Prophage induction from polysaccharide degrading bacterial strain DP203

A Dissertation for

Course code & Course Title: MBT- 651 Dissertation

Credits: 16 credits

Submitted in partial fulfillment of Master's Degree

M.Sc. in Marine Biotechnology

by

Aryan Jarkharya

22P0500004

Under the supervision of

Prof. Sanjeev C Ghadi

School of Biological Sciences and Biotechnology

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Seal of the School

DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, **"Prophage induction from polysaccharide degrading bacterial strain DP203"** is based on the results of investigations carried out by me in the **Biotechnology discipline** at the **School of Biological Sciences and Biotechnology, Goa University** under the supervision of **Prof. Sanjeev C Ghadi** and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will not be responsible for the correctness of observation/ experimental or other findings given in this dissertation.

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Date: 08/04/2024

Aryan Jarkharya

Place: Goa University

Seat No: 22P0500004

COMPLETION CERTIFICATE

This is to certify that the dissertation report **"Prophage induction from polysaccharide degrading bacterial strain DP203"** is a bonified work carried out by Mr. **Aryan Jarkharya** under my supervision in partial fulfilment of the requirements for the award of the degree of **Master's in Marine Biotechnology** in the Discipline Biotechnology at the **School of biological Sciences and Biotechnology, Goa University.**

Prof. Sanjeev C Ghadi

Date: 08/04/2024

School Stamp

Signature of Dean of the School

Dean of School of Biological Sciences & Biotechnology
Goa University, Goa-403206

Date: 8/4/24

Place: Goa University

PREFACE

This dissertation explores study of prophage induction from polysaccharide-degrading bacteria isolated from the marine environment. With a focus on understanding the mechanisms and ecological implications of this phenomenon, the research delves into the complex interplay between bacteria and their viral counterparts, bacteriophages, within the oceanic realm.

Motivated by a fascination for marine microbial ecology, this journey of discovery has been guided by the expertise and support of my supervisor, **Prof. Sanjeev C Ghadi.** Additionally, collaboration with colleagues and the unwavering encouragement of family and friends have been instrumental in shaping this research.

As this dissertation contributes to our understanding of marine microbial communities, I am grateful for the opportunity to explore the mysteries of the sea. It is my hope that this work will inspire further inquiry and deepen our appreciation for the intricate relationships that govern life in the marine environment.

Aryan Jarkharya

08/04/2024

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TABLES

Figures

ABBRIVATIONS USED

Abbreviations used	Full form
ASW	Artificial Sea Water
CAZymes	Carbohydrate Active Enzymes
C	Celsius
CO_2	Carbon Dioxide
CPs	Complex Polysaccharides
DNA	De- oxy ribose Nucleic Acid
DOM	Dissolved Organic Matter
E. Coli	*Escherechia Coli*
F	Forward
Fig	Figure
g	Gravitational Force or RCF
M	Molar
Mit C	Mitomycin C
mL	Mililiter
ng	Nano Gram
nm	Namo meter
PCR	Polymarase Chain Reaction
PFU	Plaque Forming Unit

PULs	Polysaccharide Utilization Loci
R	Reverse
RCF	Relative Centrifugal Force
RNA	Ribose Nucleic Acid
rpm	Revolutions Per Minute
rRNA	Ribosomal RNA
SEM	Scanning Electron Microscope
sp	Species
UV	Ultra Violate
ZMA	Zobell Marine Agar
ZMB	Zobell Marine Broth
°	Degree
ʎ	Lambda
µg	Micro Gram
µL	Micro Liter
µm	Micro Meter
%	Percent
ϕ	Phage

ABSTRACT

Marine ecosystem comprises of oceans, estuaries, coral reefs and coastal ecosystems which cover about 70% of Earth's surface. Complex polysaccharides (CPs) like alginate, agar, carrageenan, ulvan, laminarin, pullulan etc play a diverse role in ecological as well as biological functions in the marine environment. The bacterial strain DP203 was isolated from the coastal water of Goa, India. Strain DP203 was found to be agarolytic and carrageenolytic in nature. Furthermore, induction studies were carried out by heat shock method and Mit C treatment method. Prophages were induced by the above two methods and were isolated by double agar overlay method. The burst size was found to be very low and hence, the titre was insufficient for phage purification and concentration. Hence, new techniques need to be found out in order to get the high phage titre.

CHAPTER 1:
INTRODUCTION

1.1. Background

Water-based habitats with interactions between biotic and abiotic elements are referred to as aquatic ecosystems. Usually, they are divided into freshwater and marine environments. Less than 1% of the Earth's surface is made up of freshwater habitats. On the other hand, the marine ecosystem, which is the biggest water ecosystem, comprises of oceans, estuaries, coral reefs, and coastal ecosystems, occupying more than 70% of the Earth's surface (Bashir et al., 2020). A wide range of seaweeds can be found in aquatic environments, and the majority of these seaweeds' cell walls include complex polysaccharides (CPs). CPs such as cellulose, agar, alginate, carrageenan, pullulan, laminarin, carboxymethyl cellulose, starch etc. play an important role in ecological functions, biological activities and also have numerous pharmaceutical applications (Imran et al., 2017; Laurienzo, 2010). CPs are linear or branching chain polymers made up of repeated monosaccharide units; these units typically comprise more than ten sugar molecules connected by glycosidic linkages (Arnosti et al., 2021; Imran et al., 2017). Different functional groups modify the repeating units that form these polysaccharides, rendering them resistant. Because of this, resistant CPs are well suited to provide host marine species with structural integrity (Cumpstey, 2013; Imran et al., 2017).

About half of the world's primary output comes from the oceans, and a large amount of that organic matter is rapidly respired back into the form of CO_2. Heterotrophic prokaryotes play a major role in this fast cycle process by effectively breaking down organic materials. Certain bacteria such as *Microbulbifer* sp strain CMC5, *Saccharophagus degradans, Vibrio alginolyticus, Bacillus* sp. etc. have been isolated from various marine environments, these includes- sediments, deep sea, coastal waters, and the surfaces of crustaceans and seaweeds. (Arnosti et al., 2021; Giljan et al., 2022; Imran et al., 2017; Zimmerman et al., 2013). Carbohydrate active enzymes (CAZymes) aid in the breakdown of CPs, they are clustered

and regulated polysaccharide utilization loci (PULs) (Arnosti, 2011; Arnosti et al., 2021; Martens et al., 2008).

Bacteriophages also known as “phages” are viruses which infect diverse group of microorganisms including archaea, bacteria, and single celled eukaryotes (algae and protozoa) (Debarbieux et al., 2017; Ireland, 2023). Bacteriophages are acknowledged as the most prevalent biological agent on Earth and are present in all environments (Debarbieux et al., 2017; Kasman & Porter, 2023).

