राष्ट्रीय ध्रुवीय एवं समुद्री अनुसंधान केन्द्र
पृथ्वी प्रणाली विज्ञान संगठन
पृथ्वी विज्ञान मंत्रालय (भारत सरकार)
हेडलैण्ड सडा, वास्को-डा-गामा, गोवा-४०३ ८०४, भारत
NATIONAL CENTRE FOR POLAR AND OCEAN RESEARCH
Earth System Science Organisation
Ministry of Earth Sciences, (Goverment of India)
Headland Sada, Vasco-da-Gama, Goa - 403 804, INDIA

CERTIFICATE

This is to certify that Ms. **Nivetha janani V** student of Goa University, Taleigao Plateau, Goa, has successfully completed a winter internship from **National Centre for Polar and Ocean Research** in the field of Arctic Ecology and Biogeochemistry from 01/12/2022 to 30/12/2022 under the guidance of **Dr. K.P. Krishnan** and under the supervision of **Dr. Anand Jain** and **Ms. Yuga Ghotge.**

During the period of her internship program with us, she had been exposed to various techniques and skills in the laboratory.

We wish her every success in her life and career.

05/04/2023
Supervisor

Archana Singh / अर्चना सिंह
Scientist / वैज्ञानिक
ESSO-National Centre for Antarctic and Ocean Research
ई.एस.एस.ओ.-राष्ट्रीय अंटार्कटिक एवं महासागरीय अनुसंधान केन्द्र
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हेडलैंड सडा, वास्को डा गामा, गोवा- 403 804

Archana Singh
05.04.2023
Authorized Signature
Scientist-In-Charge
Arctic Ecology & Biogeochemistry
Arctic Operations

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REPORT OF INTERNSHIP

NATIONAL CENTRE FOR POLAR AND OCEAN RESEARCH (NCPOR)

1ST DECEMBER 2022-30TH DECEMBER 2022

SUBMITTED BY

NIVETHA JANANI V,
M.Sc.,MARINE BIOTECHNOLOGY (II YEAR),
GOA UNIVERSITY.

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Certificate of Internship

Date :

To whom it may concern

This is to certify that Ms. NIVETHA JANANI V, Student of Goa University, has successfully completed a winter internship from National Centre For Polar and Ocean Research in the field of Arctic Ecology and Biogeochemistry from 01/12/22 to 30/12/22 under guidance of **Dr. K.P. Krishnan** and under the supervision of Dr. Anand Jain and Ms.Yuga Ghotge.

We wish her every success in her life and career.

……..……..……..

Authorised Signature

Acknowledgement

I would like to begin by thanking Dr. Savita Kerkar, Dean, School of Biological Sciences and Biotechnology, Goa University, for providing me with this wonderful opportunity to work at a prestigious research institute like NCPOR. I would also like to thank Dr. Sanjeev C. Ghadi, Vice Dean, School of Biological Sciences and Biotechnology, Goa University, for allowing us to intern and for helping us nurture our interest in research and academia.

I would like to extend my gratitude to Dr. K.P. Krishnan, Scientist F, NCPOR, for allowing us to work in his prestigious lab. I would also like to thank Dr. Anand Jain, Project Scientist,NCPOR for his guidance and insightful advice and direction regarding research work duringmy internship.

I would like to thank my professors Dr. Samantha Fernandes D'Mello, Dr. Dhermendra K. Tiwari, Dr. Meghanath Prabhu, Dr. Sanika Samant and Ms. Dviti Mapari for their invaluable support.

Lastly, I would like to thank Ms. Yuga Ghotge, Junior Research Fellow, NCPOR, for her guidance during my tenure at NCPOR.

Introduction

The National Centre for Polar and Ocean Research, (NCPOR) formerly known as the National Centre for Antarctic and Ocean Research (NCAOR) is an Indian research and development institution, situated in Vasco da Gama, Goa. It is an autonomous Institution of the Department of Ocean Development (DOD), Ministry of Earth Sciences, Government of India which is responsible for administering the Indian Antarctic Programme and maintains the Indian government's Antarctic research stations, Bharati and Maitri. NCPOR operates theHimadri and IndARC Arctic research stations in Svalbard, Norway and Himansh station in Spiti, Himachal Pradesh.

