents as opposed to in vivo culture. A major advantage of cell culture is the minimized usage of animals for various experiments.

The culturing of cells requires a growth media that consists of nutrients, and other growth factors necessary to propagate cells. Generally, growth media consists of an energy source like carbohydrates, amino acids, growth hormones, fats and fat-soluble compounds, oxygen, carbon dioxide, inorganic salts, vitamins, etc (7). Scientists have developed various types of media for cell culture and they are broadly classified into natural and artificial media. Natural media includes biological fluids, tissue extracts, and clots. Artificial media is divided into four classes; Serum-containing media, Serum-free media, chemically defined media, and protein-free media. Serum-free media has several advantages such as the reduced risk of contamination, less variability, defined chemical composition, etc however, currently, serum-containing media is the preferred media as serum provides growth factors, adhesion factors, hormones, lipids, proteins, etc. It also increases the buffering capacity of the culture and protects the cells from damage. Some commonly used basal media are Dulbecco's Modified Eagle Medium, Minimum Essential Medium, Iscove’s Modified Dulbecco’s Medium, etc.

Different types of serums have been extracted from different animals but the most commonly used serum is FBS (Foetal bovine serum). FBS has low levels of antibodies and promotes cell growth rapidly. It is a rich source of proteins and provides hormones, adhesion factors,

transport proteins, and other factors required for cell growth. However, FBS has a few limitations. It is expensive, it is not chemically defined which can generate varied results in each batch. It has also been associated with various scientific and ethical issues. Due to these drawbacks, many researches have been conducted to find alternatives for this serum. Since FBS is rich in proteins researches are aimed at finding a protein-rich supplement to replace it. However, along with proteins, cell culture requires lipids, hormones, adhesion factors, vitamins, and other growth factors. Microalgae is an organism with high protein content. It is also peptides, carbohydrates, vitamins, and lipids. Further studies using microalgae can be conducted to develop a supplement to substitute FBS.

DNA barcoding is a tool used in molecular taxonomy for the identification and classification of species. This technique uses standardized DNA sequences as tags for accurate identification of a species. The standard protocol for plant identification uses PCR to amplify different regions of DNA such as *rbcL*, matK, of chloroplast DNA, or CO1 region of mitochondrial DNA. For the molecular taxonomy of microalgae, 18S rRNA genome or highly variable regions such as ITS and *rbcL* are recommended (2). DNA barcoding can also be used to identify phylogenetic relationships between different species and group unknown species to previously identified or new species.

The present work focuses on finding the potential of the microalgae *Nannochloropsis* as an alternative for FBS in growth media for animal cell culture.

2. REVIEW OF LITERATURE

Animal cell culture has become an essential tool in the field of research and development. Cell culture facilitates in vitro production of cells that are homogenous in their genetic constitution which is an ideal characteristic in research. Cells can also be modified through subculturing techniques to obtain higher performance. This technique has been used to develop drugs and vaccines, test the quality of products, study the toxicity of drugs, study the pathology of diseases, produce proteins, etc. The advancement in technology has also helped to promote the use of cell culture to higher levels of application. Nowadays, 3D cell culture models are used for research as the 3D model simulates the structure of tissues and organs more accurately than the 2D models. The 3D models are often used in cancer research and tissue engineering (3). The study of oxidative stress and study of ageing using dermal fibroblasts are also conducted using the same model (4). The cells are cultured using a growth media which has supplements necessary for their growth and division. So far in cell culture, the most prominent supplement is serum, which is a fluid extracted from the blood plasma. Foetal bovine serum is the most commonly used serum in animal cell culture. It is produced and supplied in many areas across the world as it has a great capacity to support cell growth. It consists of hormones, growth factors, vitamins, nutrients, proteins, adhesion factors, cell proliferation factors, transport proteins, protease inhibitors etc (16). It also has low levels of immunoglobulins (17). However, recent research has been directed at finding a suitable alternative for FBS due to the scientific and ethical issues affiliated with it. Extensive studies have been conducted on FBS but it is still not fully characterized (18). The unknown components in the serum may have cytotoxic effects on the culture (19). Moreover, without the complete characterization of the serum, cell culture studies using FBS may provide inconsistent results and affect the credibility of the study. These components in FBS can result in variation per batch due to which quality control is important for every batch of serum (20). FBS is prone to contaminants such as viruses, bacteria, prions, mycoplasma, etc, which will hinder cell growth and promote senescence (19). Bovine spongiform encephalopathy is a prevalent disease among cattle whose causative agent, prions, affects cattle and cattle-derived products (21). It is nearly impossible to treat the contaminated serum and free them from pathogens for cell culture. A study conducted by Guokai Chen et al (20) reported immunogenic response in cell cultures using FBS, which in rare cases have affected patients

undergoing therapeutic treatments. Another issue is the supply and availability of FBS. While FBS is used by many countries, its production is confined to a few which causes a shortfall in its supply (18). Moreover, certain countries that handle FBS production are restricted from exporting them due to the high rate of disease among their cattle. Serum extracted from such cattle will be contaminated with the disease-causing agent which will harm the culture (22). Recently, there have been markets that supply foetal bovine serum that is contaminated with adult bovine serum or newborn calves' serum. This has raised questions about the reliability of the FBS market. A major drawback of FBS is the ethical issues involved during the collection of the serum. FBS is extracted from the foetus of calves by cardiac puncture killing the foetus. Furthermore, during serum collection, the foetus is alive and the procedure will hurt the animal (18). According to Gstraunthaler et al (18) 1,000,000 foetuses are killed every year and as research fields expand, the demand for FBS will increase and more cattle will have to be killed. These procedures also don't follow the Three R concept enforced by the government to ensure that experiments are conducted by taking bioethical measures (18).

To overcome these issues, researchers pursued the development of Serum-free media. Serum-free media are of different types; chemically defined media, Xeno free media, Protein free media, and animal free media (23,24). To meet the requirements for cell culture, the factors necessary for cell growth were studied in vivo and replicated in vitro (25). Serum-free media can be chemically defined, can reduce variability, and reduce the risk of contamination. This media generally consists of basal media such as Alpha-Minimum Essential Medium, Dulbecco's Modified Eagle's Medium/F12 (1:1), Iscove's Modified Dulbecco's Medium, etc with added supplements like hormonal factors, proliferation and attachment factors, antioxidants, lipids, carrier proteins, etc (26). It also allows us to adjust the concentration of the supplements and in turn the property of the media for different stages of cell growth (27). Moreover, some differentiated cells cannot be maintained in a serum-containing media. Such cells require serum-free media for growth and proliferation (28). Serum-free media is also specific to cell type, so desired cells can be selected and cultured by using the supplements optimal for that cell line (28). Cell culture in serum-free media also facilitates large-scale production of specific cell lines without much variability and contamination (27). Several studies have been conducted to develop, test and optimize serum-free media. In one study conducted for muscle cell growth, Ham's F-12 medium containing 10 -s M fetuin, 10-6M

insulin, and 10-TM dexamethasone showed growth comparable to 10% serum (29). In study conducted by Kim and Lee in 2009 (30), the media was supplemented with hydrolysates and the growth of C11-08 cells and H-1-5 cells was analysed. In C11-08 cells supplementation with wheat gluten and soy hydrolysates increased cell growth and the viability was maximum at 50% soy hydrolysate and 50% wheat gluten hydrolysate. The H-1-5 cells showed results similar to C11-08 cells where soy hydrolysates improved cell growth, and viability was highest with 50% soy hydrolysate and 50% wheat gluten hydrolysate. The findings of the study showed that SFM can support the growth of different rCHO cell lines with the right supplementations. Al Kolman et al (31) found that chemically defined Serum-free media such as FBM, Essential8™ and TESR-E8™ supported the growth of bovine myoblast cells with an increase in the number of cells over time. According to their findings, FBM, FBM/DMEM (50:50 ratio), and Essential8™ have a great capacity for cell proliferation and need to be optimized to meet the efficiency of serum-containing medium. Most cell lines require additional supplements for their growth. B104 rat neuroblastoma cells required selenium and putrescine and Linoleic acid was required for the growth of SV40 virus transformed 3T3 cells. Moreover, attachment factors such as human plasma fibronectin and growth factors such as insulin, PDGF, hormones such as oestrogen, hydrocortisone, etc, fibroblast growth factors, platelet-derived growth factors etc can also be added to the basal media to improve cell growth (26). Although most supplements have different effects on different cell lines, insulin showed growth-inducing effects on several cell lines (28). Devireddy et al (32) developed a serum-free media and optimized it with growth factors for the growth of canine adipose-derived MSCs. The study found that the growth of these cells in the serum-free media was comparable to their growth in FBS. Arthur Luhur et al (33) conducted a study to adapt Drosophila melanogaster cell lines to serum-free conditions. The study indicated that drosophila cell lines can be grown under serum-free conditions by gradually reducing serum concentration adding and increasing the concentration of fly extract in the media. The reports of Tucker Burden et al indicated that serum-free, xeno-free and albumin-free media can maintain several cell lines. In another study, Usta et al (35), found that Neuro pure; a xeno-free chemically defined media, was able to maintain cancer cell lines and support cell growth in cancer cell lines as well as fibroblasts. There are several disadvantages to serum-free media. Except for chemically defined media, serum-free media is not always chemically defined (36). To compensate for the constituents that occur naturally in serum, many factors have to be

added separately in serum-free media which will increase the expense. Serum-free media that is chemically defined is ideal for reducing batch-to-batch variability, however, it is expensive to develop and time-consuming and therefore is not always a viable option (16). Kim and lee (30) reported that chemically defined media showed less growth and protein production than serum-containing media. Moreover, the effects of several factors present in serum are difficult to replace and replicate (16). Although some serum-free media have been formulated to provide better results than serum-containing media, and some cell lines have shown more growth in serum-free media, more studies and experiments need to be conducted for serum-free media (37). Its specific nature restricts growth to certain lines so the expense for maintaining a variety of cell lines will be high. Due to these reasons, serum-containing media is still preferred over serum-free media in the cell culture market.

