CHARACTERIZATION AND CULTURE OF SNOW ALGA *CHLAMYDOMONAS NIVALIS* FROM ANTARCTICA

Project Submitted to the Central University of Punjab

For the award of

Master of Science

IN

Life sciences with specialization in Plant Sciences

BY

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CERTIFICATE

I declare that the project report entitled "**Characterization and culture of snow alga *Chlamydomonas nivalis* from Antarctica"** has been prepared by me under the guidance of Dr. Felix Bast, Assistant Professor, Department of plant Sciences School of Basic and Applied Sciences, Central University of Punjab. No part of this project report has formed the basis for the award of any degree or fellowship previously.

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CERTIFICATE

I certify that Archana Samal has prepared her thesis/dissertation entitled **"Characterization and culture of snow alga *Chlamydomonas nivalis* from Antarctica"** for the award of Master's degree of the Central University of Punjab, under my guidance. She has carried out this work at the Department of Plant Sciences, School of Basic and Applied Sciences, Central University of Punjab.

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ABSTRACT

CHARACTERIZATION AND CULTURE OF SNOW ALGAE *CHLAMYDOMONAS NIVALIS* FROM ANTARCTICA

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Key words: *Chlamydomonas nivalis,* culture and subculture, bold basal media, vitamin B_{12}, turbidity major, zoospore, hypnospore, Flow cytometry.

The snow alga *Chlamydomonas nivalis* is the reason of colouring the snow orange-red. For current study sample was collected from Dalk Glaciers, Larsmann hills, Eastern Antarctica. This is the most studied alga of snow-fields but cytology, morphology, and taxonomy are still unsolved. Our experiment focuses on comparative growth rate study of algal culture at two different temperature one is of 4°C and other is of 20-22°C and its morphology as well as life cycle study. Experiment confirmed that algal culture at 20-22°C was growing at a higher rate than the culture at 4°C. Identification and characterization were done on the basis of light microscope and phase microspore studies. Phase contrast microscope picture signifies the presence of different types of algal cells in culture. Flow cytometry strongly support the phase contrast microscope data and signifies the presence of algal cells in different stages of its life cycle.

Archana Samal Dr. Felix Bast

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Archana Samal

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LIST OF ABBREVIATIONS

Sr. No.	Full Form	Abbreviation
1	Bold basal media	BBM
2	Normality	N
3	Serial number	Sr. No
4	Milligram	mg
5	Milliliter	ml
6	Centimeter	cm
7	Microliter	µl
8	Celsius	˚C
9	Species	*Sp.*
10	Flow cytometry	FCM

CHAPTER I
INTRODUCTION

Polar regions include Arctic and Antarctic regions covering 15% of the surface of Earth. Cold regions are among the most important and least studied ecosystems. The rich diversity of prokaryotic and eukaryotic organism make these regions important in ecological point of view. These cold climates have been colonized by kind of microorganisms referred as psychrophiles.

Psychrophilic algae and cyanobacteria contribute significantly as producers in snowfields due to the fact that environmental conditions are too limiting for terrestrial plants. *Chlamydomonas nivalis* is the most dominant unicellular green alga and a prominent representative of *Chlamydomonadaceae* on the snow surfaces. Red snow or watermelon snow is a well-known phenomenon due to the carotenoid-rich resting spores of this specialized psychrophilic green alga. Green snow is suspected to be caused by the young and metabolically active stages of the snow alga (Lutz *et al.*, 2014). *Chlamydomonas nivalis* mostly reported in the snow in form of spherical cells of 10–50 µm in diameter called hypnospore or hypnoblast. *Chlamydomonas nivalis* is being reported almost across the world ranging from the Arctic to Antarctic region, South America (Takeuchi and kohshima, *et al*., 2004), North Africa (Duval *et al.*, 1999), North America (Thomas and Duval,*et al.,* 1995), New Zealand (Novis *et al.*, 2008), Europe (Remias *et al.*,2010), Siberia (Hisakawa *et al.*, 2015; Tanaka *et al.*,2016) and Asia (Muramoto *et al.*,2010) and Antarctica.

1.1 Systematic position

Systematic position of green algae *Chlamydomonas nivalis is* of division *Chlorophyta,* class *Chlorophyceae*, order *Chlamydomonadales,* family Chlamydomonadaceae, and genus *Chlamydomona*s and species *nivalis.* Most of the snow algae are of genera *Chloromonas* and *Chlamydomonas* of class Chlorophyta. *Chlamydomonas nivalis* reported causing orange, green and pink blooms in Polar Regions (Remias *et.al* 2010) but their exact taxonomic identity is often unclear and needs to be treated as a collective taxon. *Chlamydomonas nivalis* has a resemblance with model organism *Chlamydomonas reinharditi.*

1.2 Life cycle of *Chlamydomonas nivalis*

Snow alga is the permanent resident of Arctic and Alpine snow and during summer it is exposed to high temperatures, irradiance and low nutrient content. They can tolerate low temperature, extreme light and low nutrient content which makes these microalgae to bloom on the snow. It creates its unique microhabitat to become metabolically and physiologically active. It melts the glaciers either directly via exothermic metabolism or dark patches caused by these snow algae and other related microorganism reduces albedo effect locally up to 20% (Duval *et al,* 1999; Yallop *et al.*, 2012; Lutz *et al.*, 2014, 2016).

. The metabolically active stage is called as zoospore having flagella, required liquid water between snow crystals. Flagellated stage helps these organisms to move to the depth of snow layer where suitable light, temperature, and liquid water is available. Most part of its life cycle is of an immotile stage called hypnospore which is resistant to extreme environmental conditions.