Dr. K.P. Krishnan specializes in Microbial Ecology and upon his recommendation, I was placed under Dr. Anand Jain who is a Project Scientist at NCPOR. Dr. Jain is currently working on the ecological and metagenomic studies of samples collected from fjords in Kongsfjorden in the Arctic. I worked with these samples under the guidance of Ms. Yuga Ghotge in the Polar Biology Lab, Cryobiology Lab as well the Deep Sea or F1 Lab on certainoccasions.

Kongsfjorden (Kongs Fjord or Kings Bay) is an inlet on the west coast of Spitsbergen, an island which is part of the Svalbard archipelago in the Arctic Ocean. Macroalgae are abundant in coastal Arctic habitats and contain a large amount of polysaccharides. Increasedmacroalgal productivity due to warmer temperatures and reduced sea-ice cover contribute a significant amount of polysaccharide-rich detritus in the region.

The objective of the experiments conducted was to obtain information regarding the microbial population in the fjords and co relate the growth of bacteria to the macroalgal density in their environment.

The steps involved are as follows :

1. Preparation of Slides
2. Observation of samples under fluorescence microscope
3. Visualisation of samples using LAS X software
4. Counting of bacterial cells to find population density

5. Calculation of volume of bacterial cells, both rod shaped and spherical shaped
6. Compilation of quantitative data
7. DNA extraction from samples
8. Measurement of concentration of DNA using spectrophotometer
9. PCR amplification of samples
10. Gel Elusion or cutting of gel to obtain amplified fragments
11. Measurement of DNA in eluted samples using Qubit Fluorometer
12. Preparation of Library using Ion Chef System
13. Sequencing of DNA
14. Media preparation and pouring
15. Plating of bacterial samples
16. Analysis of obtained colonies to assess effect of presence and absence of vitamins in media
17. Analysis of samples to obtain carbohydrate concentration

Principles

1. Preparation of Slides for Observation Under Fluorescence Microscope.

In order to observe the samples, the cells were stained with DAPI. DAPI is a fluorescent stain that binds strongly to adenine–thymine-rich regions in DNA. It is used extensively in fluorescence microscopy. As DAPI can pass through an intact cell membrane,it can be used to stain both live and fixed cells, though it passes through the membrane less efficiently in live cells and therefore provides a marker for membrane viability.

The cells were observed under fluorescence microscope and images were taken using LAS X software. Leica Application Suite X (LAS X) is a software platform for Leica microscopes. It integrates confocal, wide field, stereo, super resolution, and lightsheet instruments.

A fluorescence microscope uses a high intensity light source which excites a fluorescent species in a sample of interest. This fluorescent species in turn emits a lower energy light of alonger wavelength that produces the magnified image instead of the original light source.