Phages based on their life cycles are categorised into ‘lytic phages’ or ‘lysogenic phages’ (Clokie et al., 2011; Hobbs & Abedon, 2016) as shown in (Fig- 1.1.1). After infection, lytic phages cause their host cell to lyse whereas, lysogenic phages integrates into the host genome or plasmid and termed as ‘prophages’, and the bacteria contains prophages are known as lysogenic bacteria (Clokie et al., 2011; Elois et al., 2023; Howard-Varona et al., 2017; Kasman & Porter, 2023).

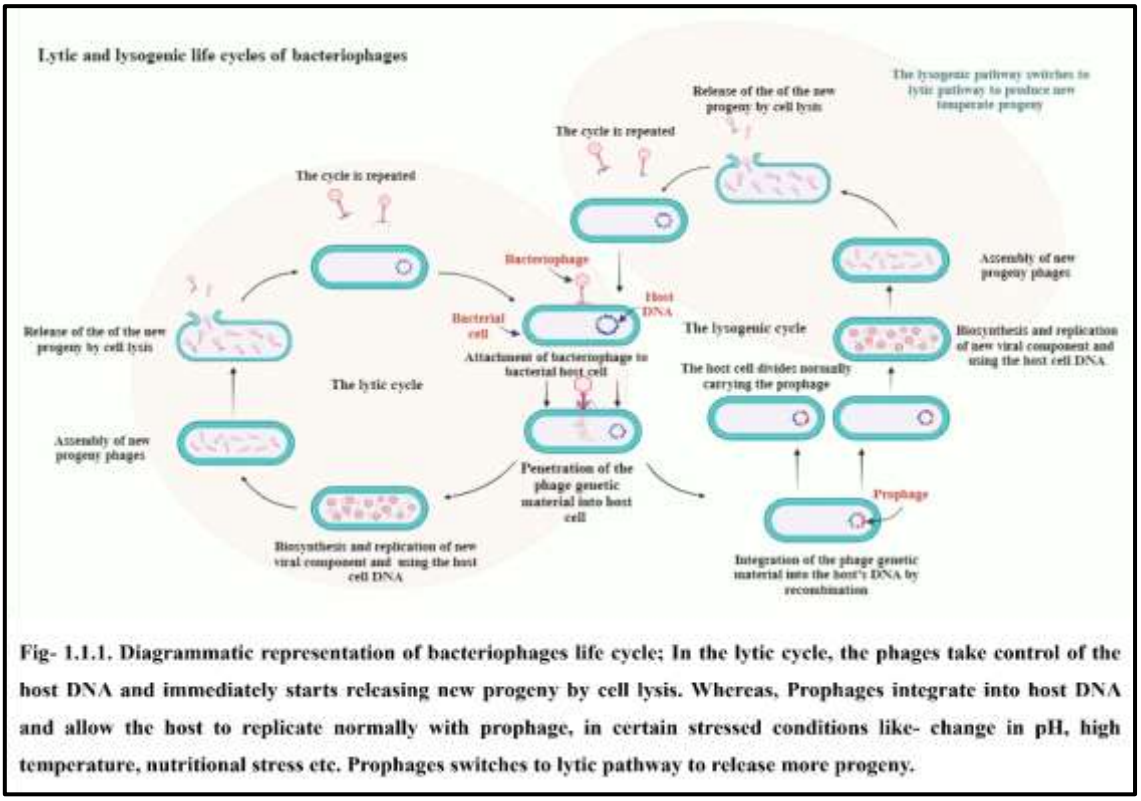

Fig- 1.1.1. Diagrammatic representation of bacteriophages life cycle; In the lytic cycle, the phages take control of the host DNA and immediately starts releasing new progeny by cell lysis. Whereas, Prophages integrate into host DNA and allow the host to replicate normally with prophage, in certain stressed conditions like- change in pH, high temperature, nutritional stress etc. Prophages switches to lytic pathway to release more progeny.

The viral lytic genes are not expressed when the virus is in the prophage stage. When the host experiences stress conditions, prophage induction takes place. As a result, the lytic genes start to express and prophage separates from the host genome. The lytic cycle then restarts, causing the lysis of the bacterial cell. Plaques are indicative of the lysis (Canchaya et al., 2004; Howard-Varona et al., 2017).

The effects of lysogeny are thoroughly studied at the cellular level, but their effects on ecosystems are not as well understood. The three stages of lysogeny progression are the creation, upkeep, and induction of productive cycles. Temperate phages 'choose' to initiate lysogenic cycles as prophages or go through lytic cycles. A number of factors, including phage density, nutritional availability, and genetic compatibility, influence the decision to enter lysogeny (Casjens & Hendrix, 2015; Silveira et al., 2021). Prophages then multiply and integrate into the bacterial chromosome. Prophages can also modify cellular functions causing evolutionary changes in the host bacteria during this stage (Bobay et al., 2013). When external stresses disrupt the host bacteria's DNA, the lytic to lysogenic switch takes place. This leads to the excision of the prophage and the activation of lytic genes, which in turn cause the host cells to lyse (Silveira et al., 2021). Changes in physiological conditions like pH, temperature, nutrients or exposure to antibiotics, foreign DNA or DNA damaging agents may lead to the induction of prophage (Banks et al., 2003; Rossi et al., 2022). Bacteriophages infect epiphytic bacterial parasite that break down polysaccharides help to maintain the steady concentration of polysaccharides in the marine environment (Imran, et al., 2017; Poduval et al., 2018).

1.2. Aims and Objectives

To study the prophage induction from polysaccharide degrading bacterial strain DP203

- Characterization of polysaccharide degrading bacteria from the coast of Goa.
- Phylogenetic studies of bacterial strain DP203.

- Induction studies of lysogen in bacterial strain DP203 by using Mitomycin C and heat shock method.

1.3. Hypothesis

In marine ecosystem CP degrading bacteria are found in abundance- their ability to hydrolyze CP into its simpler forms has piqued an interest of researchers worldwide to harness this ability to biotechnological potential. In the recent years a number of novel phages have been isolated from CP degrading bacteria which have shown potent research applications which is very less explored. This research is carried out to study the characteristics of the CP degrading bacteria isolated from the Coastal water of Goa, India and we hypothesize the presence of the novel bacteriophages which can be further studied for biotechnological applications.

1.4. Scope

Studying prophage induction from polysaccharide degrading bacteria in marine environment offers an intricate approach to understanding the complex interplay between bacteriophages and their hosts in aquatic ecosystem which includes-

- To investigate the ecological roles of marine polysaccharide degrading bacteria- plays a crucial role in marine carbon cycling.
- To understand the dynamics of prophages and their impact on their bacterial hosts in marine environment.
- To identify the novel prophage with unique genetic elements or functional characteristics which could be used for biotechnological processes like- bioprospecting or bioremediation.