The search for FBS alternatives is an ongoing process in the field of research. Several alternatives such as pituitary extracts, chick embryo extracts, earthworm coelomic fluid etc has been tested (17). Human AB serum and human platelet lysates promotes the growth of human cells and have the potential to be good alternatives for FBS for the growth of human cells (26). Karin Witzeneder et al (38) grew fibroblasts and adipose tissue-derived stem cells in human serum and human platelet lysate and found that both were good alternatives for FBS. In a study conducted by Reen Wu and Gordon H sato (39), the hormones Inc, T_3, and HC were used as serum replacement and they concluded that cell lines can be grown by replacing serum with hormones and the appropriate growth factors. Maryam Samareh Salavati Pour et al (40) showed that cord blood serum has the potential to grow umbilical cord-derived mesenchymal stem cells and replace FBS.

Microalgae is another supplement that has been studied as an alternative to serum (41). It is rich in proteins and many other nutrients such as peptides, carbohydrates, lipids and vitamins. The proteins extracted from microalgae is reported to be higher in quality than plant proteins (42). Its chemical characterization also found other constituents such as carbohydrates, lipids, astaxanthin, zeaxanthin, polyunsaturated fatty acids and other bioactive compounds (43), (44). Okamoto et al (45) reported that algal extracts had a high concentration of glucose and amino acid. About 18-20 proteogenic amino acids were extracted from C. vulgaris. Overall, algal extracts were recorded to have the same concentration or higher concentration of amino acids than normal mammalian cell culture medium. Okamoto et al (45) also found that

cell viability was higher in the medium containing algal extracts than in the medium without it . In another study conducted by Haraguchi et al (46), C2C12 muscle cells was cultured with Chlorococcum littorale extract. They found that C.littorale had a glucose concentration higher than DMEM. Moreover, its extract consisted of five proteogenic amino acids that were absent in DMEM, and the overall concentration of proteogenic amino acids in the algal extract was two times that of DMEM. The addition of algal extract to the cells improved their growth (46). A study conducted by Ashish G. Waghmare et al (47) characterized the microalgae chlorella pyrenoidosa and found high-quality protein content which on optimization can be used for large-scale production of proteins. Jian Yao Ng et al (41) found that CGF, an aqueous extract of this microalga promoted proliferation at low concentrations in certain mammalian cell lines, however at higher concentrations it showed cytotoxicity. Another microalga that is rich in proteins is *Nannochloropsis*. It also has a high content of polysaturated fatty acids, vitamins, antioxidants, other fatty acids such as palmitic acid, oleic acid and EPA, fibres, nitrates, moisture, ash, minerals and pigments (48). It has also been reported by Doan et al that *Nannochloropsis* has 37 to 60% higher lipid content than most microalgal strains (49). Moreover, proteins account for 30 to 45% of the biomass of this genus (50). Therefore, *Nannochloropsis* is rich in proteins, lipids, minerals and many other factors which deems it a good candidate as a serum alternative. Commercially, different microalgae are being produced at large scales for different applications. It has been used as a nutrient supplement, for aquaculture, animal feed, cosmetics, etc (43). However, not many studies on the effect of microalgae in cell culture have been conducted. Further research and optimization of microalgae in cell culture can facilitate the reduction of serum usage in the cell culture market and eliminate risks of contamination and variability in large-scale productions.

The molecular taxonomy of an organism is carried out with DNA barcoding. It uses specific DNA sequences of a species to identify, study and classify it by comparing it to previously identified species or by assigning them to new species. Microalgal identifications of previous studies have used the 18S rDNA as a marker ((6),(11),(12)). Other studies have also reported the use of *rbcL*, ITS1, and, ITS2 ((2),(8),(9)), where ITS1 and ITS2 have highly variable regions suitable for species-level identification (9). For identification of diatoms *rbcL* and COI has been recommended due to their highly divergent nature (10). Previous studies concerned with the

molecular identification of *Nannochloropsis* has commonly used 18S rDNA and *rbcL* as markers ((13),(14), (15)).

3. SCOPE AND OBJECTIVES

3.1 SCOPE

Foetal bovine serum (FBS) is a media supplement used in animal cell culture media. It is crucial for the growth and proliferation of cells and it is used worldwide by researchers for cell culture studies and applications. Although it is used widely, FBS has several disadvantages. It increases the risk of contamination and causes batch-to-batch variability and inconsistency. Moreover, the extraction of this serum results in the death of bovine foetuses which has raised issues concerning animal welfare. Due to these drawbacks, there has been an ongoing search for a serum alternative for animal cell culture. Development of a media supplement which is free of animal components and ensures cell growth and proliferation will be a suitable alternative reduce/replace the use of FBS in animal cell culture. Microalgae is free of animal components but rich in proteins. It is renewable and reduces the risk of contamination and inconsistency and thus could be a promising alternative to serum.

3.2 AIM

To evaluate the extract of the microalgae, *Nannochloropsis* as a media supplement to reduce or replace foetal bovine serum in animal cell culture

3.3 Objectives

1. Cultivation and molecular characterization of selected microalgae
2. Aqueous extraction of microalgae for the preparation of FBS alternative
3. Evaluation of the serum replacement potential of the microalgal extract.

4.METHODOLOGY

4.1 MATERIALS

Ammonium formate, FBS, Trypsin, Guillard's F/2 medium (Hi-Media), DMEM/F-12 media (Gibco, India), Fibronectin (Hi-Media), Trypan blue dye. The reagents used in this study are of analytical grade.

4.2 COLLECTION AND CULTIVATION OF MICROALGAE

The marine microalgae *Nannochloropsis* was selected for the present study. *Nannochloropsis* was collected from Central Marine Fisheries Research Institute (CMFRI), Kochi, Kerala. The algal culture was maintained in autoclaved artificial seawater composed of the salts with the corresponding weight provided in Table 1 with Guillard F/2 medium (Guillard, 1975). The components were thoroughly mixed using a magnetic stirrer. The conical flask was capped with a cotton plug, shaken thoroughly, and kept under lighted conditions at room temperature for two weeks.

SALTS	WEIGHT FOR 1 LITRE OF SEAWATER SOLUTION
NaCl	21.09 g
KCl	0.599 g
NaF	2.8 mg
NaBr	0.08 g
H_3B0_3	0.23 g
$NaHCO_3$	0.174 g
$Na_2 SO_4$	3.55 g
$MgCl_2. 6H_2O$	9.5g
$CaCl_2. GH_2O$	1.344 g
$SrCl_2. 6H_2O$	0.0218 g

Table 1. Salts for seawater preparation

Nannochloropsis was harvested after fourteen days by centrifugation. The solution was centrifuged at 10,000 rpm for 5 minutes. The biomass obtained was further washed with 0.5M ammonium formate thrice, and washed with distilled water twice to remove the salt and the pellet was collected. The biomass collected was collectedwas used for molecular identification and cell culture studies.

4.3 MOLECULAR IDENTIFICATION OF MICROALGAE

4.3.1 EXTRACTION OF DNA

DNA was extracted from *Nannochloropsis* using Nucleosieve® Total DNA extraction kit (Primordia Lifesciences, Kochi). The steps involved in DNA extraction are described below:

- 0.1g of the harvested microalgae was frozen with liquid nitrogen and ground to a fine powder with a mortar and pestle using liquid nitrogen.
- 900 µl PDE I buffer was added to the powder, mixed, and transferred to a fresh microcentrifuge tube.
- 3-5 glass beads were added to the suspension and mixed by vortexing for 5 minutes
- 200 µl PDE II buffer and proteinase K was added and the formed suspension was incubated in a water bath for 10-15 minutes at 60°C with intermediate vortex.
- To this 10 µl Rnase was added and incubated at 37°C for 10 minutes, followed by centrifugation at 4000 rpm for 10 minutes.
- The supernatant formed was transferred to a fresh microcentrifuge to which 500 µl PBB and 360 µl isopropanol was mixed by inverting.
- The sample was transferred to a spin column and centrifuged at 10,000 rpm for 1 minute.
- The filtrate was discarded and 500 µl wash buffer was added and centrifuged at 10,000 rpm for 1 minute. This step was repeated.
- The column was centrifuged for 10,000 rpm for 1 minute and transferred to a new microcentrifuge tube.
- 30-50 µl EBA buffer was added, the column was incubated at room temperature for 1 minute and centrifuged at 10,000 rpm for 1 minute.
- The elute was collected.

4.3.2 AGAROSE GEL ELECTROPHORESIS

- Agarose gel of 1.2 % concentration was prepared by dissolving 0.24 g of Agarose in 20 ml 1X TAE buffer.
- 2 µl Ethidium bromide was added and the solution was poured to a gel boat, the comb was inserted and solution was allowed to set.
- Once set, the comb was removed, a mixture of 1 µl microalgal DNA and 2 µl bromophenol blue dye was loaded in one well and ladder was loaded to an adjacent well.
- The gel was run at 100 V for 20 minutes.

4.3.3 POLYMERASE CHAIN REACTION (PCR)

In a PCR tube, forward primer, reverse primer, DNA sample, distilled water and Taq mix was added according to the volumes provided in Table 2. The tube was spun to mix the contents and placed in the PCR machine. The reaction was initiated and the cycles were repeated 35 times under the conditions provided in Table 3.