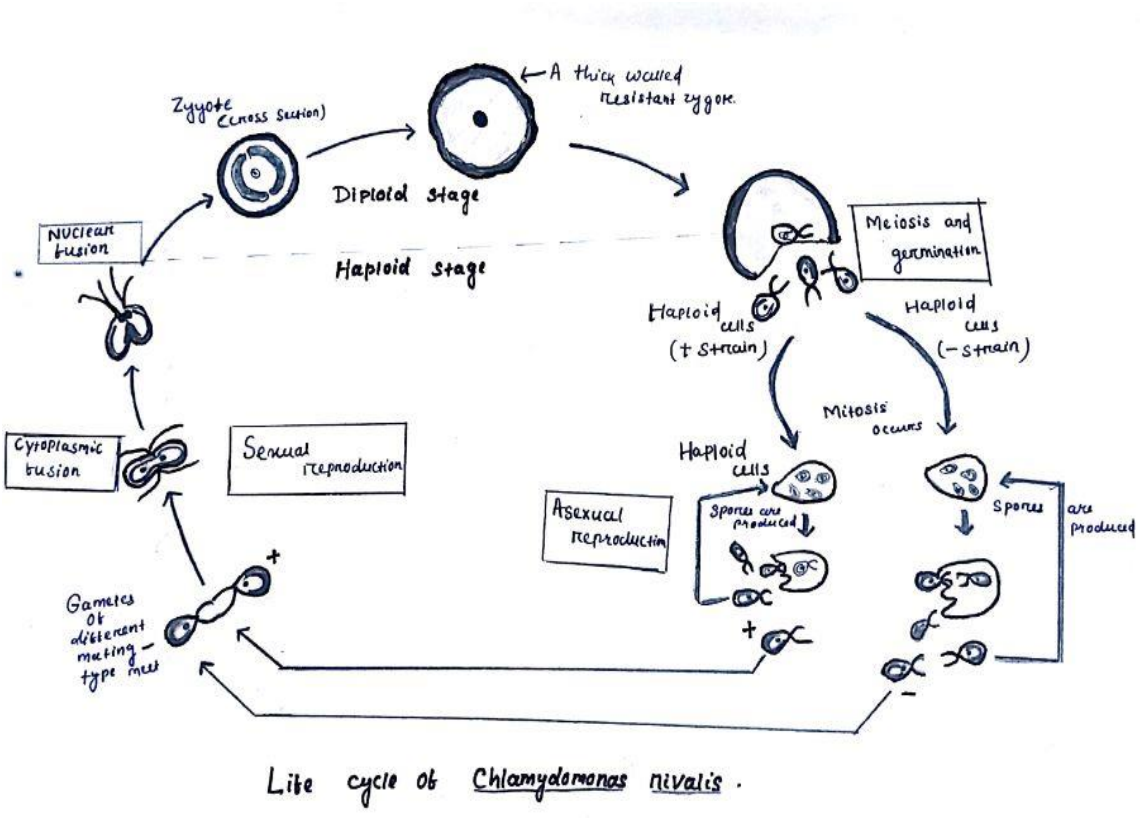

Figure 1: Life cycle of *Chlamydomonas nivalis*

Chlamydomonas nivalis transforms from one form to another form during its life cycle (Fig; 1) according to the physiological environment and nutrient availability. The complex life cycle and adaptation capability of this alga makes it common inhabitant of various snowfields. Transformation potential of zoospore into hypnospore is one of the adaptation strategies of the alga. Fragmentation of chloroplast takes place in order to increase the surface to volume ratio during the transformation process (Remias *et al.,* 2010). Another characteristic feature during transformation is the accumulation of reserve materials like lipids, sugars and secondary carotenoid. Characteristic pink to brownish-orange colour of the hypnospore is due to astaxanthin, a secondary carotenoid (Hoham and Mullet 1977; Leya 2004; Nedbalová *et al.* 2008). Astaxanthin with other phenolic compounds act as cryoprotectant which augment its UV protection ability.

SIGNIFICANCE OF THIS STUDY

Sample was collected from Dalk Glacier, Larsemann Hills, Eastern Antarctica, First goal is to identify the sample. To my best knowledge there is no previous report of presence of *Chlamydomonas nivalis* over that place. Morphology of alga will be studied in different stages of its life cycle.

According to (Hoham *et al.,* 1975) species that normally do not grow above 10ºC are called true snow algae. So another goal is to study growth of this particular snow alga on and above 10ºC in controlled laboratory condition and to compare its growth in various temperature. Culture of this alga is a bit hard with BBM culture media, media used for culture of green microalga. Microalga is B_{12} dependent hence contaminated by bacterium *Mesorhizobium* species (Kazamia *et al.,* 2011) and fungi, by keeping in mind this fact a complementary goal is to study significance of vitamin B_{12} in growth of the alga.

OBJECTIVES

1. To culture *Chlamydomonas nivalis* in controlled laboratory condition in different photoperiodicity and temperature to identify optimal growth, also morphology and life cycle.
2. To extract DNA for further molecular study.

CHAPTER II
REVIEW OF LITERATURE

Chlamydomonas nivalis is the most studied alga of snow-fields but still there is a lack of physiological and cytological data. This alga is of commercial interest due to its high antioxidant and phenolic content (Li *et al.*, 2007), in addition to its ability to produce astaxanthin (Rezanka *et al.*, 2008). A few recent studies have measured chlorophyll fluorescence, from sample of Czech Republic (Stibal *et al.,* 2003), measurement of gas exchange between snow containing *Chlamydomonas nivalis* (William *et al.*2003). Use of oxygen evolution for photosynthetic determination of *Chlamydomonas nivalis* in Alpine region of Australia (Remias *et al.*2005), genetic and morphology of sample from Japanese mountains (Muramoto *et al.* 2008). Other studies are based on the conductivity of living cells and electron microscopy (Muller *et al.*, 1998; Muller *et al.*, 2001) and culture collection of this alga (Leya, 2004). Previously its distribution, taxonomic and pigmentation has been studied throughout the Spitsbergen archipelago (Muller *et al.*, 1998; Leya, 2004). A broader survey of this species is necessary in order to understand its life cycle and survival strategies in extreme habitat like snow. This species has gained a lot of interest after scientists reported that these microalgae are one of the reasons of melting glacier.

During spring and summer when the snow starts melting the green and red algal blooms appear in the Alpine and Antarctic snow. These psychrophiles grow in the extreme environment such as high light intensity, the temperature below freezing point and desiccation (Hoham and Duval, *et al.,* 2001). Melting snow support a group of microbial community, including fungi and heterotrophic bacteria dependent on algae for carbon (Williams *et al.*, 2003; Mannisto and Haggblom, 2006; Hodson *et al.*, 2010; Zawierucha *et al.*, 2015; Hamilton and Havig, 2017). Other than carbon fixation snow algae also fix nutrient like iron, manganese, and phosphate (Hamilton and Havig, *et al.,* 2017).

Green algae, golden algae and cyanobacteria and other heterotrophic invertebrates, fungi, and bacteria are also found in snow (Remias *et al.*, 2013). These species coexist in mutualism and parasitism (Little *et al.,* 2008; Konopka et al., 2009). The habitat of snow algae must be considered in their physiological

measurement (Hoham and Duval, 2001; Muller *et al.*, 2001). Psychrophilic is an all-time choice for the study of adaptation to extreme environment and diversification of life (Siddiqui *et al.*2013). They can also provide a clue on how life might be possible on other planets (exobiology) (Gao *et al.,* 2013), it became the most interesting topic after the discovery of liquid water on Mars. These kind of snow algae are solely responsible for the inorganic carbon fixation and climate change (Lyon and Mock *et al.,* 2014). Psychrophilic green algae has a higher level of lipids compared to mesophilic one so, it's the best option for biofuel production (Mou *et al.* 2012).

Growth temperature, physiological and environmental conditions for adaptations are very interesting topic to know. Confusion always exists between psychrophiles and psychrotrops. Psychrotrops are the permanent residents of the cold environment and can survive below freezing point but their maximal growth temperature is above 20ºC. But psychrophiles can survive at low temperature but grow optimally in the range of 15ºC-20ºC (Morita *et al*., 1975). Keeping in mind their remarkable diversity and variable habitats it's not easy to group them under a single definition of psychrophily. The alga we are discussing here comes under photopsychrophiles, the term is used in order to differentiate it from heterotrophs psychrophiles as it is photosynthetic in nature (Layboume-Parry and Pearce *et al.,* 2007). Culture-based study of snow algae or other psychrophilic algae requires knowledge of maximum temperature for its survival and growth. Psychrophilic verification can be done with the knowledge of their optimum growth temperature and upper survival temperature.

The upper survival temperature of psychrophilic alga depends on many factors, first factor is depends on the exposure time which is one day for unicellular species where it is three months for multicellular ones (Wiencke *et al.* 1994), development and reproduction of these organisms also depend on temperature. For example, the fungus *Myriosclerotinia borealis* produces vegetative hyphae during low temperature and when the temperature is high and non-permissive then it produces resting bodies (Newsted *et al.*1985; Newsted and Huner *et al.,* 1987). So it's complicated to understand the temperature ranges for survival of psychrophiles and their optimum growth temperature. Third maximal growth and temperature limits also depends on the growth media particularly its salt concentration, as in case of

green alga *Chlamydomonas sp.* (Eddie *et al.* 2008). The final factor which can affect the growth rate and survival of psychrophilic algae is their natural environment.