CHAPTER 2:
LITERATURE REVIEW

2.1. Oceanic polysaccharide

The oceans provide half of the world's primary output, the majority of which is swiftly respired back into CO_2. This fast cycle is mostly caused by heterotrophic prokaryotes resulting efficient breakdown of organic resources. The knowledge of marine nitrogen, phosphorus, and sulphur cycles far surpasses that of the microbially driven organic carbon cycle. Compared to other key molecule classes including proteins, lipids, and nucleic acids, little is known about polysaccharides and their biogeochemical processes. The separation and analysis of polysaccharides are particularly difficult due to analytical issues with polysaccharide solubility, size, and lack of easily tracked spectroscopic properties. However, recent research on prokaryote genes, transcripts, and metagenomes have demonstrated the significance of polysaccharides and their modifications for ocean carbon metabolism (Teeling et al., 2012). Ocean acidification is the term used to describe the progressive decrease in seawater pH caused by the buildup of anthropogenic carbon dioxide (CO_2). The ability of the ocean to store CO_2 is mostly determined by biological processes like Photosynthesis, respiration and biological pump. Heterotrophic bacteria's breakdown and subsequent respiration of organic molecules is the primary source of CO_2 in the ocean. whereas, few studies have been conducted about how ocean acidification might affect the activity of microorganisms that degrade materials (Piontek et al., 2010).

2.2. Polysaccharide degrading microbiological communities in marine environment

In order for bacteria to utilize polysaccharides, they must first hydrolyse them into oligo- and monomers. This is done by excreting hydrolytic enzymes that have varying substrate specificities (Zimmerman et al., 2013). Although the use of biopolymers has been continuously associated with the phylum *Bacteroidetes*, other marine bacterial taxa, such as *Gamma proteobacteria*, are also capable of producing hydrolytic enzymes

(Fernández-Gómez et al., 2013; Tang et al., 2012; Wietz et al., 2015). Numerous marine bacterial species exist that may break down the polysaccharides listed in (Table 2.2.1)

Table 2.1.1. List of CP degrading bacteria in marine ecosystem having different CAZymes

S. no	Bacteria	CAZymes present in polysaccharide degrading bacteria	references
1.	*Agarivorans* sp.	Agarase, alginate lyase	(Fu et al., 2008; Hu et al., 2009; Kobayashi et al., 2009)
2.	*Alteromonas* sp.	Alginate lyase, ulvan, agarase	(Koch et al., 2019; Sawabe et al., 1997; Wang et al., 2006)
3.	*Bacillus* sp.	Agarase, carrageenase	(Kang & Kim, 2015; Prabha et al., 2012)
4.	*Microbulbifer* sp.	Agarase, Alginate lyase, carrageenase, chitinase, cellulase, Xylanase, carboxymethyl cellulose, pectinase	(Gonzalez et al., 1997; Jonnadula et al., 2009; Vashist et al., 2013; Yoon et al., 2003)
5.	*Saccharophagus degradans*	Agarase, Alginate lyase, chitinase, cellulase, fucosidase, laminarin, pectinase, polysaccharide lyase, Xylanase	(Ekborg et al., 2005; Huang et al., 2022)

Bacteria need CAZymes with the right structural specificity in order to use polysaccharides as substrates. These enzymes' structural and functional specificities dictate which organic matter is hydrolyzed to smaller sizes and then taken in by a cell for additional metabolism. (Arnosti, 2011). The CAZymes that catalyze the hydrolysis of a particular polysaccharide are frequently grouped and controlled inside PULs (Martens et al., 2008). PULs have the mechanisms required for detecting, binding, depolymerizing, and absorbing polysaccharides (Martens et al., 2009; McKee et al., 2021; Sichert & Cordero, 2021). They also show great specificity for the polysaccharide they are trying to uptake. A few enzymes encoded in compact PULs can effectively break down simple polysaccharides like starch or laminarin (Becker et al., 2020; Martens et al., 2009), but a cascade of up to 21 debranching enzymes, each acting in a particular sequence before reaching the polysaccharide backbone, is needed to break down CPs like pectin or ulvan. Fucoidan, a highly branched and sulfated polysaccharide found in brown algae, is extremely complicated and requires hundreds of enzymes to break down (Reisky et al., 2019; Sichert et al., 2020; Sichert & Cordero, 2021).

2.3. The abundance of bacteriophages in the marine ecosystem

Like bacteria, a large diversity of bacteriophages is present in the ocean. The ocean is thought to be home to 10^{30} viruses (Weynberg, 2018), with an average of 10^{7} viruses per mL of surface seawater (Vincent & Vardi, 2023). Ten times as many viruses as prokaryotes exist, and each virus causes about 10^{28} new infections per day. Marine viruses are found in a variety of habitats. While viruses in open waters have a concentration of 10^{4}–10^{5} particles per mL, those in coastal waters have a higher concentration of 10^{6}–10^{7} particles per mL because of upwelling currents and terrestrial runoff (Suttle, 2007; Weynberg, 2018). With 10^{7}–10^{10} particles of viruses per gramme of dry sand, viruses are equally prevalent in sediments as they are in the aqueous column

(Jacquet et al., 2010). Marine phages influence community makeup, global biogeochemical cycles, and bacterial abundance through lysing their bacterial hosts (Breitbart, 2012).

2.4. Biochemical effects of bacteriophages

In order to proliferate, phages- try to eradicate their bacterial hosts. Most of the viruses identified in the oceans are assumed to be phages. Phage killing affects global biogeochemical cycles, releasing dissolved organic materials (DOM), and controlling microbial abundance. (Breitbart, 2012). The production rates of marine viral communities vary daily, ranging from 10^8-10^{11} viruses per litre. This results in turnover times varying from 0.09-3.5 days (Jacquet et al., 2010). The viral shunt is facilitated by the daily release of about 10^9 tonnes of carbon from approximately 10^{28} viral infections (Suttle, 2007). This method eliminates carbon from the traditional food chain and also functions as a marine microbial recycling program encouraging the cycling of nutrients and energy. 25% of the carbon fixed by sunlight is thought to cycle via the viral shunt (Breitbart, 2012). Viral-infected systems tend to retain more nutrients in the euphotic zone than non-infected systems, where the nutrients are eventually absorbed by larger species that descend to the deep sea (Finke et al., 2017).