REAGENTS	VOLUME
NC rbcL F	1 µl
NC rbcL R	1 µl
DNA	1 µl
DH_2O	11.55 µl
Max Taq mix	15 µl
100 mm $MgSO_4$	0.45 µl

Table 2. Components of PCR reaction mixture

STEPS	TEMPERATURE	TIME
Denaturation	94 °C	2 minutes
Denaturation	94 °C	30 seconds
Annealing	53°C	30 seconds
Extension	72°C	1 minute
Final extension	72°C	10 minutes

Table 3. PCR conditions for *rbcL* amplification

Agarose gel was prepared as mentioned above and the PCR product was loaded and run at 100 volts for 20 minutes.

4.3.4 PURIFICATION OF PCR PRODUCT FROM AGAROSE GEL

The amplified DNA was purified from agarose gel for sequencing. The following are the DNA purification steps.

1. Excise the DNA fragment from the agarose gel using a scalpel or other device and transfer it into a 1.5/2ml microcentrifuge tube.

2. Add 2 volumes of Solubilizing Buffer to each volume of agarose excised from the gel (e.g., for 100 mg of agarose gel slice add 200 µl of solubilizing buffer).

3. Incubate at 55-60°C for 10-15 minutes until the gel slice is completely dissolved. Mix by vortexing the tube intermittently during the incubation.

4. Transfer the melted agarose solution to a Spin Column in the collection tube.

5. Centrifuge at 10000 rpm for 1min. Discard the flow-through.

6. Add 500 µl of Wash Buffer (WB2) to the column and centrifuge at 10000 rpm for 1 min. Discard the Flowthrough and place the column back to the same collection tube.

7. Repeat step 6.

8. Centrifuge the empty spin column for an additional 1 min at 10000rpm to remove residual Wash Buffer. Discard the collection tube.

9. Place the spin column in a clean 1.5/2ml microcentrifuge tube. Add 20-50ul of Elution buffer (EBA) and incubate for 1-3 min at Room temperature. Centrifuge at 10000rpm for 1min.

Agarose gel was prepared as previously mentioned and the purified product was loaded. The product was sent for sequencing at GeneSpec Pvt. Ltd at Kakkanad, Kochi, Kerala.

4.3.5 SEQUENCE ANALYSIS

The sequence obtained was analyzed by Nucleotide BLAST for the molecular identification of the algal species.

4.4 EXTRACTION OF MICROALGAE FOR MEDIA SUPPLEMENT FORMULATION

The microalgal biomass obtained by centrifugation was weighed and mixed with distilled water to prepare a decoction of 10% concentration. The decoction was boiled for 30 minutes at 60 °C, centrifuged at 10,000 rpm for 15 minutes, and the supernatant formed was collected and filtered. The filtrate obtained was used as a media supplement for cell culture studies.

4.5 CELL CULTURE EXPERIMENTS

4.5.1 EXPANSION OF CELL LINES

The effect of *Nannochloropsis* extract as FBS alternative was studied on CHO-K1 cell lines procured from NCCS, Pune.

Cryopreserved CHO-K1 cell lines were thawed and cultured in T-25 flasks with DMEM/ F-12 media with 10% FBS at 37^{o} C with 5% CO_2. These cells were maintained in a CO_2 incubator and sub-cultured to conduct the experiments.

4.5.2 CULTURING OF CHO-K1 CELLS IN BASAL MEDIA SUPPLEMENTED WITH NANNOCHLOROPSIS EXTRACT AND FBS

To evaluate the effect of algal extract on the morphology, adherence, and proliferation, CHO-K1 cells were trypsinized using 1X Trypsin- EDTA solution and seeded into six well plate with DMEM with different concentrations of FBS and microalgal extract as shown in Table 4.

EXPERIMENT NUMBER	FOETAL BOVINE SERUM (FBS) CONCENTRATION	MICROALGAL EXTRACT CONCENTRATION
1	10 %	0%
2	1%	0%
3	0.5 %	0%
4	0%	10%
5	1%	10%
6	0.5 %	10%

Table 4. Concentration of FBS and *Nannochloropsis* used for the culture

The wells were observed using an inverted microscope on the first and fourth day to visualize the morphology and adherence of different treatments.

The cells were trypsinized on the fifth day and cell count was taken by trypan blue staining using Hemocytometer. 50 µl of trypsinized cell was mixed with 10 µl 0.4 % trypan blue. Mixed for 5 min and counted the unstained cells using hemocytometer to determine the viable cells.

4.5.3 DETERMINATION OF CELL PROLIFERATION CAPABILITY OF ALGAL EXTRACT UNDER REDUCED SERUM CONDITION

To determine the proliferation capacity of *Nannochloropsis*, cells were seeded in DMEM with FBS and *Nannochloropsis* extract. Cells grown in DMEM with 10% FBS was taken as control. Cell counting was performed on the 5^{th} day using trypan blue dye as mentioned in previous experiment.

4.5.4 EVALUATION OF FIBRONECTIN AS ATTACHMENT PROMOTING FACTOR FOR SUPPLEMENT FORMULATION.

Fibronectin was used as an additional supplement along with the algal extract to find whether it supports cell adherence property of *Nannochloropsis* extract. Cells were seeded in two petri plates, named A and B. Cells were seeded in DMEM with algal extract and fibronectin in A. In Petri dish B cells were seeded in DMEM with FBS. The morphology, adherence and proliferation of cells were observed using an inverted microscope on Day 3 and Day 5.

4.5.5 EVALUATION OF PRIMORDIA COCKTAIL AS MEDIA SUPPLEMENT

Three cocktails formulated by Primordia Lifesciences; Kochi (CC1, CC2, CC3) were evaluated as an alternative to FBS. Cells were grown in DMEM media with microalgal extract (AE) and three cocktail formulations under reduced serum conditions as shown in Table 5. The concentration for microalgal extract with cocktail was 10%. Cell morphology and adherence were visualized using inverted microscope as in previous experiments. The wells were counted using trypan blue dye and a hemocytometer with an inverted microscope on day 4.

EXPERIMENT NUMBER	CULTURE CONDITIONS
1	0.5% FBS + No cocktail + AE
2	0.5% FBS + CC1 + AE
3	0.5% FBS + CC2 + AE
4	0.5% FBS + CC3 + AE
5	1% FBS + No Cocktail + AE
6	1% FBS + CC1 + AE
7	1% FBS + CC2 + AE
8	1% FBS + CC3 + AE
9	0.5% FBS + CC1 without AE
10	0 .5% + CC2 without AE
11	0 .5% + CC3 without AE

Table 5. Concentration of FBS and cocktails with AE used for the culture.

4.5.6 SERUM REPLACEMENT ASSAY

The cells grown in DMEM supplemented with reduced serum condition and algal extract were taken for serum replacement studies. The cells from experiments with 0.5% FBS and 1% FBS containing AE with cocktails (Experiment numbers: 3, 4, 7, and 8) in the previous experiment were reseeded into fresh six well plate under new conditions. The cells of the 3rd and 4th experiments were reseeded in DMEM with CC2 and CC3 respectively under serum-free conditions. Similarly, cells from the 7th and 8th experiments were reseeded with 0.1% FBS

along with cocktails. 0.1% FBS without cocktail was taken as control. Cells were counted using trypan blue on 4th day as in previous experiments.

EXPERIMENT NUMBER	CULTURE CONDITIONS
1	0 % FBS + CC2 + AE
2	0 % FBS + CC3 + AE
3	0.1 % FBS + CC2 + AE
4	0.1 % FBS + CC3 + AE
5	0.1 % FBS

Table 6. Conditions for Serum replacement assay

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5. RESULT

5.1 COLLECTION AND CULTIVATION OF MICROALGAE

Nannochloropsis collected from CMFRI was transferred to seawater of salinity 30 ppt with Gulliard F/2 media. The biomass was harvested after 14 days by centrifugation at 5,000 rpm for 10 min and washed with 0.5M ammonium formate to remove salts and contaminants. The washed microalgal pellet was used for all experiments except molecular identification.

Fig.1. *Nannochloropsis* cultured in 1 litre seawater with 10 ml f/2 Guillard's supplement

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5.2 MOLECULAR IDENTIFICATION OF MICROALGAE

5.2.1 DNA EXTRACTION

The DNA extracted from *Nannochloropsis* was visualized by agarose gel electrophoresis. An intact DNA band was observed.

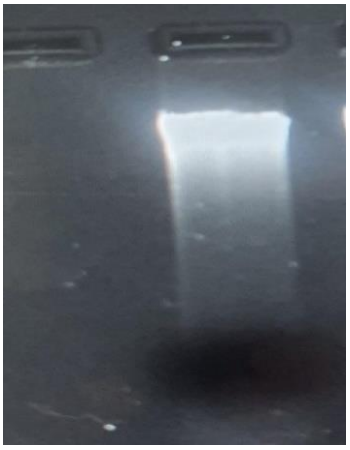

Fig.2. DNA extracted from *Nannochloropsis* and observed using Gel documentation system

5.2.2 PCR AMPLIFICATION

The *rbcL* gene of *Nannochloropsis* was amplified using PCR and the PCR product was loaded in agarose gel and observed as a band of 1533 base pairs.