The traditional method to study taxonomy of snow algae was only based on light microscopy study of field sample (Fritsch *et al.* 1912). Recent taxonomic study of alga is done on the basis of multiple aspects like culture methods, light microscopy, electron microscopy and molecular phylogeny (Ling 2001, Hoham *et al.* 2006). Few genetic and taxonomic evidences are known as it's not an easy task to culture the field collected Zygotes. (Fukushima *et al., 1963*) had made an effort towards light microscopy based study of various snow algae from the collected sample of Japanese mountain. But no re-examination were done on those snow algae afterwards.

CHAPTER III
MATERIAL AND METHODS

3.1 Sample collection

The sample was collected from Dalk Glacier of Larsemann Hills, Eastern Antarctica on 12th January 2017 by Dr. Felix Bast. The place from where the sample was collected is placed above 116ft and of S 69° 23.628' E 76° 14.378' latitude longitude. The sample was collected in zip locked sample collection bag with pieces of ice. During collection and transport, it was kept in a mini refrigerator.

3.2 Light microscopy

Collected sample was observed under Olympus CX21i light microscope. Red zygotes were observed and clump of unicellular cells seen around mucilage sheet. Photograph of different stages after were taken under Magnus MSZ-TR microscope. Petri-plates and culture flask photos were taken using Canon EOS 60D camera.

3.3 Optimisation of media and growth conditions

Culture was grown under axenic condition. Pure culture was obtained by petri-plate culture method before liquid culture. 3N BBM + agar (12g/L) was mixed and poured in the Petri-plates and kept for solidification, covered with Para-film. After 24 hours algae were streaked on the plates and kept in sterile growth condition at low temperature. 3N BBM media contain the nutrients mentioned in (Table 1) and pH of media must be in between 6.4-6.8.

Inoculum was taken from petri-plate for liquid culture of 250ml Borosil flask. Flask was containing sterile media 100ml BBM media [3NBold's basal medium (Bischoff and Bold, 1963) kept in culture room in lower temperature. Different flasks were kept in different temperatures to optimise temperature for best growth. After two or three trial of culture procedure, culture media was slightly modified with 0.01gm/L vitamin B_{12} (Algenity 2016). Continuous light may harm the culture in initial stages so, light and dark cycle was maintained in duration of 12:12 hours. First successful liquid culture (A-6) was maintained at 20-22°C temperature range, 12:12 light and dark cycle in aseptic condition.

Table 1. Concentration of different nutrients in the 3N BBM media and amount of stock solutions used for media preparation.

	Type of nutrient	Weight	Quantity of stock solution (In ml)	Stock used for media preparation (In ml)
1	$NaNO_3$	3.75gm	100	10
2	$MgSO_4$	0.375gm	100	10
3	NaCl	0.125gm	100	10
4	K_2HPO_4	0.375gm	100	10
5	KH_2PO_4	0.878gm	100	10
6	$CaCl_2.2H_2O$	0.125gm	100	10
7	H_3BO_3	0.1142gm	10	1
8	$ZnSO_4$ MOD_3 $CO(NO_3)2.6H_2O$ $MnCl_2.4H_2O$ $CuSO_4.5H_2O$	0.0882gm 0.0071gm 0.0049gm 0.0144gm 0.0157gm	10	1
9	$FeSO_4.7H_2O$ H2SO_4 Concentrated	0.049gm 10ul	10	1
10	EDTA KOH	0.5gm 0.31gm	10	1

3.4 Cell culturing

For sub-culturing purpose inoculum was taken from pure culture flask (A-6), which is known as parent culture. Aseptic condition was maintained for every culture. Two Sub-cultures were maintained at two different temperatures one was 4ºC (A-10) and another is 20º-22ºC (A-11) for comparative growth study. Before sub-culture cell counting must be done so that each flask must contain an equal number of unicellular algae approximately. Haemocytometer count showed each ml of culture contains approximately 1×10^6 cells. During cell counting the number of cells in the culture was very high, so dilution was needed. After cell count dilution factor was multiplied by total cell count.

10µl of inoculum was taken for sub-culture of 250ml of flask. Cell count was done every time at an interval of 10 days in order to know growth rate. Repeated culture and subculture process were tried to obtain the best temperature range for optimal growth of alga.

3.5 Cell turbidity measure

The turbidity of cell signifies the growth of the culture, so absorbance can be the measure of cell growth. During analysis of spectrophotometer data more absorbance signifies more growth. Parent culture flask A-6 reading was taken in interval of 10 days, from 10^{th} day to 40^{th} days. Reading was taken at three wavelengths 664nm, 684nm, and 687nm in 96 well micro plate's reader. 100µl of a sample of 3 replicas was used to measure absorbance. Subcultures A-10 (maintained at 4°C), A-11 (maintained at 20-22°C) reading was taken in 5 days interval, from 5^{th} day up to 25 days. Same wavelengths were used that was used for A-6. Growth graph was plotted with the help of excel sheet.

3.6 Morphology analysis by phase contrast-microscope

Slides of algal culture were made by taking a drop of culture media containing unicellular alga. The slide was observed under phase contrast microscope and photograph was taken at focus of 10x, 40x, and 100x.

3.7 Flow cytometer analysis (FCM)

Flow cytometer analysis was done in order to study the stages of life cycle of the unicellular alga. DNA content of the unicellular alga can also be determine by FCM. DNA content and cell cycle stages analysis were done by treating algal cells with propidium iodide in BD C6 accuri flow cytometer.

3.7.1 Sample preparation: (propidium iodide staining)

Sample prepared into two aliquots one is for control purpose and other one is for staining purpose (treated).

1. Sample was placed in 2ml centrifuge tube and centrifuged for 5mins at 720g in room temperature.
2. Media was removed and the cell suspension was divided in to two aliquot.
3. Cell mass was re-suspended in 1ml PBS and the washing was done.
4. Then again PBS was added and centrifuged. Finally the supernatant removed, process repeated twice.
5. Then fixation of sample was done with 1ml mixture of 100% ethanol diluted with PBS in ratio of (1:1) and stored at 4°C for 40 minutes.
6. The sample was centrifuged at 200g for 10 minutes at 4°C and the supernatant was discarded.
7. Washing of cell was done for two times (repeat of step 3 and 4).
8. 50µl RNase (200µg/ml) was added, incubated for 30 minutes.
9. 100µl of propidium iodide was added to one of the aliquot (treated one) and the other was untreated.

3.7.2 Sample Run and Data analysis

The sample was run in flow cytometry. First, the untreated sample was run then treated one. Data analysis was done for gated population in reference to untreated one. Data was obtained in form of graph which shows different DNA content of cells.

3.8 DNA extraction

After we successfully managed to get enough cell mass through culture method, next step was the DNA extraction for molecular study. Proper protocol was followed for DNA extraction. Before DNA extraction sample harvesting was done.

3.8.1 Sample harvest process

Total cell mass in 250ml flask was used for a single DNA extraction process. 150mg of sample was harvested from a single flask for DNA extraction. Sample from the flask was directly poured to the centrifuge tubes and centrifuged by the help of ultracentrifuge machine. Supernatant was discarded and the mass of cells on the bottom of tube was harvested. By onetime centrifuge all cell mass could not be obtained so, process to repeat many times. Then sample extracted from the bottom of the tube with the help of sterile needle directly transferred in to the mortar-pestle.