2.5. Life cycle of bacteriophages

Bacteriophages are composed of protein and nucleic acid components that initiate a phage generation cycle when they infect a bacterial cell. Through the use of phage-specific proteins to start several pathways, an infected bacterial cell is reprogrammed to become a factory for phage production. The production of structural proteins usually happens after nucleic acid replication. Through cell lysis, new phage particles are assembled and expelled from the cell. Approximately hundred phages can be produced during an entire cycle, which takes forty minutes. These type of phages are called as temperate phages (Campbell, 2003). Conversely, lysogeny's molecular understanding is derived from a small number of

E. coli phages, including λ and Mu. They integrate into the bacterial chromosome by random transposition or targeted recombination (Casjens & Hendrix, 2015; Harshey, 2014) while others have circular (like- P1 (Łobocka et al., 2004) or linear- like N15 (Ravin, 2015)) genomes that are maintained extra chromosomally. For infection, some temperate phages, such P4, depend on other phages, like P2 (Christie & Calendar, 2016). Some, such as the cholera-causing Vibrio phage CTXphi, continuously infect their host and merge during lysogenic cycles (McLeod et al., 2005). Lysogeny typically involves establishment, maintenance, and possibly induction of productive cycles.

2.6. Lysogeny in marine environment

Prophage DNA is incorporated into bacterial chromosomes in a process known as lysogeny, which is a symbiotic interaction between bacteria and prophages. Prophages have the ability to cause host cells to lyse, releasing the phage particles. Lysogeny is common in marine bacteria with around 28-71% of isolates containing prophages. Through horizontal gene transfer, prophages are known to have a significant impact on the genomic diversification of bacteria (Zhao et al., 2010). These prophages also help in regulating gene expression and function (Feiner et al., 2015). Prophages might grant the host new functions, which could change the physiology of the cell (Hargreaves et al., 2014). Like lytic phages, induced prophages are capable of infecting nearby bacterial colonies. By modifying the patterns of gene expression, they can affect the host's physiology and release nutrients. This affects the local marine ecosystem by causing DNA transfer or recombination events and by supplying virulence traits (Kieft & Anantharaman, 2022; Nanda et al., 2015).

2.7. Isolation of prophage

Despite being quiescent, prophages have the ability to explode, much like molecular time bombs, causing host lysis in high-stress situations (Paul, 2008; Zhao et al., 2010). A significant proportion (28–71%) of marine bacterial isolates were found to have prophages

that can be induced on by Mitomycin C (Mit C) or UV radiation (Jiang & Paul, 1998; Stopar et al., 2003; Zhao et al., 2010). The activation of the lytic-lysogenic switch can occur spontaneously at a low frequency (10^8–10^5 per cell for λ (Czyz et al., 2001) or in response to external stressors that cause CI inactivation, such as those that trigger the cell's DNA damage response (the SOS response). Changes in nutrition, pH, or temperature, as well as exposure to antibiotics, hydrogen peroxide, alien DNA, or chemicals that damage DNA, are examples of stressors (Casjens & Hendrix, 2015; Howard-Varona et al., 2017; Mell & Redfield, 2014). Likewise, the heat induction method has demonstrated potential in comprehending λ genome regulation and has been employed to generate prophages. It has also been used to investigate temporal gene expression. (Rokney et al., 2008). All prophages are not amenable to standard methods such as Mit C, and induction frequently requires chemical or physical therapy (Paul, 2008). Marine phage isolation is a potent technique that aids in the identification and interpretation of unknown sequences in viral metagenomics and aids in the discovery of new bacteriophages (Zhan et al., 2016). Bacteriophages specific to marine bacteria belonging to genera *Vibrio, Psychrobacillus, Acinetobacter, Erythrobactor* have been reported (Kokkari et al., 2018; Lin & Tsai, 2022; Liu et al., 2022; Lu et al., 2017; Yang et al., 2010). Also, bacteriophages infecting CP degrading bacteria have been previously studied in different contexts including phage H17- 5c and phage H17- 9b belonging to *Vibrio harveyi* and *Vibrio alginolyticus* (Kitamikado et al., 1992; OKANO et al., n.d.; Suginta et al., 2000), ϕ RIO- 1 belonging to *Pseudoalteromonas marina* (Hardies et al., 2013), ϕ 40:2 belonging to *Flavobacterium, cellulophaga baltica* (Holmfeldt et al., 2007; Nam et al., 2007), ϕMC1 belonging to *Microbulbifer* strain CMC- 5 (MTCC 9889) (Poduval et al., 2018).

CHAPTER 3:
METHODOLOGY

3.1. Sample collection

Bacterial strain DP203 was obtained from the culture collection (Discipline of Biotechnology, SBSB, Goa University). Strain DP203 was previously isolated from DonaPaula beach, Goa, India. The geographical location is mentioned in the (Table 3.1.1) and sampling site location is mentioned in (Fig 3.1.1).

Table 3.1.1. Geographical location of sampling site

Date and Time of the year	Location of sampling site	Description of site	Sample type	Co- ordinates
20/07/2021	DonaPaula Beach	Sandy	Coastal water	15°27’17.4” N 73°48’17.5” E

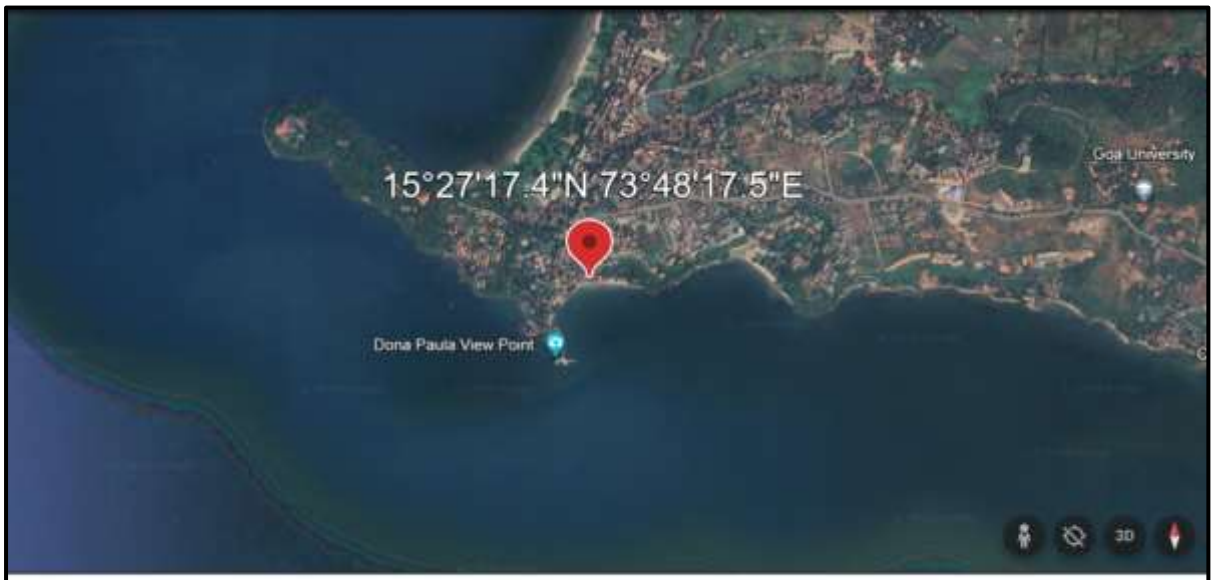

Fig- 3.1.1. Sampling site location in Goa, India

3.2. Growth medium for the isolation of host bacteria

The strain DP203 was isolated using Artificial Sea Water (ASW) supplemented with 2% agar (Appendix- II).