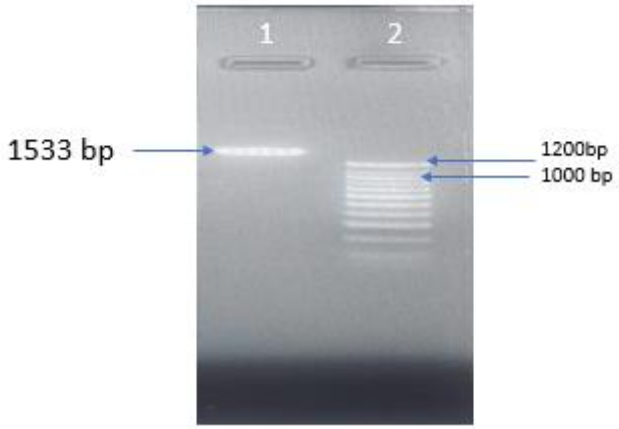

Fig.3. PCR amplification of rbcL gene of the microlgal sample

Lane 1: 1533bp PCR amplicon

Lane 2: 100 bp DNA Ladder

5.2.3 GEL EXTRACTION

The *rbcL* gene was extracted from agarose gel and observed as a thick clear band.

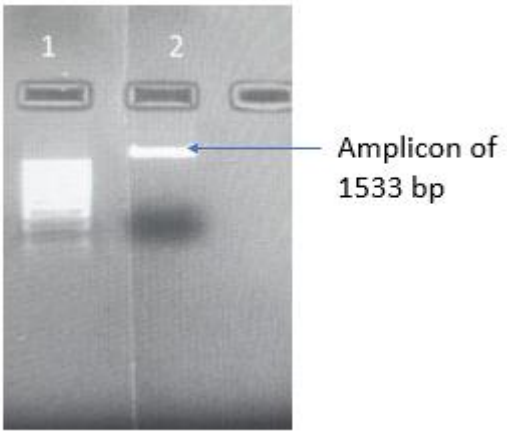

Fig. 4. PCR product

Lane 1: 100 bp DNA Ladder

Lane 2: Purified PCR product

5.2.4 DNA SEQUENCE ANALYSIS

The PCR amplicon was purified and sequenced and on blast analysis it was observed to have 100% similarity with the microalga *Nannochloropsis oceanica* (Fig.5)

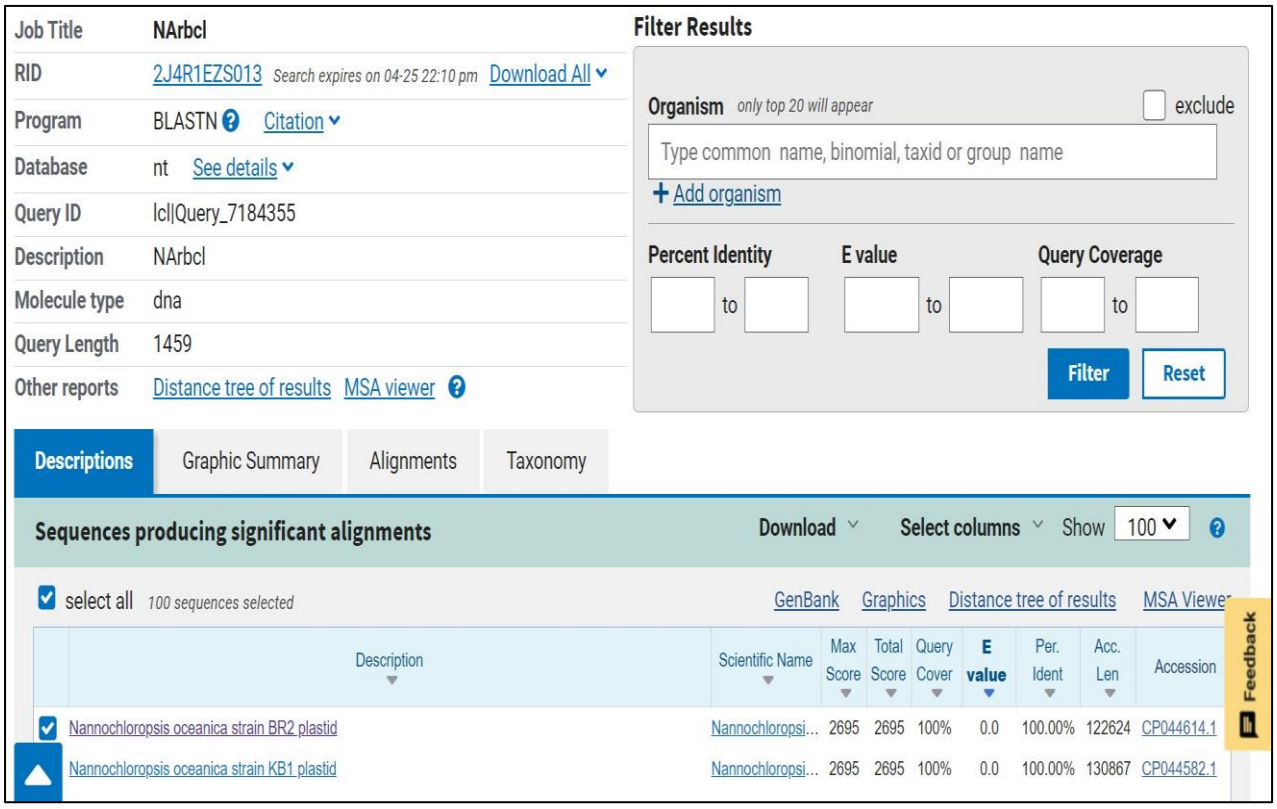

Fig 5a.BLAST analysis of *rbcL* gene of *Nannochloropsis*

AAACGAACGTTATGAATCAGGTGTAATCCCTTACGCTGAAATGGGTTACTGGGATGCAAACTACACA
ATTAAAGATACTGATGTACTTGCATTATTCCGTATCACTCCACAACCAGGGGTTGATCCAATTGAAGC
TGCGGCTGCCATCGCTGGTGAATCTTCAACAGCTACATGGACTGTAGTATGGACTGACTTATTAACA
GCTTGCGATGTTTATCGAGCAAAAGCTTACAAAGTTGATTCAGTTCCAGGTACTAGCGACCAATACT
TCGGTTACGTTGCATATGAATGTGATCTTTTTGAAGAAGGTTCAATTGCTAACTTAACAGCTTCAATT
ATCGGTAACGTATTTGGATTCAAAGCTGTAAAAGCATTACGTCTTGAAGATATGCGTATGCCTTACG
CTTACTTAAAAACATTCCAAGGTCCAGCTACTGGTGTGATTGTTGAACGTGAGCGTTTAGACAAATTC
GGACGTCCTTTATTAGGTGCAACTGTAAAACCTAAACTTGGTTTATCAGGTAAAAACTATGGACGTG
TTGTATACGAAGGTTTAAAAGGTGGTTTAGACTTCTTAAAAGATGACGAAAACATTAACTCTCAACC
ATTCATGCGTTGGCGTGAACGTTTCTCGTACGTAATGGAAGGTGTTAATAGATCAGCTGCAGCTTCT
GGTGAAGTTAAAGGTTCTTACCTTAACGTTACTGCAGCAACTATGGAAGAAATGTACGAACGTGCTG
AATTTGCTAAACTTGTTGGTTCAGTAATTATCATGATCGACTTAGTAATTGGTTATACTGCAATTCAAT
CGATGGCTGTTTGGTCTCGTAAGAATGATATGATCCTTCACTTACACCGTGCAGGTAACTCAGCATAT
GCTCGTCAAAAGAACCATGGTATTAACTTCCGTGTAATTTGTAAATGGATGCGTATGGCTGGTGTTG
ACCACATCCATGCAGGTACAGTTGTAGGTAAATTAGAAGGTGACCCTCTAATGGTTAAAGGTTTCTA
CAACGTATTATTACAAACATCACTAGATATTAACTTACCACAAGGTATCTTCTTCGAACAAGACTGGG
CTTCTTTAAGAAAAACACTACCTGTAGCTTCTGGTGGTATCCATTGTGGACAAATGCACCAGTTACTT
AACTATCTAGGTGAAGACTGTGTATTACAATTTGGTGGTGGTACAATTGGTCACCCTGATGGTATCG
CTTCTGGTGCGACTGCTAACCGCGTAGCTATGGAGTCAGTGCTTTTAGCTAAATATGAAGGTAAAGA
TTACATTAACGAAGGACCAAAAATTTTACGTGCGGCGGCAGAAAGTTGTGCGCCATTACGTTCTGCT
TTAGATCTTTGGAAAGATATTGCTTTCAACTATACATCAACAGATACTGCTGATTACATTGAAACTGC
AACTAAACAGTAATCGTATAAAAACAATATCAATCAGTTTAAC