3.8.2 DNA Extraction Protocol

1. 3ml of STE buffer was added to 6µl B.M.E, 6mg BSA and dissolved for 1 minute.
2. 1ml of S.T.E buffer was added to pre-chilled mortar-pestle and 150ml (cold stored) sample was added to it.
3. Silica beads were added to it and then crushed. 2ml S.T.E buffer was added again.
4. Crushed slurry was added to high shredder tubes
5. The sample was then centrifuged at 13,000 rpm for 2mins. Supernatant was discarded and pellet kept for the next processes.
6. 200µl of CTAB solution buffer added to each tube containing pellets and kept in rotospiner for 10 minutes.
7. 20µl RNAase was added to it, mixed and incubated for 10 minutes.
8. Next step was incubation of sample at 65°C for 30 minutes in water bath.
9. 400µl of Chloroform: Isoamyl alcohol (24:1 ratio) was added and mixed for 10 minutes. Centrifuged for 10 minutes at 13,000 rpm.
10. Three phases were separated. The upper phase was DNA, middle phase debris and lower phase is organic phase.
11. Only the upper phase was taken out which contained DNA.

12. Double volume (DNA volume) of ethanol (96-100%) Sand 0.1 volume of 3M sodium acetate of PH 5.2 was added and incubated at -20°C for 10 minutes.
13. Again centrifuged at 13,000 rpm for 10 minutes and supernatant was discarded.
14. 500µl of 70x chilled ethanol was added and centrifuged for 10 minutes at 13,000 rpm.
15. Supernatant was discarded and pellet kept for air dry for 1 hour to remove ethanol trace.
16. Lastly pellet was dissolved in 200µl elution buffer.
17. For short-term storage it will be kept in 2 to 8°C and for long-term storage in -20° to -50°.

3.8.3 Quantitative measurment of DNA content

DNA content was measured by taking reading at spectrophotometer (Nanodrop 2000 spectrophotometer).

3.8.4 DNA quality assessment

Quality of DNA was measured with the help of gel electrophoresis. A mixture of 60ml 50X T.A.E buffer and 0.48gm agarose (0.8% agarose) was made. The solution was gently mixed and boiled in micro-oven for 2 minutes. Then 2.4µl EtBr was added when sample cooled down slightly. Then solution was poured into gel casting tray and the appropriate comb was inserted into and left for solidification. When gel became solidified place it in running buffer in the Electrophoresis equipment. The mixture of genomic DNA (5µl) and loading dye (3µl) was added to wells formed on the gel. Then voltage, current and time were set into 100v, 85mv, 40 minutes respectively. After gel run, the final step was gel doc imaging.

CHAPTER IV
RESULTS

4.1 Significance of standardised media and culture conditions

4.1.1: Importance of adding B_{12} in media

Initially, BBM culture media was used for culture purpose but no significant growth was seen. During the morphological study, some contaminations was observed with our desired culture cells that might be some kind of fungi or bacteria. This alga requires vitamin B_{12} for cobalt requirement (Williams *et al*., 2003) so gets attached to fungi or bacteria. These bacteria and fungi might be the reason for inhibition of growth of alga. When cyanocobalamin was added from outer source in order to satisfy cobalt requirement, contamination gradually decreased. Cells started growing after reduction of contamination. hypnospore converted in to motile green zoospore and cell culture started looking green. In order to compare how Vitamin B_{12} affect the growth of *Chalamydomonas nivalis* one petri plate with agar and 3NBBM and another petri plate with agar, BBM and vitamin B_{12}. In comparison to without vitamin B_{12} medium, a better growth was seen in petri plate with vitamin B_{12} (Figure 3), although I don't measured the growth.

4.1.2 Importance of growing culture in different temperatures:-

Snow algae normally grow at a temperature below 10°C. So parent cultures were grown at low temperatures and gradually temperature was increased. Direct exposure to high temperature may cause degeneration of algal cells. Other reason was to do comparative growth rate study of algal culture in two different temperature one at 4°C and other at 20-22°C. Gradual increase in temperature made the culture habitual to high-temperatures. Exposure of snow algae culture to temperatures above 25°C resulted in cell death. The best growth was observed when the algal growth was maintained in dark: light cycle of 12:12 hours.

4.2. Morphological assessment of sample:-

Morphological assessment of collected samples, based on light-microscopic study shows that the initial stage of the sample were immobile, red, rounded zygotes of size 8-16µm. Chloroplasts were masked by a red coloured pigment which covers almost the whole surface of the cell. Aggregation of many cells was seen around

mucilaginous envelopes. Based on the characteristic features, this alga was identified as *Chlamydomonas nivalis*, according to Kol (1968), (Ettl *et al.* 1983) and (Komarek & Komarek *et al.,* 2001). After 45 days of culture in particular media, the whole culture became green. Morphology study under light microscopy and phase contrast microscopy showed different types unicellular alga cells in different stages of their life cycle, where some were small green flagellated cells, others were round and big green. Some unicellular cells fused with each other by their anterior portion and some cells were seen in clumps. In parent culture, there was no evidence of chloroplasts in red cells, while in cultured cells maximum fraction contained chloroplast. Some dividing cells were also noticed under phase contrast microscope**.**

4.3 Growth rate measurement

Turbidity measurement of cell culture was done in two phases, one was parent culture (A-6) and another culture was for sub cultures (A-10 and A-11). Growth of particular alga can be visualised from the (Fig; 8). Peti-plates culture growth can be compared from (Fig; 9), although I can't measure the growth.

4.3.1 Turbidity measure of parent culture

Growth rate of algal cultures was measured by turbidity measurement method at three wave lengths after 10 days interval.

Table 2: Turbidity measurement of parent culture A-6 sample with spectrophotometer.

Serial no	No of days	Absorbance at 664nm	Absorbance at 684nm	Absorbance at 687nm
1	10	0.006±0.005	0.006±0.0005	0.006±0.0001
2	20	0.050±0.009	0.055±0.009	0.055±0.007
3	30	0.069±0.015	0.071±0.0143	0.073±0.001
4	40	0.106±0.0251	0.110±0.025	0.107±0.027