3.3. Dye based screening method for detection of polysaccharide degradation

For the detection of selective polysaccharide breakdown, dye- based plate assays are a popular and highly specific method (Ruijssenaars & Hartmans, 2001). The agarolytic activity of the bacterial strain was detected by streaking the culture DP203 on ASW agar plates. Likewise, ASW gel rite plates containing 2% of alginate and carrageenan respectively, were streaked and incubated for 48 hours at 30°C for the detection of alginolytic and carrageenolytic activities. Furthermore, the plates were flooded with Lugol's iodine solution, 10% sulphuric acid and phenol red dye on ASW agar plates, ASW-alginate and ASW- carrageenan plates respectively.

3.4. Scanning Electron Microscopy (SEM) analysis

In order to analyse host bacteria by SEM, 2 mL of a 24 hours grown culture was used. Bacterial pellet of DP203 was rinsed with saline and resuspended in 2 mL sodium phosphate buffer. A loopful of reconstituted bacterial culture was spread onto a glass coverslip and allowed to air dry. this was followed by an overnight fixation with 25% glutaraldehyde, then washed with 0.1 M sodium phosphate buffer and a series of ethanol treatments (20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100%) given at 10 minutes intervals. The sample was analyzed after being sputter coated in gold.

3.5. Biochemical and carbohydrate utilization tests

Biochemical tests were performed for strain DP203 by using [KB002 HiAssorted™ Biochemical Test Kit (for Gram- negative rods)]. Similarly, Carbohydrate utilization tests for strain DP203 were also employed by using [KB009 HiCarbo™ Kit (KB009A / KB009B1 / KB009C)].

3.6. Gram staining

In order to check the gram character, overnight grown culture DP203 was Gram stained by using Gram Stains- kit K001- 1KT and then observed under compound microscope under 100X objective lens.

3.7. Isolation of genomic DNA

1.5 mL of overnight grown culture was used and DNA was isolated by using (HipurA@ Bacterial Genome DNA Purification Kit MB505). The eluted DNA was run on 0.7% agarose gel electrophoresis and the bands obtained were visualized using UV transilluminator. The concentration of isolated DNA was determined using Qubit 2.0 Fluorometer.

3.8. Polymerase Chain Reaction (PCR)

PCR was performed to amplify the 16S rRNA gene. the PCR reaction mixture components and parameters are mentioned below (Table 3.8.2 and 3.8.3). DNA obtained was amplified by using the bacterial universal primers as mentioned in (Table 3.8.1).

Table 3.8.1. Bacterial universal primers (Dos Santos et al., 2019)

27F	5’- AGAGTTTGATCCTGCCTCAG- 3’
1492R	5’- TACGGTTACCTTGTTACGACTT- 3’

Furthermore, agarose gel electrophoresis was carried out of the amplified PCR product and the bands obtained were visualized under a UV transilluminator.

Table 3.8.2. Reaction mixture of the PCR

PCR components	Volume (µL)
Sterile miliQ water	40
10X Taq buffer	5
dNTPs	1
Template DNA (50 ng)	1
Forward primer	1
Reverse primer	1
Taq polymerase	1
Total volume	50

Table 3.8.3. PCR parameters

Steps	**Temperature**	**Time**	**Total number of cycles**
Initial denaturation	95°C	2 minutes	
Denaturation	95°C	30 seconds	30 cycles
Annealing	51.9°C	30 seconds	
Annealing	72°C	1 minute and 33 seconds	
Final Elongation	72°C	8 minutes	

The amplified PCR product was sent for sequencing to Eurofins Genomics India Pvt. Ltd.

3.9. Induction of prophage

3.9.1. Heat shock method (Rokney et al., 2008)

The bacterial strain DP203 was grown overnight, and then diluted to a ratio of 1:100 in fresh ZMB and incubated at 30°C. After 1 hour 30 minutes, the culture was subjected to heat shock treatment at 42°C for 1, 2 and 3 minutes respectively. The culture was

transferred to 50 mL falcon tubes and centrifuged at 10,000g for 10 minutes at 30°C following each time interval, and the untreated culture was used as control. Additionally, the cell pellet was resuspended in 200 µL of sterile ZMB, which was then used as phage lysate, and stored at 4°C.

3.9.2. Mit C treatment method (Rokney et al., 2008; Zheng et al., 2014)

Overnight grown DP203 culture was diluted to 1:100 using a fresh and sterile ZMB and incubated at 30°C at 200 rpm in a shaker incubator until the absorbance reached 0.2. 100 mL of culture was then supplemented with 1 µg/mL of Mit C, and the incubation was carried out at 30°C with 120 rpm in a shaker incubator. One flask with untreated culture was used as control. Absorbance was continuously measured every hour for a period of 6 hours, 2 mL of aliquots from the Mit C treated culture were taken in microfuge tubes and centrifuged at 10,000 rpm for approximately 2 minutes at 30°C. Additionally, the supernatant was removed, and the filtrate was kept at 4°C for later use after being filtered using 0.22 µm syringe filter.

3.10. Isolation of induced prophage by double agar overlay method

Double agar overlay method was used to isolate the induced prophages (Kropinski et al., 2009; Stachurska et al., 2021) by Mit C treatment and heat shock method. 4- 5 mL Zobell Marine Agar (ZMA), which containing 0.8 % agar, was made and heat blocked at 100°C in a water bath. Additionally, 1mL of host culture was carefully mixed with 100 µL of phage lysate and 4- 5 mL of ZMA. The mixture was poured aseptically onto the ZMA plate, allowed to solidify and incubated for 24 hours at 30°C.