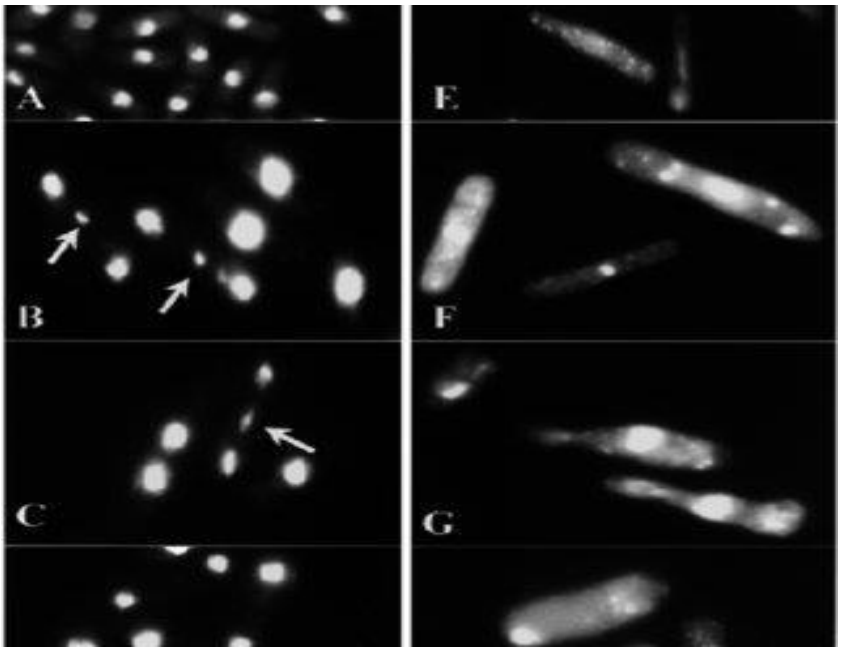

Figure showing bacterial cells under fluorescent microscope after staining with DAPI

2. Cell Volume Calculation

Reliable calculation of biomass of marine bacterioplankton is based on accurate measurement of bacterial cell size. In much of the work done to date (Fergu- son and Rublee, 1976; Bowden, 1977; Watson et al.,1977; Zimmermann, 1977; Fuhrman and Azam, 1980;Fuhrman et al., 1980) average cell volume has been used to estimate bacterial biomass. Cell sizes are determined by a variety of microscopic methods, including measurement of wet preserved cells by epi-fluorescence microscopy, measurement of dried preserved cells or carbon replicas by electron microscopy,and visual classification into specific size categories;
the accuracy of these methods has not been critically evaluated. Such studies have also tended to ignore the size distribution of bacterioplankton cells, except for classification into a few broad size categories; it is desirable to know size distributionmore accurately for studies of selective grazing by bacteriovores and the determination of the bacterioplankton contribution to optical properties of seawater. Bacterioplankton size was measured with a magnifier marked at 0.1 mm intervals.
Cells were categorized into spheres (volume= 4/3 n r3) and rods (cylinders with hemispherical caps, volume = rc r2 [L - 2/3 r]).
The data obtained was entered in the system.

3. DNA Extraction

DNA Extraction was done using a commercially available kit. There are five basic steps of DNA extraction that are consistent across all the possible DNA purification chemistries: 1) disruption of the cellular structure to create a lysate, 2) separation of the soluble DNA fromcell debris and other insoluble material, 3) binding the DNA of interest to a purification matrix, 4) washing proteins and other contaminants away from the matrix and 5) elution of the DNA.

1. Creation of Lysate

The first step in any nucleic acid purification reaction is releasing the DNA/RNA into solution. The goal of lysis is to rapidly and completely disrupt cells in a sample to release nucleic acid into the lysate. There are four general techniques for lysing materials: physicalmethods, enzymatic methods, chemical methods and combinations of the three.

2. Clearing of Lysate

Depending on the starting material, cellular lysates may need to have cellular debris removed prior to nucleic acid purification to reduce the carryover of unwanted materials (proteins, lipids and saccharides from cellular structures) into the purification reaction, which can clog membranes or interfere with downstream applications. Usually clearing isaccomplished by centrifugation, filtration or bead-based methods.

3. Binding to the Purification Matrix

4. Washing

Wash buffers generally contain alcohols and can be used to remove proteins, salts and other contaminants from the sample or the upstream binding buffers. Alcohols additionally help associate nucleic acid with the matrix.

5.Elution

DNA is soluble in low-ionic-strength solution such as TE buffer or nuclease-free water. When such an aqueous buffer is applied to a silica membrane, the DNA is released from thesilica, and the eluate is collected. The purified, high-quality DNA is then ready to use in a

wide variety of demanding downstream applications, such as multiplex PCR, coupled invitro transcription/translation systems, transfection and sequencing reactions.