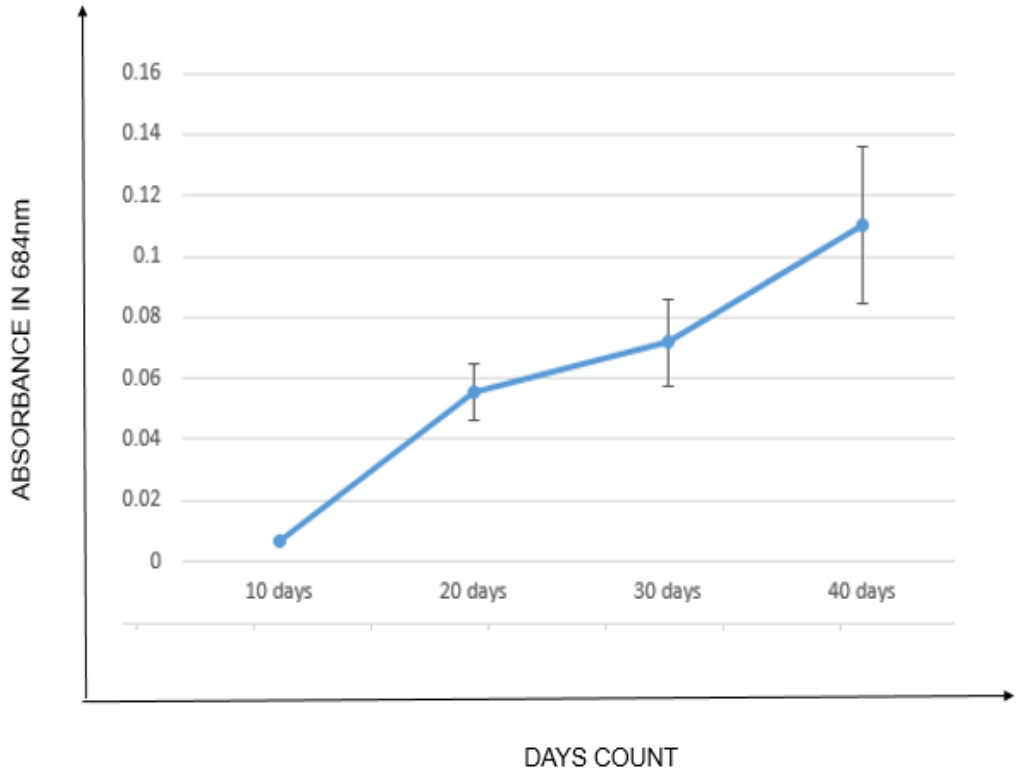

Figure 2: Growth curve for parent culture A-6.

According to the growth curve, initially growth rate was high for first 20-30 days but after that, growth became slow, for unknown reasons. After 30 days growth again increased, and maximum growth was seen up to 30-40 days. Out of three wave lengths, 684nm, and 687nm both were found suitable to optimally measure the turbidity of culture. Most appropriate wave length was 684nm.

4.3.2 Turbidity measure of sub-culture:-

Turbidity measure of two subcultures A-10 and A-11 signifies growth rate comparison for two temperature ranges. Comparing the growth of subcultures and parent cultures, we can conclude a better growth was obtained in subcultures than parent culture. Growth of subculture A-10 on 25^{th} day was more than growth of parent culture on 30^{th} day. Initial growth was quite slow for both the culture, growth rate was remarkably high in between 20-25 days for both the subcultures.

Table 3. Turbidity measurement of algal sub-culture A-10 (4°C sample) and A-11 (22°C sample) with spectrophotometer**.**

Serial no	No of days	Absorbance at 684nm (subculture in 4°C)	Absorbance at 684nm (subculture in 20-22°C)
1	5	0.00633±0.0005	0.0066±0.0057
2	10	0.00866±0.003	0.0113±0.0032
3	15	0.0133±0.0115	0.036±0.0115
4	20	0.042±0.0131	0.088±0.013
5	25	0.105±0.0165	0.2626±0.016

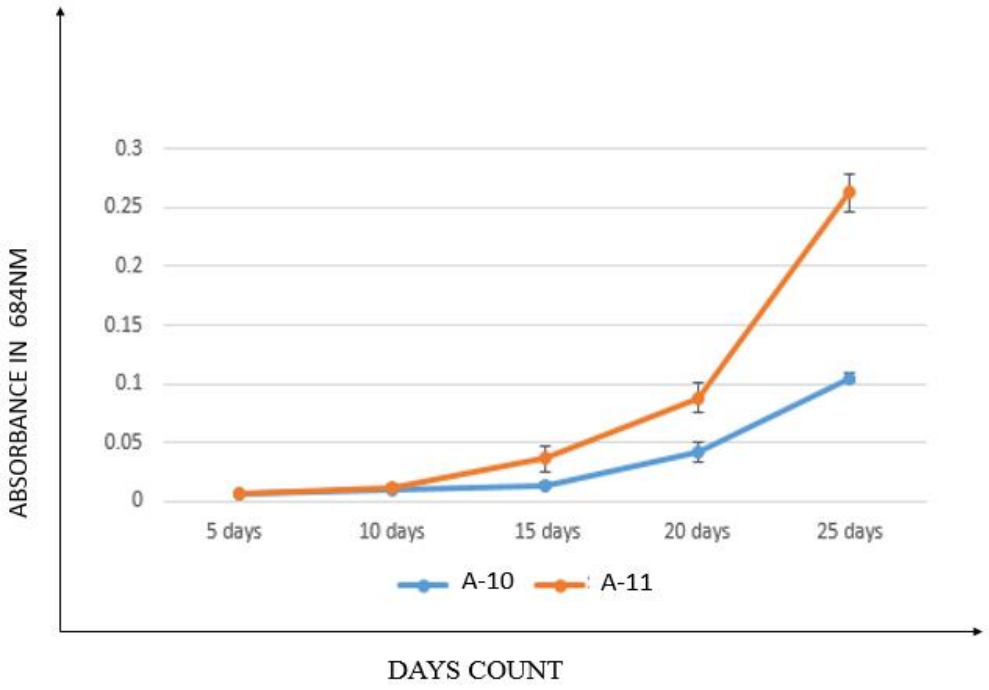

Figure 3: Comparative growth curve of subculture A-10 and A-11 at 684nm.

Data of (Table 2) and (Fig; 2), signifies that the culture was growing at normal temperatures like 20-22° C, so it might grow in normal room temperature in Polar regions during winters (when room temperature is ≤22°C). Data of (Table 3) and (Fig ; 3) graph of indicating towards the fact that better growth was obtained in case of sample A-11 (subculture in 20°C-22°C) than A-10 (subculture 4°C). Intermediate temperatures could not be obtained because we failed to maintain particular laboratory temperature for a long time. From close observation, it is clear that actively dividing green zoospore need a higher temperature than 10°C. Very Slow growth was seen below 4°C and below 0°C no actively dividing green cells were seen. On the basis of the comparative study, we can conclude that growth rate was increasing with increase in temperature but after 25°C it was seized. This result was due to gradual increase in temperature during successive subcultures, snow alga was becoming gradually habitual to higher temperatures. Up to our observation maximum temperature tolerance was 25°C.

4.4 DNA content and Ploidy level measure of cultured sample

The microscopic images (Figure 7.D) made it clear that cells of culture contain different types of cells. One is smaller in size, oval in shape and having less chlorophyll content and another one is bigger, round in size and contains more

chlorophyll. So we assume that sexual reproduction and asexual reproduction may be going on in the life cycle of *Chlamydomnas nivalis*, the different type of cells might be zoospore and zygote. In order to have further insights, DNA content of cells was measured to know ploidy level. We tried flow cytometry analysis of the cultured sample by staining with propidium iodide (Fig; 4.A) shows the unstained cell population and the (Fig; 4.B) is for stained one. Analyzed data shows three peaks, which are signifying three different ploidy level or different DNA content of cells. The peaks show different C values (C value is the haploid genome content of the cell). The first (highest) one show 1C value means haploid algae, intermediate one is 2C and last one 4C both represent hyperhaploid algae or ripened algae (Gerashchenko et.al 2010). From the data of gated population 1C is of 45.67%, 2C, and 4C are 6.45% and 4.97% respectively. From flow cytometry data it is clear that culture contains a different type of cells.