Fig 5b. Sequence of rbcL gene of *Nannochloropsis*

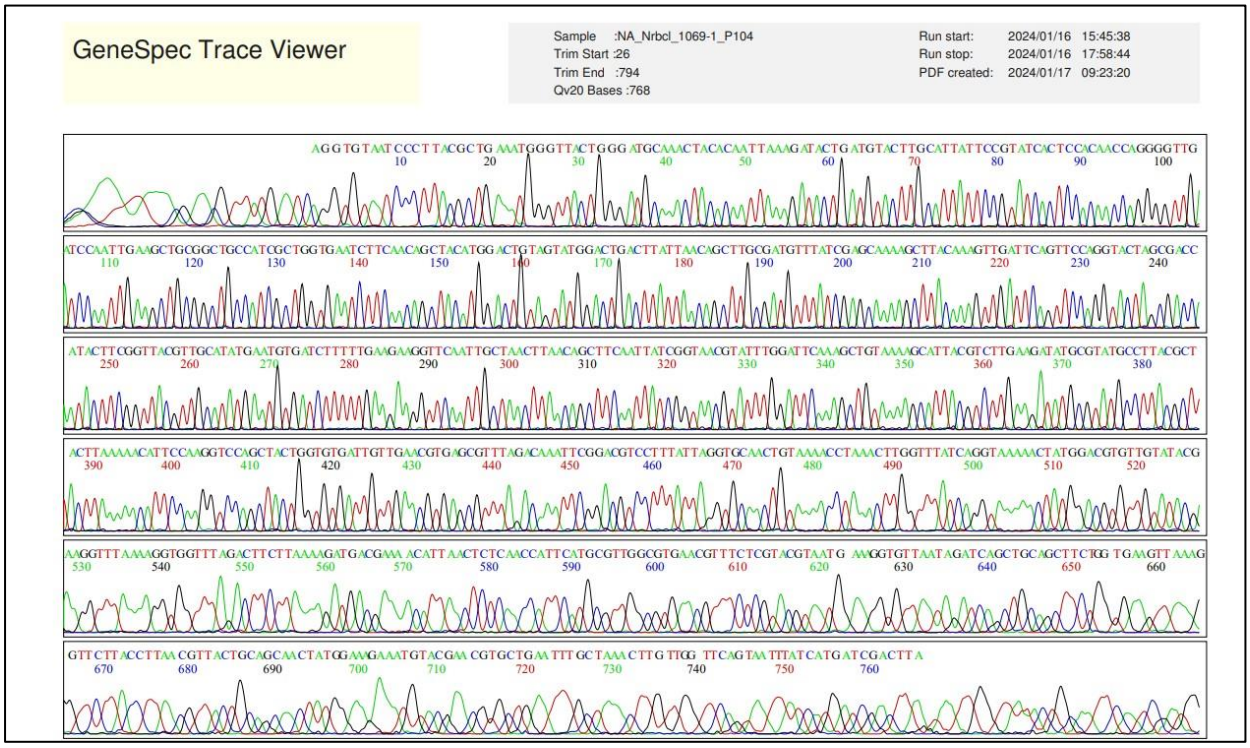

Fig 5c. Chromatogram of *rbcL* gene sequence of *Nannochloropsis* (Forward Primer)

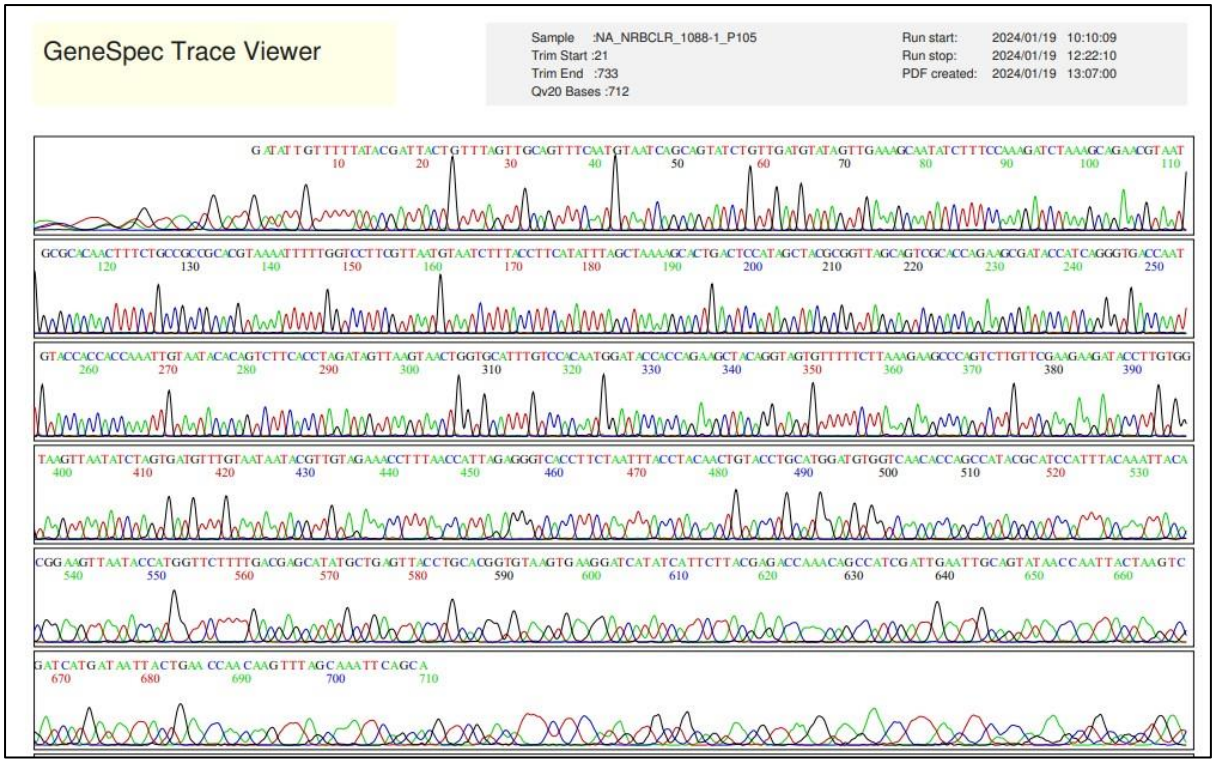

Fig 5 d. Chromatogram of *rbcL* gene sequence of *Nannochloropsis* (Reverse primer)

5.3. CELL CULTURE STUDIES

5.3.1 EXPANSION OF CELL LINES

The cryopreserved cells were thawed, cultured to T-25 flasks with DMEM/ F-12 media with 10% FBS at 37° C with 5% CO_2. The cells grew and were maintained and subcultured for experiments.

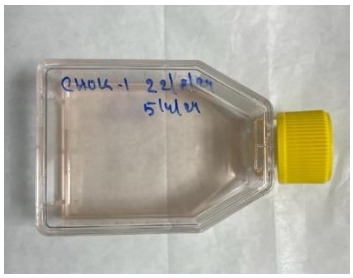

Fig.6. CHO-K1 cells cultured in T-25 flask

5.3.2 CULTURING OF CHO-K1 CELLS IN DMEM MEDIA SUPPLEMENTED WITH NANNOCHLOROPSIS EXTRACT AND FBS

Cells cultured in DMEM with FBS as well as with *Nannochloropsis* extract (AE) were observed under an inverted microscope. Images are shown in Fig. 7 and Fig 8

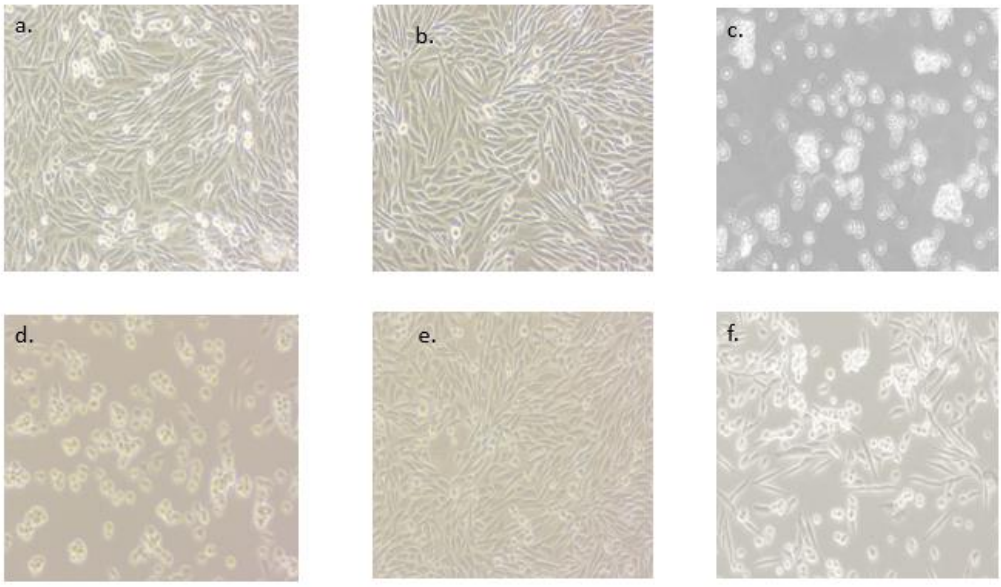

Fig.7. Day 1: Microscopic images of CHO-K1 cells grown in DMEM with a. 10% FBS b.1% FBS c. 0.5% FBS d. 10% AE e. 1% FBS + AE f. 0.5 % FBS +10% AE

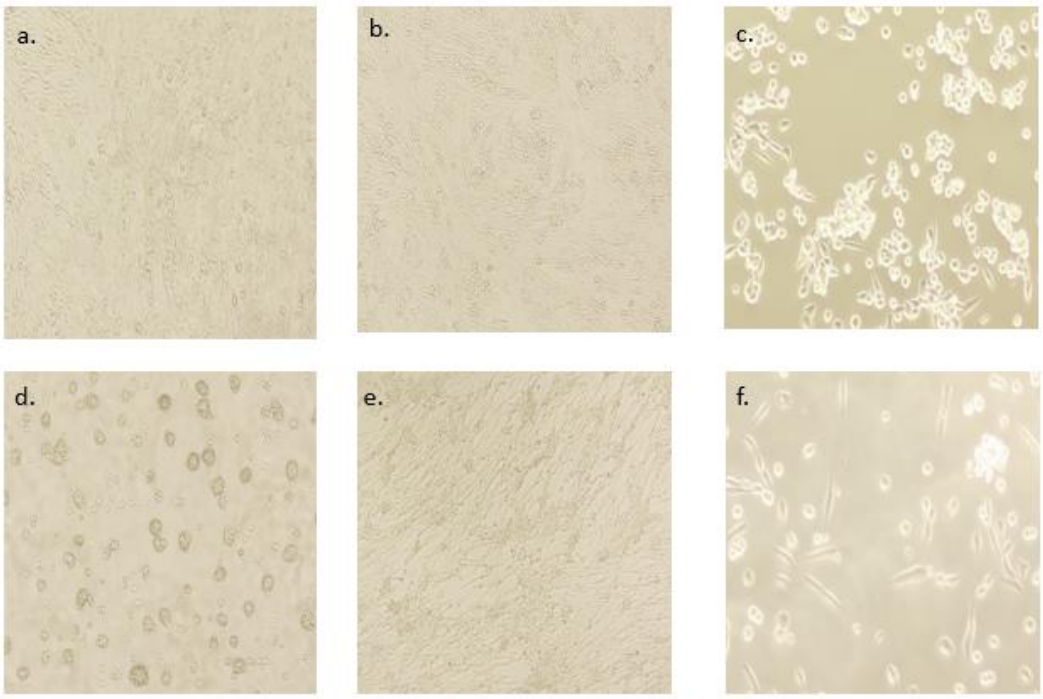

Fig. 8. Day 4: Microscopic images of CHO-K1 cells grown in DMEM with a. 10% FBS b. 1% FBS c. 0.5% FBS d. 10% AE e. 1% FBS + AE f. 0.5 % FBS + 10% AE

The cells grown in DMEM with 0.5% FBS +AE showed better proliferation than the cells with 0.5% FBS alone.The cells when seeded with the algal extract alone did not show sufficient growth and attachment so additional supplements were evaluated.