CHAPTER 4:
ANALYSIS AND CONCLUSION

4.1. Host bacteria

The host bacteria DP203 was grown on ASW agar and ZMA plates as shown in (Fig- 4.1.1) and the colony characteristics were observed on ZMA plate are listed down in (Table4.1.1)

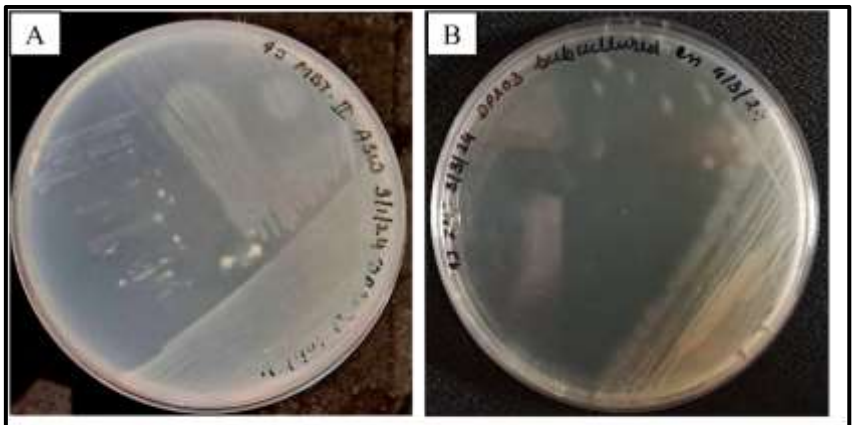

Fig- 4.1.1. Growth of bacterial strain DP203 on ASW and ZMA agar plates; (A) Growth of bacterial strain DP203 on ASW agar plate at 30°C; (B) Growth of bacterial strain DP203 on ZMA plate at 30°C

Table 4.1.1. Colony characteristics of bacterial strain DP203

Colony Characteristics	Size	Form	Color	Elevation	Margin	Opacity	Consistency
Observations	2mm	Irregular	Cream	Flat	Irregular	Opaque	Smooth

4.2. Dye based screening

Dye- based screening of bacterial strain DP203 was performed, strain DP203 was showing clear zone on ASW agar plate after flooding with Lugol's iodine which suggests that the strain DP203 is agarolytic in nature. Similarly, the strain DP203 had shown clearance zone on ASW gel rite plate containing 0.2% of carrageenan after flooding with Lugol's iodine which suggests that the strain DP203 is also carrageenolytic in nature as shown in (Fig- 4.2.1).

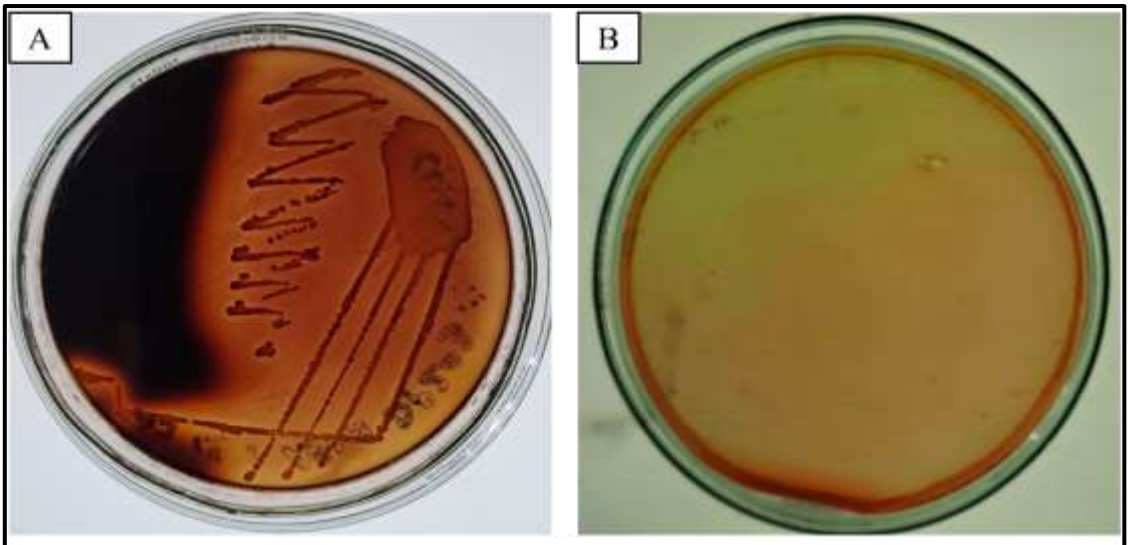

Fig- 4.2.1. Screening of polysaccharide degradation ; (A) Plate A is showing agarolytic activity on ASW agar plate after flooding with Lugol's iodine of strain DP203; (B) Plate B is showing carrageenolytic activity on ASW carrageenan plate after flooding with Phenol red dye.

4.3. SEM analysis of host bacteria

SEM analysis was performed by following the above-mentioned protocol. Small rods can be observed at 20,000X magnification and the bar is corresponding to 2 µm as shown in (Fig- 4.3.1).