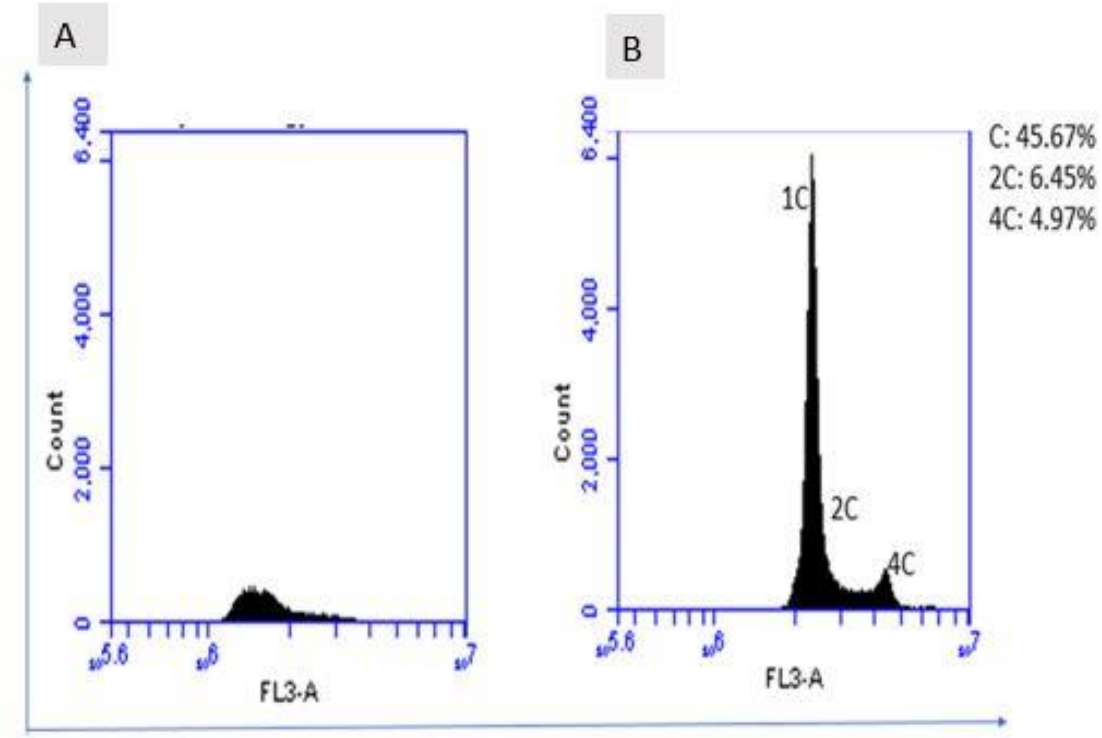

Figure 4: Flow cytometry data of DNA content analysis (A) unstained cell population (B) stained cell population.

1C DNA content is haploid type of cells and others 2C and 4C content cells are different from them. 2C and 4C were hyperhaploids. But exact stages of the cells

were not known. To the best of my knowledge the 2C content cells were zygotes, but there is need of further examination in order to prove they were zygote or not. From flow cytometry data it is clear that culture contains a different type of cells.

4.4 DNA quality and quantity analysis

Nano-drop reading showed 68.2 ng/µl nucleic acid concentration and A260/280 ratio was 1.6, which signified good quantity of DNA. Gel doc image clarified quality was not so pure because DNA didn't move far from well of gel. Further purification is needed to improve quality of DNA content.

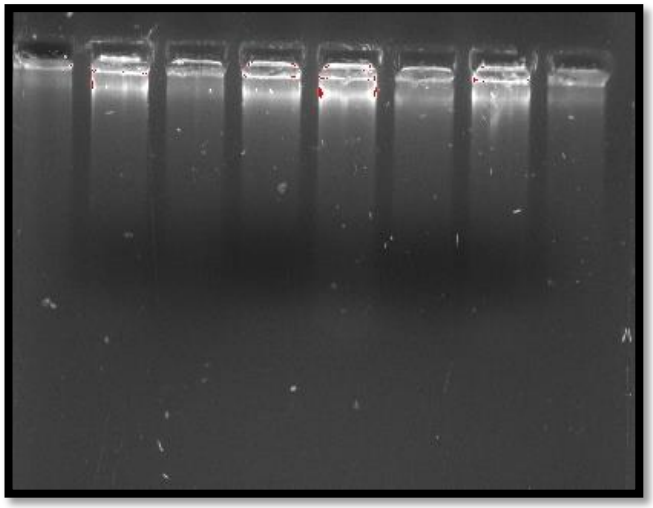

Figure 5: Image of Gel Doc contains genomic DN

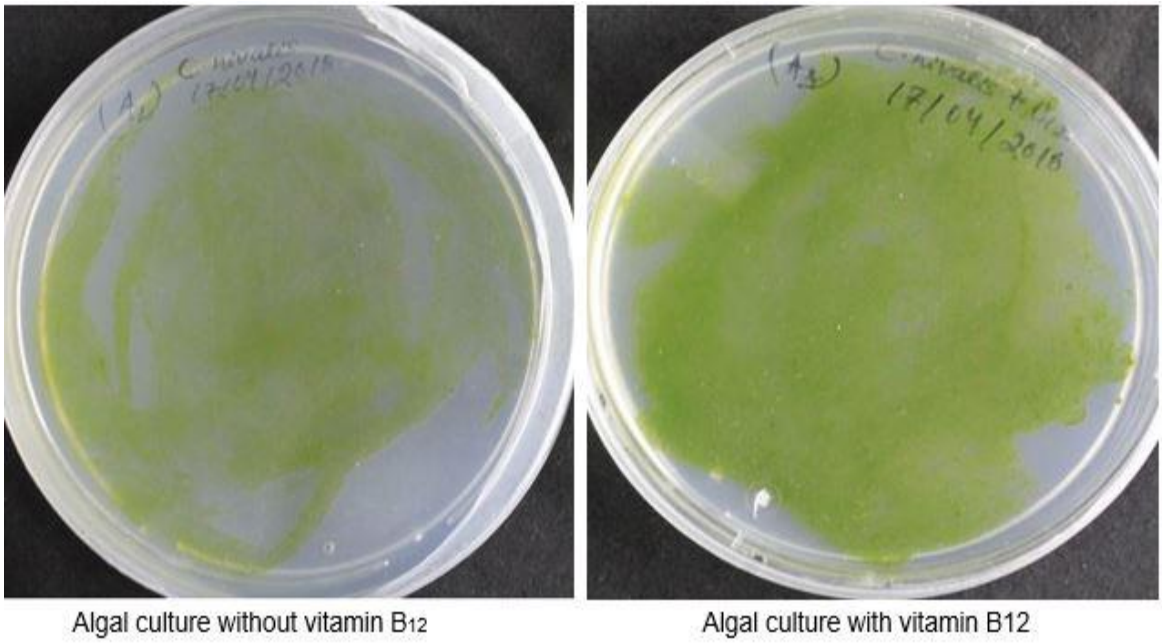

Figure 6: Growth difference of alga with and without presence of vitamin B_{12}.