5.3.3 DETERMINATION OF PROLIFERATION CAPABILITY OF ALGAL EXTRACT UNDER REDUCED SERUM CONDITION

Cells that were seeded in DMEM, FBS, and microalgal extract showed more growth and proliferation than the cells that were seeded only with DMEM and FBS. The cells grown in DMEM without FBS did not show growth, and cell death was observed.

CULTURE CONDITIONS	CELL COUNT (X 10^4)
FBS + MICROALGAL EXTRACT	106
FBS ALONE	96
ALGAL EXTRACT ALONE	6
WITHOUT FBS	0

Table 7. Cell count to determine proliferation capability of *Nannochloropsis*

5.3.4 EVALUATION OF FIBRONECTIN AS ATTACHMENT PROMOTING COMPONENT

Fibronectin was added to microalgal extract to promote cell adhesion. Cells were cultured in FBS and DMEM (B) as well as with DMEM and *Nannochloropsis* extract +Fibronectin (A). Morphology and proliferation of the cells were observed on Day 3 (Fig.9a and Fig.9b) and Day 5 (Fig.9c and Fig.9d)

On day 3, the cells were observed. In A (Fig.9a), containing cells, DMEM and the microalgal extract, the cells were not attached. In (Fig.9b) cell attachment and growth was observed.

On day 5, the petri dishes were again observed. The cells were not attached in A (Fig.9c) and cell death was observed. But in B (Fig.9d), cell attachment as well as growth was observed.

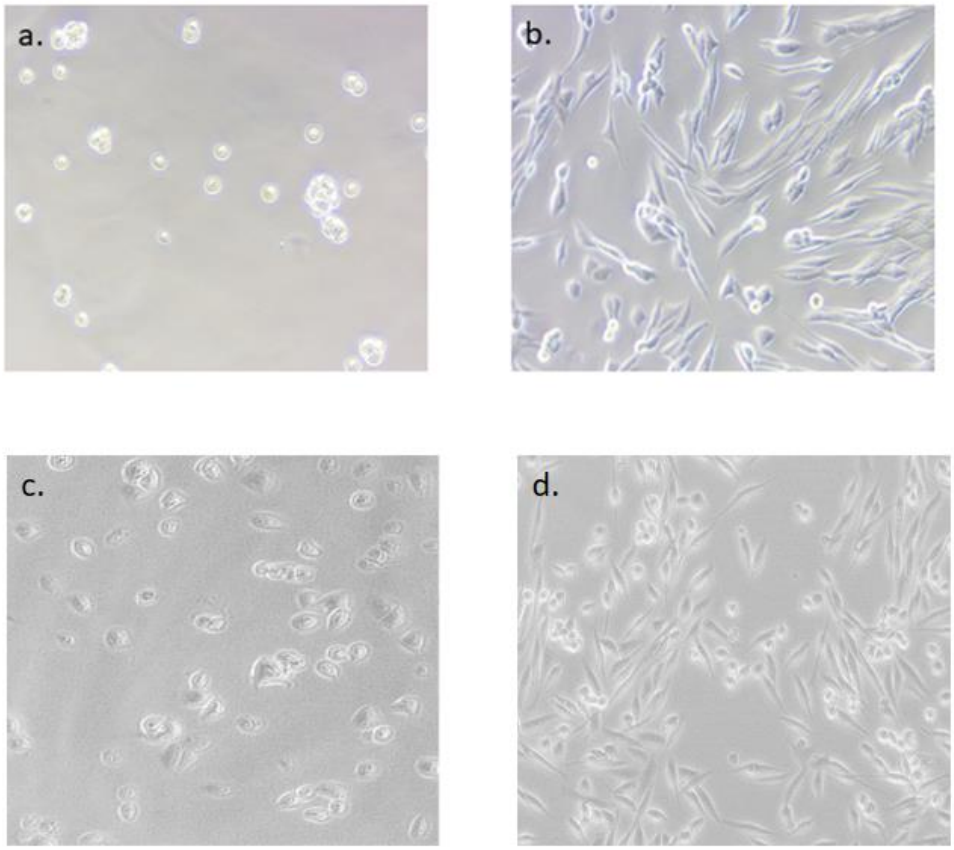

Fig. 9 Microscopic images of CHO-K1 cells grown in DMEM with

a. AE + Fibronectin (DAY3) b. 1% FBS (DAY 3)

c. AE + Fibronectin (DAY 5) d. 1% FBS (DAY 5)

5.3.5. EVALUATION OF PRIMORDIA COCKTAIL AS MEDIA SUPPLEMENT

The algal extract + FBS combination along with CC2 (Fig.10A c and g) and CC3 (Fig 10A d and h) respectively showed the most growth and attachment of cells. However, the same combination with CC1 had comparatively poor growth. Experiments only with FBS (no cocktail and no microalgae) showed some growth and attachment. However, in the absence of the FBS and AE (Fig.10B), no growth and attachment was observed even after the addition of the cocktails . The cells were counted on Day 4. The cell count as shown in Table 8 showed that the cocktail formulations promote the cell adherence and proliferation of CHO-K1 cells under reduced serum conditions.

CULTURE CONDITIONS	CELL COUNT ($X10^4$)
0.5% FBS +AE	28
0.5% FBS +CC1 +AE	31
0.5% FBS +CC2 + AE	49
0.5% FBS +CC3 + AE	56
1% FBS +AE	75
1% FBS + CC1 + AE	106
1% FBS + CC2 + AE	210
1% FBS + CC3 +AE	250
0.5 % FBS + CC1 without AE	4
0.5 % FBS+ CC2 without AE	8
0.5 % FBS+ CC3 without AE	10

Table 8. Condition for cell growth and cell count

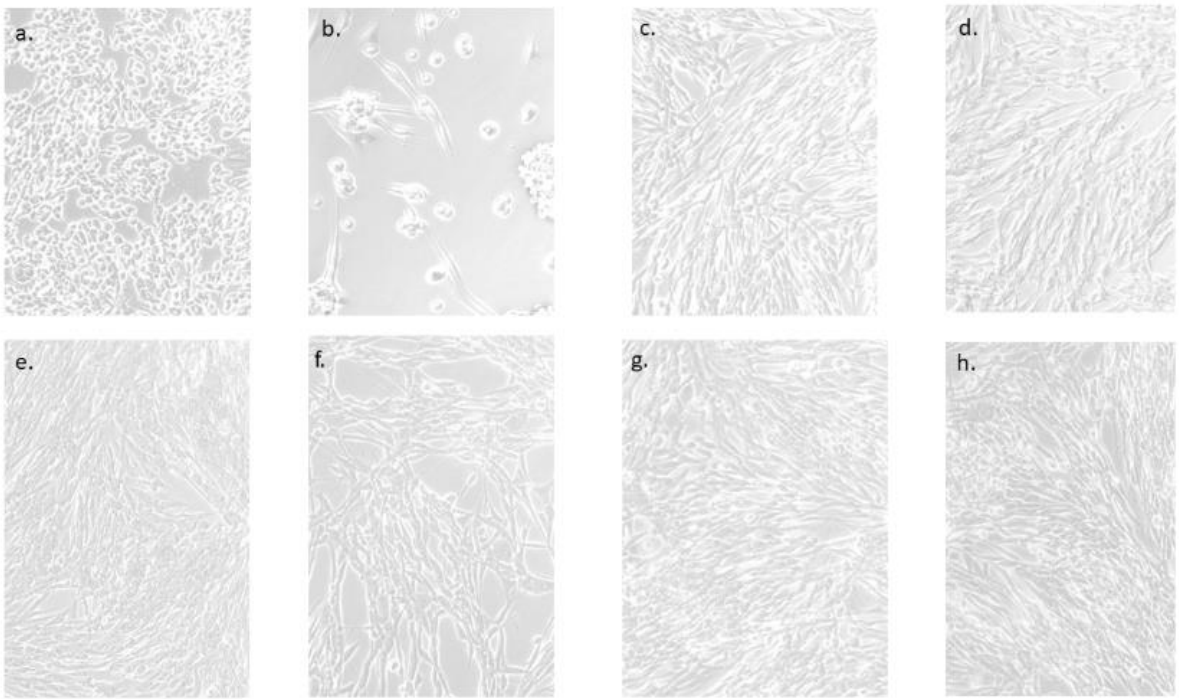

Fig.10A Microscopic images of CHO-K1 cells grown in DMEM with a. 0.5% FBS + 10% AE b. 0.5% FBS +CC1 +10% AE c. 0.5% FBS +CC2 + 10% AE d. 0.5% FBS +CC3 + 10% AE e. 1% FBS + 10% AE f. 1% FBS + CC1 + 10% AE g. 1% FBS + CC2 + 10% AE h. 1% FBS + CC3 + 10% AE