DNA Extraction

4. PCR Amplification of DNA

The DNA obtained was first measured using spectrophotometer, following which it was amplified using PrimeSTAR PCR kit by Takara Bio. PrimeSTAR is an optimized mixture composed of PrimeSTAR HS DNA Polymerase developed by TaKaRa Bio Inc, reaction buffer and dNTP mixture as 2 fold concentration. As this product offers quick preparation ofreaction mixture and reduction of contamination risk, it is also useful for high throughput application. PrimeSTAR HS DNA Polymerase has a matchless proof reading activity due to very strong 3' to 5' exonuclease activity, and besides it's amplification efficiency is higher than that of Taq DNA Polymerase. Furthermore an antibody mediated hot start formulation prevents false inititation events during the reaction assembly due to mispriming and primer digestion. When used with Takara Bio's optimized reaction buffer, PrimeSTAR HS achievesthe high fidelity, high sensitivity, high specificity required application such as DNA amplification from cDNA library.

Step	Temperature	Time	Cycle
Initial denaturation	98°C	5 min	1 cycle
Denaturation	98°C	10 sec	30 cycles
Annealing	xx°C	5 sec	
Extension	72°C	1 min	
Final Extension	72°C	5 min	1 cycle
End/Hold	4°C	To hold	-

Figure showing typical PCR cycles for PrimeSTAR HS DNA Polymerase kit

5. Gel Cutting or Elution

Gel Elution was performed using the Wizard SV Gel and PCR Clean Up System made by Promega.

The Wizard® SV Gel and PCR Clean-Up System is based on the ability of DNA to bind to silica membranes in the presence of chaotropic salts. After electrophoresis to separate the DNA fragments, the band(s) of interest is excised and dissolved in the presence of guanidine isothiocyanate (Membrane Binding Solution). Alternatively, after amplification, an aliquot ofthe PCR is added to the Membrane Binding Solution and directly purified. The system allowsa choice of methods for isolation of DNA from the dissolved agarose gel slice or PCR amplification. DNA can be isolated using microcentrifugation to force the dissolved gel slice or PCR product through the membrane while simultaneously binding the DNA on the surfaceof the silica.

6. Measurement of eluted DNA using Qubit Fluorometer

The Invitrogen Qubit 4 Fluorometer accurately and quickly measures the concentration of DNA, RNA, or protein in a single sample. It can also be used to assess RNA integrity and quality. The easy-to-use touchscreen menus make it easy to select and run the assays you need, with results displayed in just a few seconds.

The Qubit fluorometer uses fluorescent dyes to determine the concentration of either nucleic acids or proteins in a sample. The most common method of measuring the concentration of nucleic acids and protein is the UV-absorbance method. This method uses a spectrophotometer to measure the natural absorbance of light at 260 nm (for DNA and RNA) or 280 nm (for proteins). The Qubit system requires purchasing of additional fluorescent dyes that bind specifically to analytes of interest such as double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), RNA, or protein providing more specific quantification with higher costs.