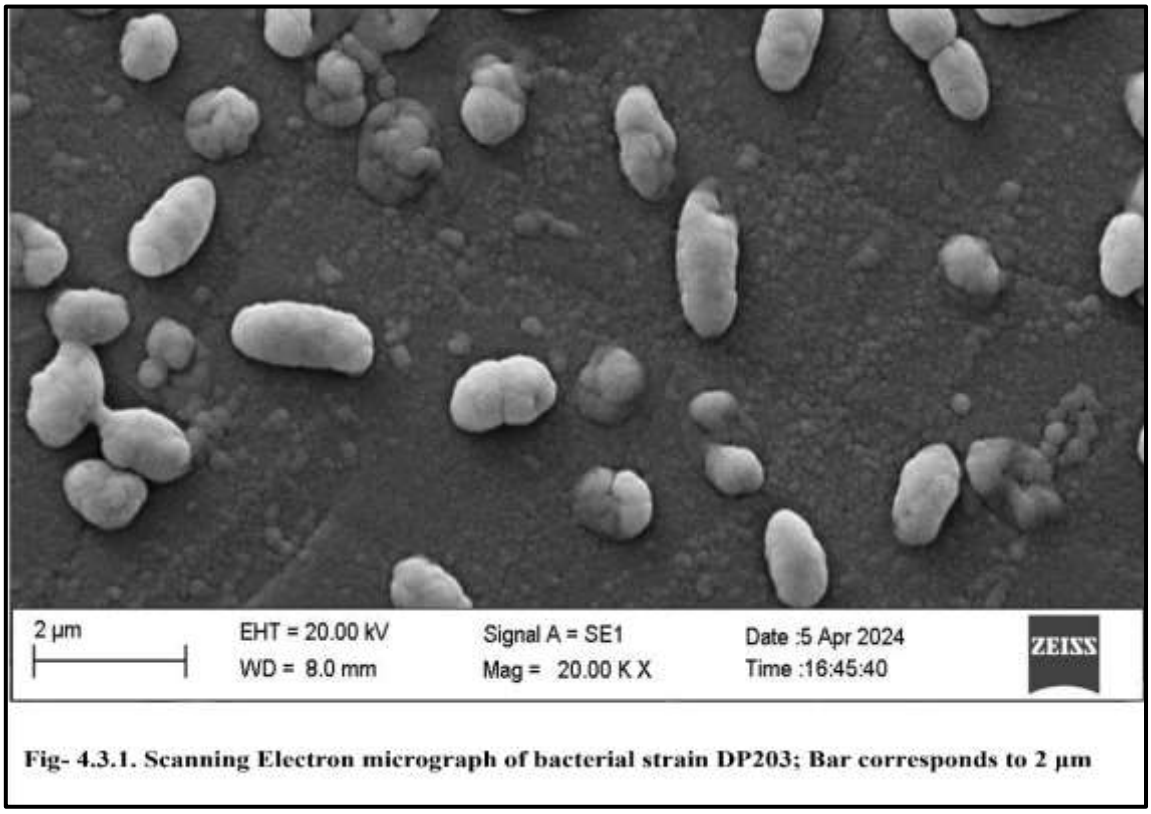

Fig- 4.3.1. Scanning Electron micrograph of bacterial strain DP203; Bar corresponds to 2 µm

4.4. Biochemical and carbohydrate utilization tests

Biochemical test for strain DP203 were performed and results were interpreted as per the interpretation chart provided in the kit (Fig 4.4.1) and results are listed down in (Table 4.4.1).

Fig- 4.4.1. Biochemical test of strain DP203

Table 4.1.1. Observation table for biochemical test results

S. No	Test	Observations
1	Citrate utilization	-
2	Lysin utilization	-
3	Ornithine utilization	-
4	Urease	-
5	Phenylalanine Deamination	-
6	Nitrate reduction	+
7	H_2S production	-
8	Glucose	+
9	Adonitol	v
10	Lactose	-
11	Arabinose	v
12	Sorbitol	v
Note: (+) sign represents the positive results; (-) sign represents the Negative results; (v) sign represents the 11- 89 % positive results		

Bacterial strain DP203 is showing only two positive results as mentioned in observation table. The results obtained are not similar to any of the species mentioned in the interpretation chart provided with the kit.

Similarly, carbohydrate utilization test for strain DP203 were performed and results were interpreted as per the interpretation chart provided in the kit (Fig 4.4.2.) and results are listed down in (Table 4.4.2.).

Fig- 4.4.2. Carbohydrate utilization test of strain DP203; (A) KB009 part-A of the test kit contains 12 Carbohydrate utilization test; (B) KB009 part-B of the test kit contains 12 Carbohydrate utilization test; (C) KB009 part-C of the test kit contains 11 sugar utilization tests and one control

Table 4.4.2. Observation table for carbohydrate utilization test results

S. No.	Test	Observations
1.	Lactose utilization	-
2.	Xylose utilization	V
3.	Maltose utilization	+
4.	Fructose utilization	+
5.	Dextrose utilization	+
6.	Galactose utilization	v
7.	Raffinose utilization	v
8.	Trehalose utilization	+
9.	Melibiose utilization	-
10.	Sucrose utilization	v
11.	L- Arabinose utilization	v
12.	Mannose utilization	+
13.	Inulin utilization	v

14.	Sodium gluconate utilization	+
15.	Glycerol utilization	+
16.	Salicin utilization	+
17.	Dulcitol utilization	v
18.	Inositol utilization	v
19.	Sorbitol utilization	v
20.	Mannitol utilization	+
21.	Adonitol utilization	v
22.	Arabitol utilization	v
23.	Erythritol utilization	-
24.	alpha- Methyl-D- glucoside utilization	-
25.	Rhamnose utilization	v
26.	Cellobiose utilization	+
27.	Melezitose utilization	v
28.	alpha- Methyl-D- Mannoside utilization	-
29.	Xylitol utilization	v
30.	OPNG test	-
31.	Esculin utilization	-
32.	D- Arabinose utilization	v
33.	Citrate utilization	-
34.	Malonate utilization	-
35.	Sorbose utilization	-
36.	Control test	-

Note: (+) sign represents the positive results; (-) sign represents the Negative results; (v) sign represents the 11- 89 % positive results

4.5. Gram characteristics of host bacteria

Gram staining was performed of bacterial strain DP203 to check their Gram character. Gram negative rods were observed under compound microscope under 100X objective lens.

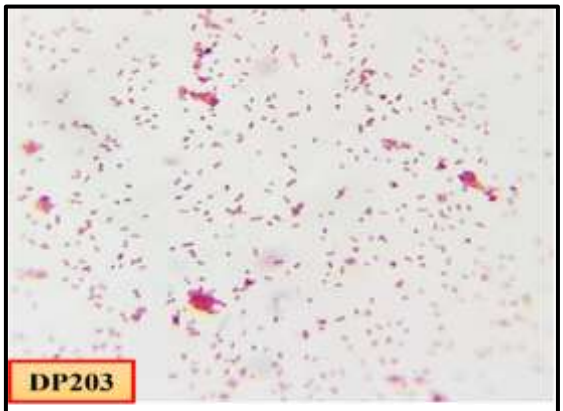

Fig- 4.5.1. Gram character of strain DP203; Gram negative rods were observed under compound microscope at 100X magnification

4.6. Isolation of genomic DNA of host bacteria

Bacterial DNA was successfully isolated and observed under UV transilluminator as shown in (Fig- 4.6.1.) and the DNA concentration was 54.4 ng/ µL.

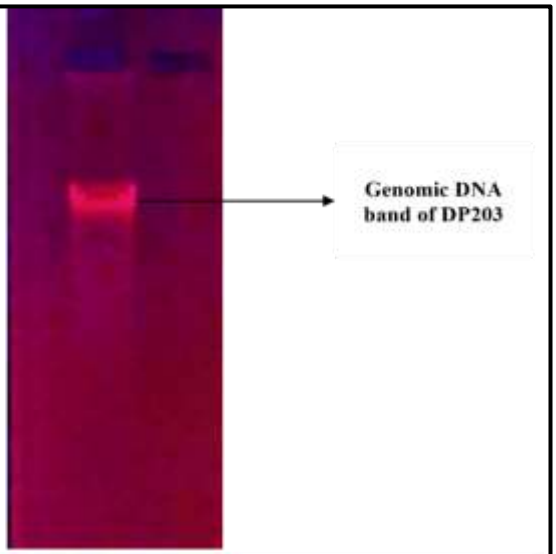

Fig- 4.6.1. Genomic DNA band of Bacterial strain DP203; The isolated DNA was electrophoresed and observed under UV transilluminator and the concentration of the isolated DNA was 54.4 ng/ µL.