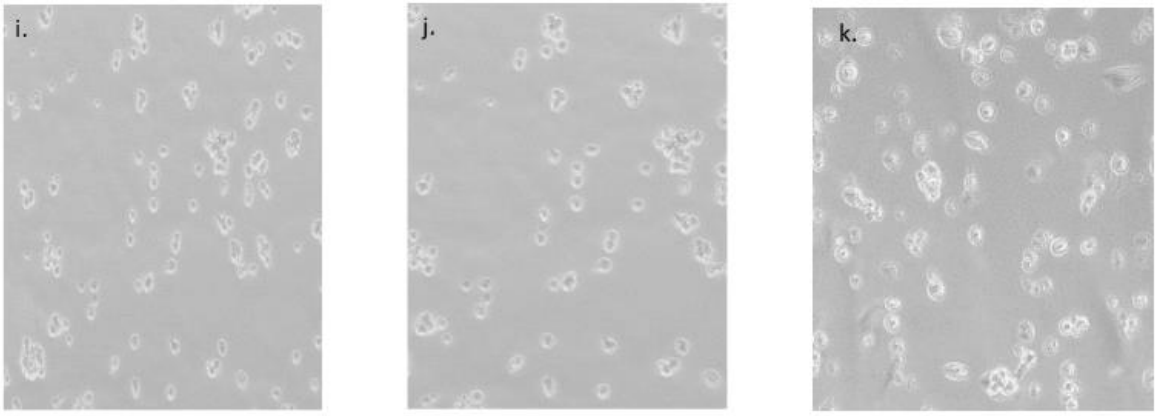

Fig.10B Microscopic images of CHO-K1 cells grown in DMEM with 0.5% FBS + cocktails without AE i. CC1 j. CC2 k. CC3

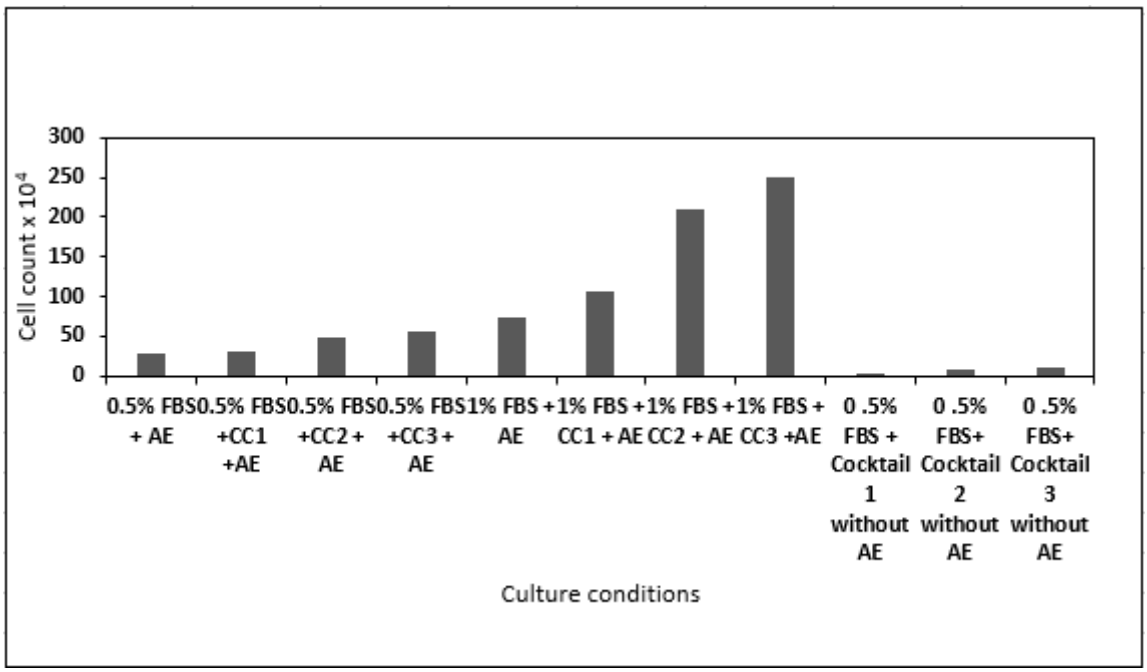

Fig.11 Bar diagram showing cell count of CHO-K1 cells grown in different cocktails with AE under different FBS concentration

The cocktails CC2 and CC3 showed significant proliferatio and cell adhesion than CC1 sowe took CC2 and CC3 for seum replacement assay.

5.4 SERUM REPLACEMENT ASSAY

Cells were centrifuged from the previous experiment (optimization of primordia cocktail as a media supplement) and seeded under serum free, and serum reduced conditions with the addition of microalgal extract +cocktails 2 (Fig.12a and Fig.12c) and microalgal extract + CC

3 (Fig.12b and Fig.12d). Cell growth, attachment, and proliferation was observed under both serum reduced and serum free conditions with the addition of the cocktails and the microalgal extract. The cells were observed on days 4,and 7, shown in Fig.12, and Fig 13 respectively. The cells were counted on day 9 (Table 9).

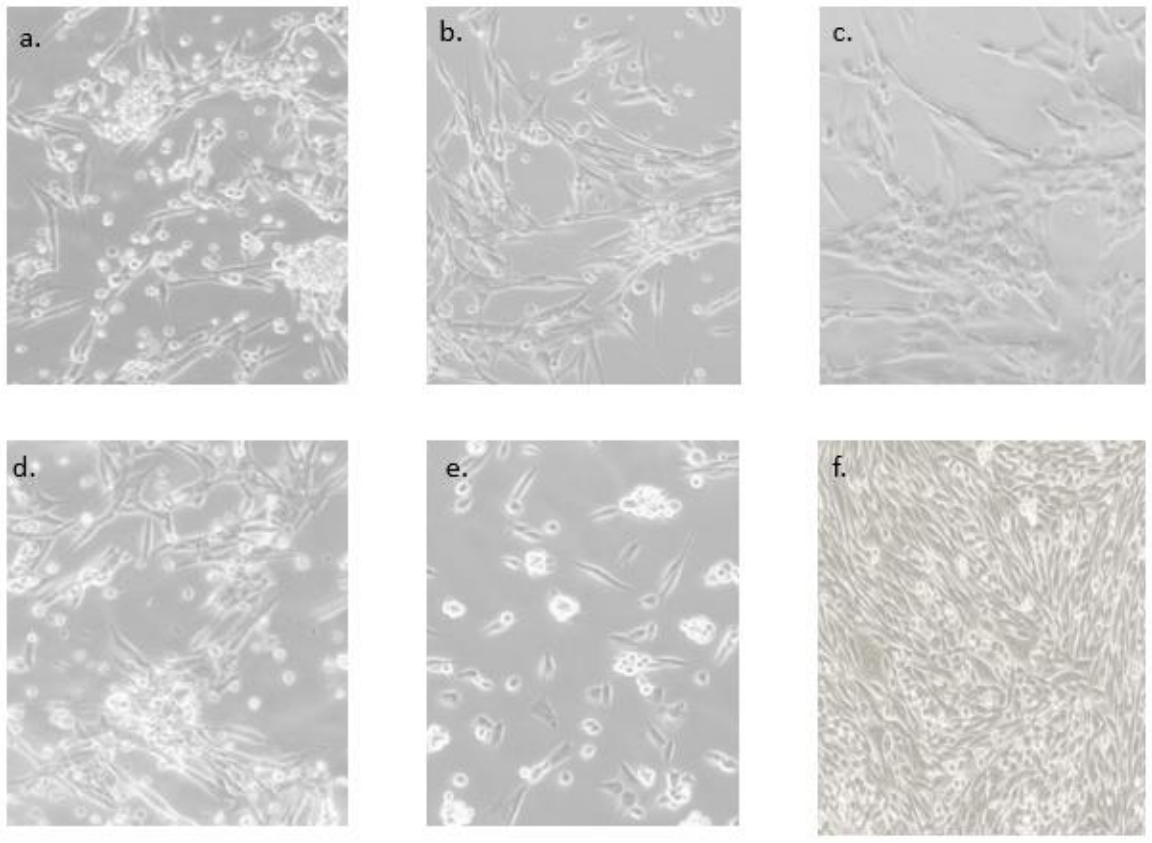

Fig 12. DAY 4: Microscopic images of CHO-K1 cells grown in DMEM with a. 0% FBS + CC2 + AE b. 0% FBS + CC3 + AE c. 0.1% FBS + CC2 + AE d. 0.1% FBS + CC3 + AE e. 0.1% FBS + 0 % AE + 0% cocktail f.10% FBS