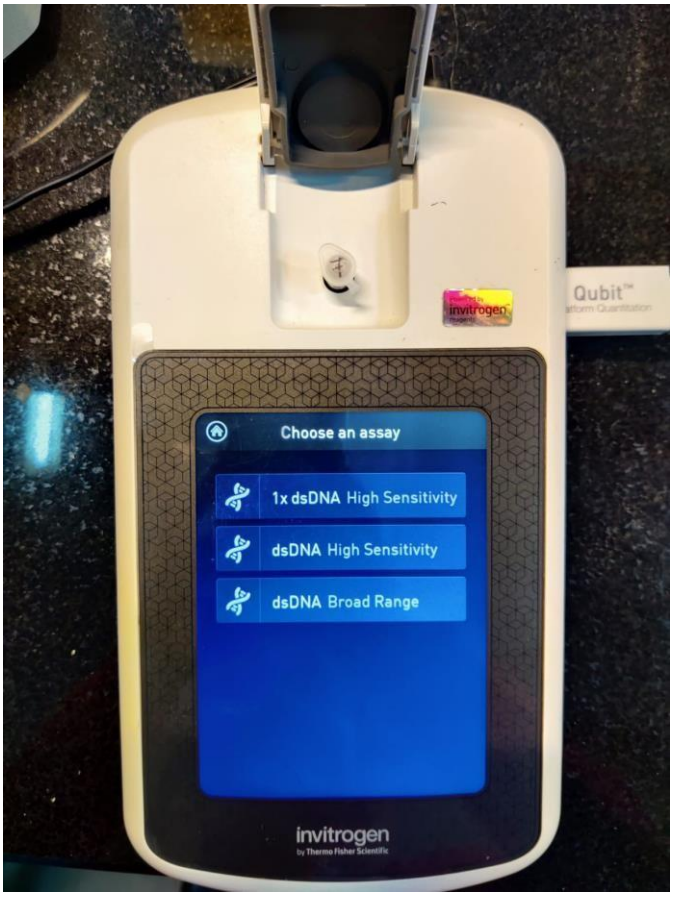

Figure showing Qubit Fluoro

7. Preparation of Library Using Ion Chef System

The Ion Chef System is the next generation of workflow simplification products for the Ion GeneStudio S5 Systems. The Ion Chef System provides automated library preparation, template preparation, and chip loading for users at any experience level. In less than 15 minutes of up-front hands-on time and with the use of pre-packaged library preparation reagent kits, the Ion Chef System provides a convenient walk-away workflow resulting in equalized, pooled libraries ready for templating. The automated workflow supports Ion AmpliSeq one- and two-pool designs. Automating sample preparation workflows on the IonChef System results in significantly higher productivity.

8. Media Preparation and Pouring

Modified Basal Medium was used to grow the bacterial cultures. Basal media, also known as simple media, are the growth media that support the growth of non-fastidious bacteria. They are also called general purpose media. Generally, basal media are useful for primary isolationof microorganisms.

Basal media contain amino acids, glucose, and ions (calcium, magnesium, potassium, sodium, and phosphate) essential for cell survival and growth. l-glutamine is an amino acidthat is essential for protein and nucleic acid synthesis and energy production in cell culture.Most media either contain l-glutamine or are substituted with it at the time of use.

8 samples from 8 locations each were taken. After preparing the MBM, different components were added as nutrient sources. Laminarin, Mannitol, and Sodium Alginate were added to different units of media in order to obtain growth.

The components of media were as follows:

Composition	Gram /litre	600
NaCl	25	15
KCl	0.75	0.45
$CaCl_2$	0.2	0.12

$MgSO_4$	7	4.2
NH_4Cl	0.5	0.3
Yeast extract	2	0.12
Peptone	0.3	0.18
K_2HPO_4	7	4.2
KH_2PO_4	3	1.8
Trace Metal Solution	1	0.6
Vitamin Solution	1	0.6
Distilled Water	1000 ml	600 ml
pH	7.0	7.0

9. **Quantitative Analysis of Carbohydrate In SamplesCollected**

Estimation of carbohydrate was performed using phenol sulfuric acid method in the Cryobiology Lab under the guidance of Mrs. Archana Singh who is currently working on mineral nutrition and biogeochemistry of polar samples.

The phenol-sulfuric acid technique is a fast and easy method for determining the amount of carbohydrates present in an experiment sample. It can detect virtually all kinds of carbohydrates including di-, mono-the -, and even oligo and polysaccharides. Although the method is able to detect nearly every type of carbohydrates, their absorptivity for the variouscarbohydrates differs. So, unless the sample is proven to have only one type of carbohydrate,the results should be calculated arbitrarily using one carbohydrate.

In this process, the concentrated sulfuric acid is able to break down all polysaccharides and disaccharides and oligosaccharides into monosaccharides. These pentoses (5-carbon substances) are later dehydrated to furfural and the hexoses (6-carbon substances) to the hydroxymethyl furfural. The compounds react with phenol, resulting in an orange-gold hue. If the product is extremely rich in xylose (a pentose) like bran from wheat or corn it is suggested to use xylose to create the standard curve used in the test, and then determine the absorption at 480 nanometers. If the product is high in hexose sugars, glucose is often used tobuild the standard curve. its absorption can be measured as 490nm. The color produced by

this reaction lasts for a long time and the precision of the procedure is within +-2% in appropriate conditions.