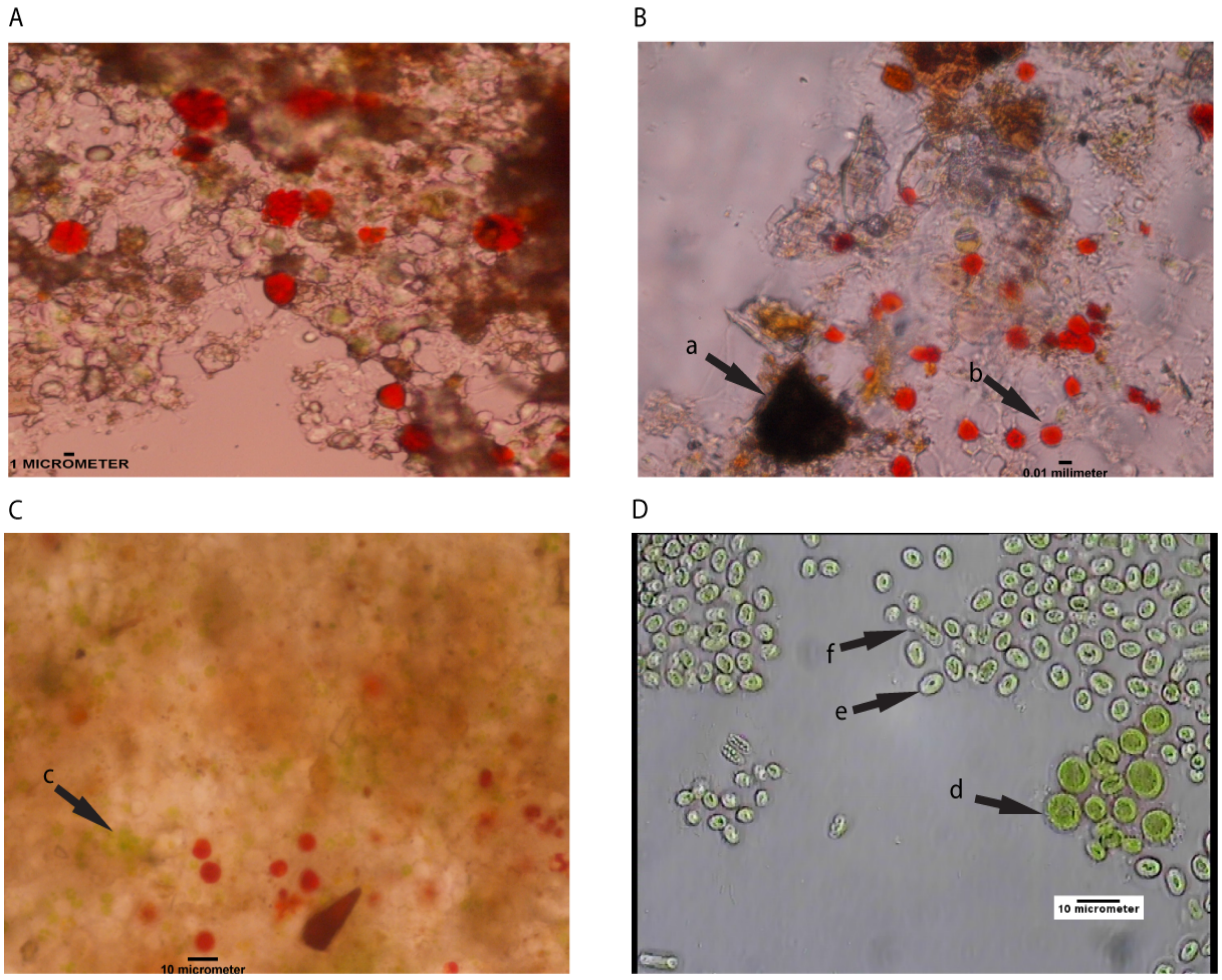

Figure 7: Image of (A) hypnospore of *chlamydomonas nivalis* with mucilaginous sheath of scale bar 1um (B) a. contamination of fungi during culture process (C) c. green cells produced from germinating of hypnospores.(D) after 45 days culture showing e) zoospores d) zygote, f) two zoospore undergoing for sexual reproduction.

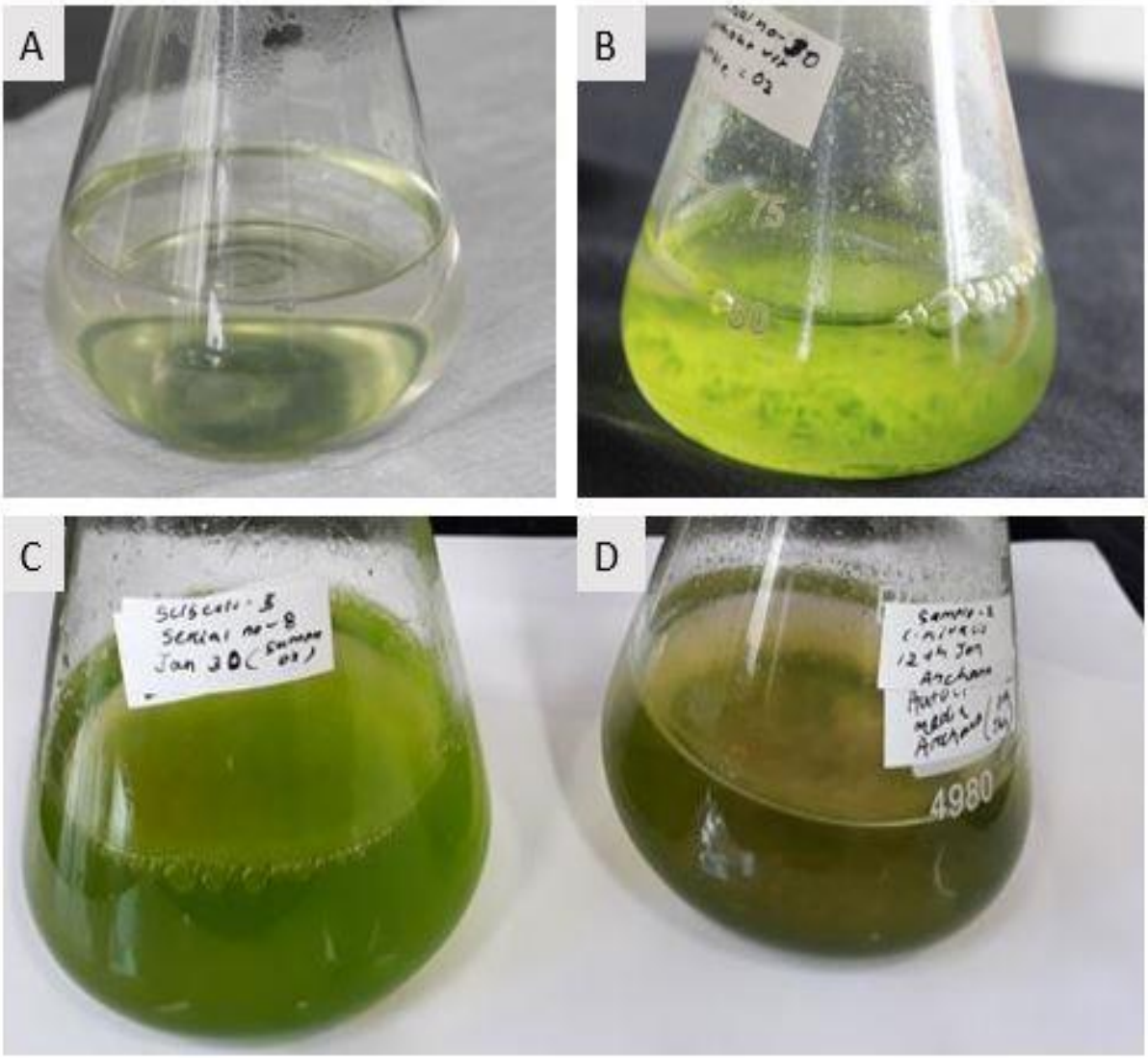

Figure 8: Image of liquid culture of alga (A) 5 days culture (B) 25 days culture (C) 45 days culture and (D) 65 days culture.