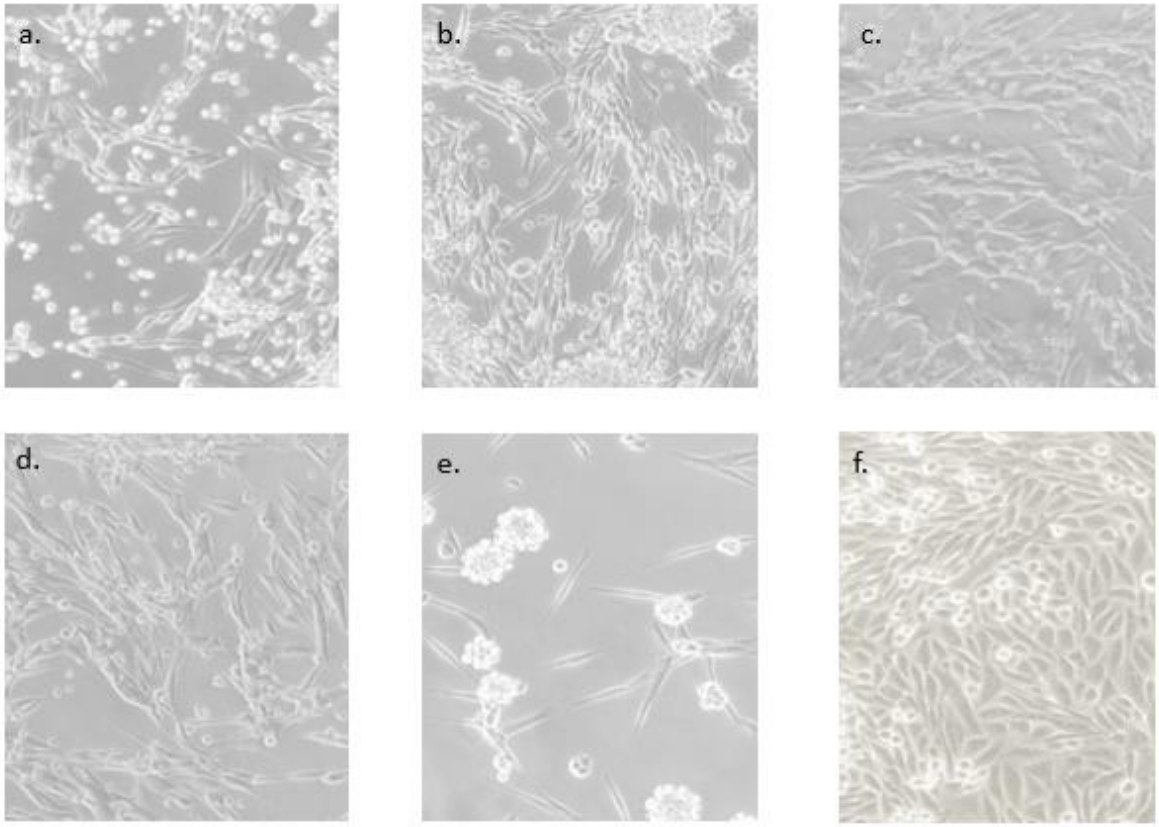

Fig 13. DAY 7: Microscopic images of CHO-K1 cells grown in a. 0% FBS + CC2 + AE b. 0% FBS + CC3 + AE c. 0.1% FBS + CC2 + AE d. 0.1% FBS + CC3 + AE e. 0.1% FBS + 0 % AE + 0% cocktail f. 10% FBS

CULTURE CONDITIONS	CELL COUNT (X 10^4)
0% FBS + CC2 + AE	70
0% FBS + CC3 + AE	150
0.1% FBS + CC2 +AE	229
0.1% FBS + CC3 + AE	231
0.1% FBS + 0 % AE + 0% cocktail	48
10% FBS	293

Table 9. Serum replacement assay- condition for cell growth and cell count

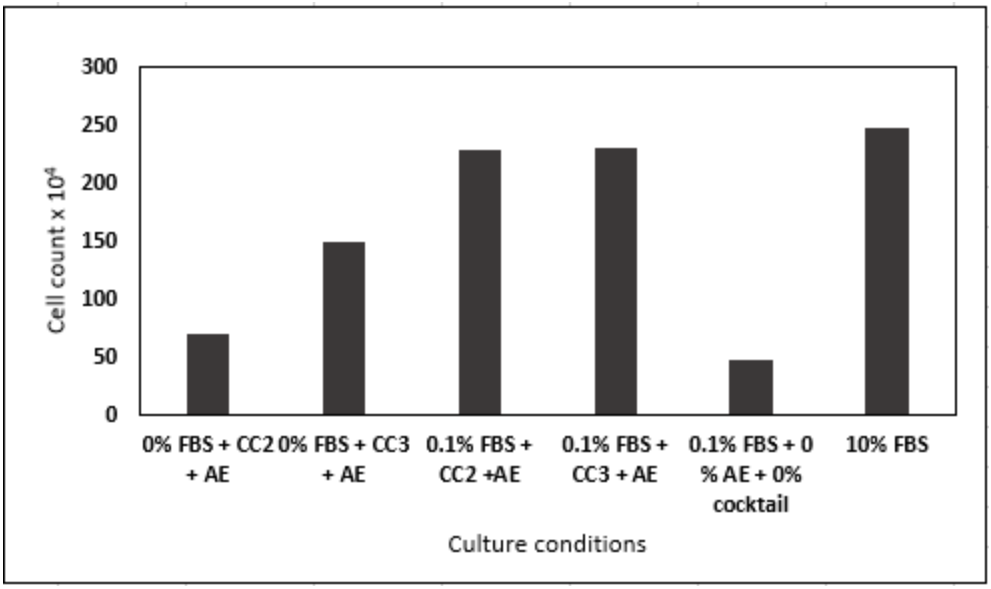

Fig 14. Bar diagram showing cell count of CHO-K1 cells grown in different cocktails with AE under serum-reduced and serum-free conditions

6. DISCUSSION

Animal cell culture has become a fundamental technique in pharmaceutical and biomedical fields where it is used to, study, develop, and test various products including drugs, vaccines, proteins, etc. Cells require a variety of nutrients to support their growth. In cell culture, these nutrients are provided through a basal media (DMEM, MEM, RPMI etc) supplemented with additional proliferating factors. The commonly used supplement is Foetal Bovine Serum (FBS) at a concentration of 2% to 10%. The basal media consists of carbohydrates, amino acids, inorganic salts, vitamins, etc, and FBS is rich in proteins, lipids, hormones, cell adhesion and proliferative factors all of which aid in the culturing of cells. Although it is used widely, FBS has several disadvantages including variability in the consistency of composition and susceptibility to contamination which affects the quality of protein production. It also raises animal welfare issues as the serum extraction results in the death of the bovine foetus.

Finding an alternative using natural resources that is sustainable and renewable can address the issues caused by FBS and effectively replace it. Microalgae seems as a good choice for media supplement formulation as it is rich in several nutrients such as proteins, lipids, carbohydrates, etc that can support cell growth. *Nannochloropsis* was selected for the study as it is reported to be rich in proteins where proteins constitute 45% of its biomass. The microalgae was collected from Central Marine Fisheries Research Institute, Kochi, Kerala. On sequencing the *rbcL* gene of *Nannochloropsis* it was identified to have hundred percent similarity to the species *Nannochloropsis oceanica.* The algal extract was obtained by aqueous extraction and was used for media supplement formulation.

The cell line CHO-K1 was selected for this study as these cells are commonly used in industrial biotechnology. They are derived from the epithelial cells of the ovary of an adult Chinese hamster.CHO-K1 cells are used for recombinant protein production, toxicology research, and other pharmaceutical applications. In this study, the potential of microalgae to reduce or replace foetal bovine serum was evaluated. When the cells were cultured with the *Nannochloropsis* extract, a potential for cell proliferation was recorded,. *Nannochloropsis* also showed cell adherence capability when seeded along with FBS. However, *Nannochloropsis* extract alone was not sufficient to support cell growth and adherence and proliferation similar to 10% FBS. To enhance the cell adhesion, cells were seeded with *Nannochloropsis* and

fibronectin, an attachment factor commonly used in cell culture. Fibronectin with algal extract did not show significant cell adhesion. *Nannochloropsis* extract in combination with three cocktails consisting of growth and attachment factors formulated by Primodria Lifesciences, (CC1, CC2, and CC3) were evaluated for cell culture under reduced serum conditions. The *Nannochloropsis* extract fortified with cocktails supported cell growth, cell adhesion and proliferation in low serum condition. Cells grown in reduced serum conditions were counted by Trypan Blue staining using a hemocytometer. Trypan blue is an azo dye that can distinguish between live and dead cells. It works on the principle that live cells with intact membranes are impenetrable for trypan blue whereas the porous membranes of dead cells allow the dye to penetrate through and stain them. Cell counting results showed that CC2 and CC3 with AE has a significant potential for cell growth and adherence under reduced serum conditions. Serum replacement assay was carried to evaluate the efficiencies of cocktails for cell culture without the use of FBS.

Serum replacement assay was carried out with the cells grown in *Nannochloropsis* extract with CC2 and CC3 were reseeded with 0.1% FBS and 0% FBS respectively. The results of serum replacement assay showed that the formulated cocktail with algal extract showed similar attachment and proliferation compared to medium with 10% FBS. The results of the present study shows that the cocktail formulation with *Nannochloropsis* extract is a promising alternative for Foetal Bovine Serum (FBS) for supplementing the basal media for cell culture studies.

7. CONCLUSION

Animal cell culture is an essential tool in several pharmaceutical, and biomedical applications and it is commonly used for recombinant protein production. The cells are commonly cultured in Foetal bovine serum (FBS), an animal product that is rich in proteins, proliferation factors, adhesion factors, and several other nutrients required for cell growth and proliferation. However, it has many disadvantages. This study evaluated the potential of the microalgae *Nannochloropsis*, reported to have 45% of proteins in its biomass, to replace or reduce Foetal bovine serum for cell culture. CHO-K1 cells were cultured with the extract of *Nannochloropsis* along with cocktails formulated by Primordia Lifesciences under reduced and serum-free conditions. These formulations showed potential for cell attachment and proliferation, under reduced serum conditions as well as serum-free conditions. The morphology and growth of the cells under these conditions were comparable to FBS. Due its renewable and sustainable nature and its capacity to support cell growth, the formulations are promising alternatives for Foetal bovine serum for cell culture studies.

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