The results obtained were analysed using Agilent Biotek Gen 5 software.

Materials And Methods

1. Preparation of Samples

a. Preparing Solutions

1. Add 2 mL of deionized water (diH2O) or dimethylformamide (DMF) to the entire contents of the DAPI vial to make a 14.3 mM (5 mg/mL) DAPI stock solution. Note: DAPI has poor solubility in water, so sonicate as necessary to dissolve. The 5 mg/mL DAPI stock solution may be stored at 2–6°C for up to 6 months or at ≤–20°C for longer periods.

2. Add 2.1 µL of the 14.3 mM DAPI stock solution to 100 µL PBS to make a 300 µM DAPI intermediate dilution

3. Dilute the 300 µM DAPI intermediate dilution 1:1,000 in PBS as needed to make a 300 nM DAPI stain solution.

b. Labelling fixed cells

1. Wash the cells 1–3 times in PBS as needed.

2. Add sufficient 300 nM DAPI stain solution to cover the cells.

3. Incubate for 1–5 minutes, protected from light.

4. Remove the stain solution.

5. Wash the cells 2–3 times in PBS.

6. Image the cells.

2. Cell Volume Calculation

The cells were observed under fluorescence microscope and the dimensions of the cells were recorded using the LAS X Software. This data was entered in an excel sheet and the volume was calculated for various samples collected at different stationsin both macroalgae dense regions and less dense regions.

3. PCR Amplification

1. Add required reagents or mastermix and template to PCR tubes.
2. Mix and centrifuge.

   *Add mineral oil to prevent evaporation in a thermal cycler without a heated lid.
3. Amplify per thermo cycler and primer parameters.
4. Evaluate amplified DNA by agarose gel electrophoresis followed by ethidium bromide staining

- PCR Conditions:

.

(A) 3 step PCR

98℃	10 sec	⎫
55℃	5 or 15 sec	⎬ 30 cycles
72℃	1 min/kb	⎭

(B) 2 step PCR

98℃	10 sec	⎫
68℃	1 min/kb	⎭ 30 cycles

First try 3 step PCR when using PrimeSTAR HS DNA Polymerase.

· Denaturation conditions:	98℃, 5 - 10 sec Alternatively, if a denaturation temperature lower than 94℃ is used, set the denaturation time to10 - 15 sec.
· Annealing temperature:	Initially, try 55℃ (optimization may be required.)
· Annealing time:	Annealing time is dependent upon primer Tm values. Calculate primer Tm values using the following formula (*) ; When Tm ≧ 55℃ Set for 5 sec When Tm < 55℃ Set for 15 sec

(*) Tm value (℃) = [(the number of A and T) x 2] + [(the number of G and C) x 4] - 5

The above Tm value formula is valid for primers whose lengths are ≦ 25 mer. For primers longer than 25 mer, an annealing time of 5 sec should be used.

- Optimization of Parameters:

To obtain the best PCR results, it is important to optimize reaction parameters when using PrimeSTAR HS DNA Polymerase.

1) Enzyme amount

In general, 1.25 units of enzyme per 50 μl reaction is recommended. Depending upon the amplified fragment size, purity, and amount of template, the amount of enzyme may be adjusted accordingly.

2) Template DNA amount

Recommended template DNA amounts (assuming a 50 μl reaction):

Human genomic DNA:	5 - 200 ng
E. coli genomic DNA:	100 pg - 100 ng
cDNA library:	1 - 200 ng
λ DNA:	10 pg - 10 ng
Plasmid DNA:	10 pg - 1 ng

Avoid excess template DNA, which can lower enzyme reactivity.

Templates containing Uracil, such as bisulfite-treated DNA, cannot be amplified with this enzyme.