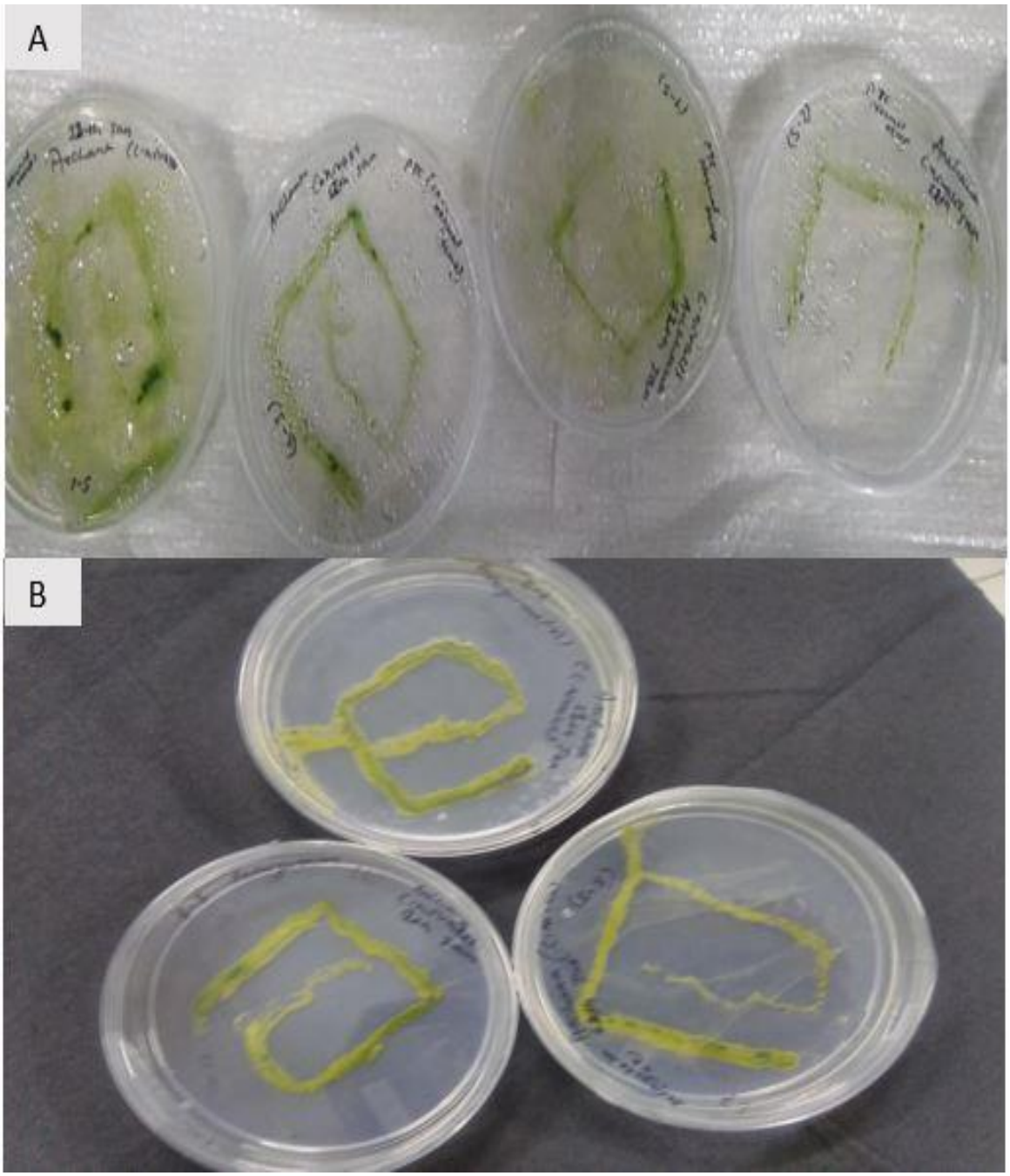

Figure 9: Image of petri plates of cultured alga initial stage A. on 10th day and B. on 50th day.

CHAPTER V
DISCUSSION

We able to grow this fastidious alga in two temperatures like 4 ºC and 20-22 ºC in normal laboratory conditions. Sample was collected in form of hypnospore (cyst stage) and we were able to germinate zoospores from the cyst in presence of proper media and suitable environmental condition. Previous studies field material was used for physiological and morphological study because *C. nivalis* is difficult to culture. To my best knowledge never been successfully grow in laboratory conditions (Remias *et al.* 2010). Many times algal culture became contaminated by heterotrophic bacteria (Kazamia et al 2012) and fungi (Williams et al., 2003) the probable reason might be the vitamin B_{12} requirement by alga. Vitamin B_{12} addition from outer source was fruitful and we were able to establish the importance of vitamin B_{12} for algal culture experimentally. Subcultures were maintained in different temperatures and light condition and turbidity measurement test was done. According to (Hoham 1975), Species normally do not grow above 10ºC are called true snow algae but interestingly that parent culture and one subculture were successfully grown at temperature more than 20°C-22°C. Best growth was seen in our experiment establishes the fact that optimal growth temperature and best growth period are 20°C-22°C and 30-40 days respectively. From cells morphology study under phase contrast microscope two kinds of cells were seen, expected cells zygotes and zoospores. To confirm this assumption flow cytometry was done and the result showed three peaks, which signifies three different DNA content of cells. One peak among three (the major one) represents haploid algae (1C). While two other peaks (the minor ones) represent hyperhaploid algae or ripened algae (2C and 4C) (Gerashchenko et.al 2010). From the FCM result, it is clear that our culture was growing and showing different stages of its life cycle but we are not enough confident enough to call the 2C as zygote. But yes, our culture has cells of different DNA content, so that in different stages of their life cycle. The haploid ones are zoospores and its population was high that means the culture was in the actively growing state. Enough cell mass also obtained for molecular study.

To the best of my knowledge there is no report of culture and characterization of *Chlamydomonas nivalis* from India, making this study the first report from the nation. Secondly, the sample was collected from Larsemann Hills Eastern Antarctica, there is no previous report of the presence of this alga in that region. Lastly, we were able to grow this alga at 20°C, so it can grow not only in low temperature but also in normal room temperature during winters. Stages of complicated life cycle of *Chlamydomonas nivalis* is bit clear now. We can say zoospores germinates from hypnospore or hypnoblast in the presence of proper nutrient, light and liquid medium.

CONCLUSION

Chlamydomonas nivalis, fastidious snow alga successfully grew in controlled laboratory condition. This alga grows at temperature above 20°C, signifies its novel adaptation strategy. Turbidity measurement infers that sub-culture grew at a faster rate than the initial culture because it gradually adapted to higher temperatures. We experimentally established the fact that Vitamin B_{12} is required for successful growth of this alga. FCM study showed different DNA content suggesting that cells were in different stages of its life cycle.

FUTURE PROSPECTIVE

Multilocal phylogeny reconstruction can be done using 18S rRNA, COX2-3 spacer to ascertain evolutionary relationship with other Antarctic and Arctic samples. Flow cytometry data can be obtained for different treated sample to analyze DNA content as well as the cell cycle stages. The growth of algae can be measured at more temperatures followed by its morphological study.

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