3) dNTP and Mg^{2+} concentration:

Because dNTPs have a chelating effect, excess dNTPs will lower the effective Mg^{2+} concentration in the reaction mixture. The supplied 5X PrimeSTAR Buffer provides 1 mM final Mg^{2+} concentration that has been optimized for use with a 200 μM final dNTPs concentration in the reaction mix. Thus the concentration of dNTPs should not be modified.

Note: Do not substitute dUTP for dTTP in the PrimeSTAR HS reaction mix. The use of dUTP drastically decreases enzyme reactivity.

4) Primer and PCR condition

The use of commercially available primer design software, such as OLIGO Primer Analysis Software (Molecular Biology Insights) is recommended for obtaining appropriate primer sequences that follow general primer design guidelines and can be tailored specifically for your template DNA.

Guidelines for Primer Design:

a) Primer length:	For general amplification of DNA fragments, 20 - 25 mer primers are suitable. Exact PCR conditions should be determined by referring to "VI. PCR conditions".
b) Modified bases:	Never use primers containing inosine (I) with PrimeSTAR HS DNA Polymerase.
c) Degenerate primers:	Degenerate primers may be used with PrimeSTAR HS DNA Polymerase.

5) Annealing conditions

Annealing conditions should be determined by referring to "VI. PCR Conditions". When low yield of amplified PCR product is obtained, troubleshoot as follows:

<Smearing and/or extra bands appear on agarose gels>

i) Shorten the annealing time. For example, decrease time from 15 to 5 sec.
ii) If the annealing time is already 5 sec, then raise the annealing temperature to 58 - 65℃.
iii) Try 2 step PCR.

<Target product is not amplified.>

i) Extend the annealing time. For example, increase time from 5 to 15 sec.
ii) Lower the annealing temperature to 50 - 53℃.

- Electrophoresis, Cloning and Sequencing of Amplified Products

1) Electrophoresis of the amplified product

TAE Buffer is recommended for agarose gel electrophoresis of amplified products that are obtained using PrimeSTAR HS DNA Polymerase.

Note: Use of TBE Buffer may result in DNA band patterns which are enlarged at the gel bottom.

2) Termini of amplified products

Most PCR products amplified with PrimeSTAR HS DNA Polymerase have blunt end termini. Accordingly they can directly be cloned into blunt-end vectors (if necessary, phosphorylate before cloning), but can not be cloned directly into T-vectors. Mighty Cloning Reagent Set (Blunt End) (Cat. #6027) is recommended for cloning into blunt-end vectors.

3) Restriction enzyme reaction

Prior to performing restriction enzyme digestion of amplified PCR products, remove all traces of PrimeSTAR HS polymerase from the reaction mix by phenol/ chloroform extraction or DNA extraction by NucleoSpin Gel and PCR Clean-up (Cat. #740609.10/.50/.250). In particular, the restriction site produced with enzymes arising 3' -protruding cleavage sites, such as *Pst* I, may be digested by residual 3' → 5' exonuclease activity of PrimeSTAR HS DNA Polymerase, resulting in deletions.

4) Direct sequencing

Perform phenol/chloroform extraction or DNA extraction using NucleoSpin Gel and PCR Clean-up (Cat. #740609.10/.50/.250) of PCR products prior to direct sequencing and ensure inactivation of 3' → 5' exonuclease activity.

4.Media Preparation And Plating Of Samples

The following steps were involved:

1. The various components were weighed and dissolved in water
2. The media was autoclaved
3. Laminarin, mannitol and alginate were not autoclaved but filter sterilised
4. The media was poured in the plates and the plates were allowed to cool
5. Bacterial samples were spread on the plates and the plates were incubated for 1 week in order to observe growth

Results

Cell Volume Calculation : The bacterial cells indigenous to macroalgae dense regions had larger volume than the cells collected from macroalgae poor regions. The number of cells was higher in the case of the former as opposed to the latter. Thus, there seems to be a direct correlation between the macroalgal content in the area and the bacterial population. This is due the fact that the bacteria utilise the macroalgae as a source of nutrition and therefore the population density is higher in geographical areas having greater amount of macroalgae

**The remaining figures were not shared with us in order to maintain confidentiality.*