4.7. PCR amplification of host bacteria

Bacterial genomic DNA of strain DP203 was PCR amplified. Agarose gel electrophoresis showed a distinct band corresponding to 1.5 kb was obtained. as shown in (Fig- 4.7.1). The PCR amplified band was sent to Eurofins Genomics India Pvt. Ltd. for 16S sequencing.

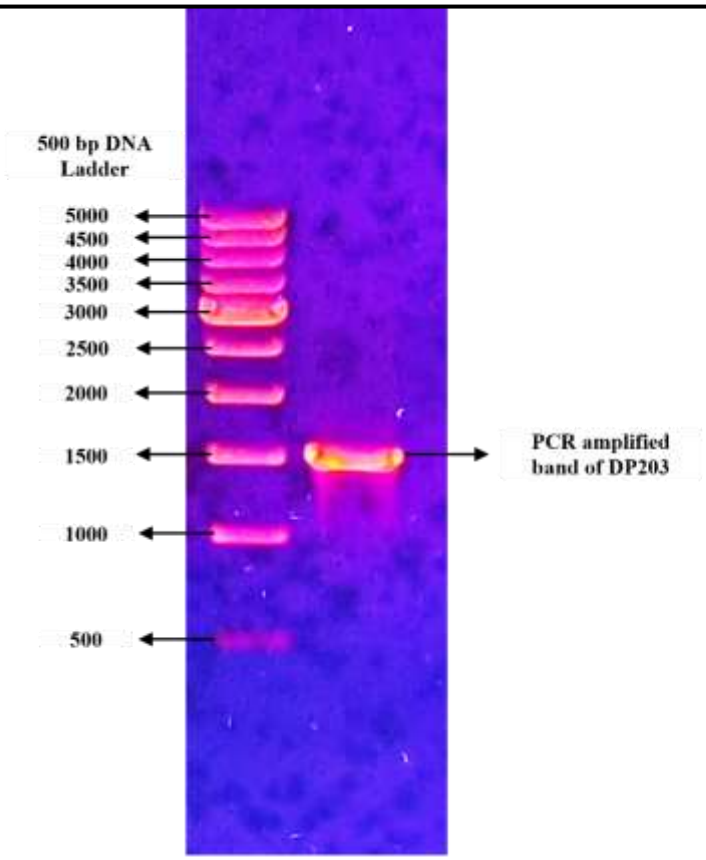

Fig- 4.7.1. PCR amplified band of Bacterial strain DP203; PCR amplification and agarose gel electrophoresis was performed and gel was observed under UV transilluminator and the PCR amplified band was approximately found to be 1.5 kb

4.8.Prophage induction

4.8.1. By heat shock method

In the present study prophage induction was carried out by heat shock method for 1, 2 and 3 minutes respectively. Further to check whether prophages are induced or not, double agar overlay method was performed and the results obtained from double agar overlay method are shown in (Fig- 4.10.1).

According to (Rokney et al., 2008) the enhanced green fluorescent protein plasmids (pEGFP) fusion test indicates that CII activity results from 5 minutes of heat treatment of the lysogen. Furthermore, they described that the CII protein made during the first 5 minutes at 42°C is eliminated during the next 5 minutes and hence, this elimination explains the lack of CII activity after 10 or 15 minutes of heat shock.

4.8.2. By Mit C treatment method

In the present study prophage induction was carried out for 12 hours by treating with Mit C. Further to check whether prophages are induced or not, double agar overlay technique was performed and the results obtained from double agar overlay method are shown in (Fig- 4.10.2). Graph was also plotted as shown in (Fig- 4.9.2.1) to determine whether the bacterial growth is decreasing or not after treating with Mit C. The significant growth can be observed in graph in comparison with control group which suggests the induction of prophages.

Many prophages were previously induced by Mit C treatment and have been reported (Chen et al., 2006; Rokney et al., 2008; Zheng et al., 2014). According to (Balasubramanian et al., 2019) Mit C is the most frequently used chemical that damages DNA and induces SOS response in the host bacteria. These stimulating factors cause the bacterial RecA protein to become activated, which in turn cleave the main repressor protein CI of the prophage and release the phages at early and middle genes. According

to (Chen et al., 2006) Following the Mit C treatment, TM1040 produced a significant number of phage particles within the first eight hours.

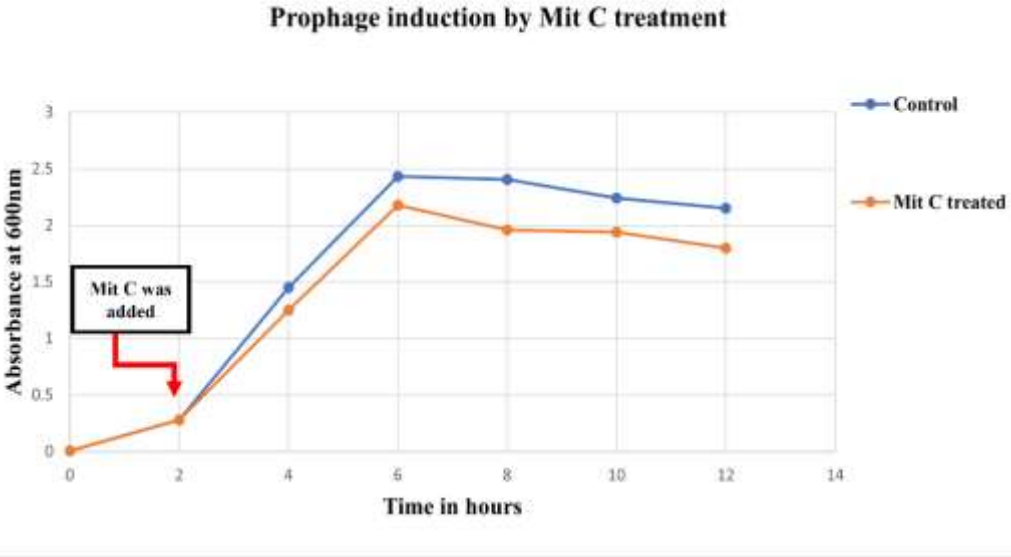

Fig- 4.8.2.1. Prophage induction by Mit C treatment method; Blue line in the graph is showing the growth of strain DP203 untreated served as (control); Orange line is showing the growth of strain DP203 treated with Mit C

4.9. Isolation of prophage by double agar overlay method

Isolation of induced prophages by both heat shock and Mit C method was carried out by double agar overlay method. The clear plaques were observed after the 1, 2, and 3 minutes of heat shock as shown in (Fig- 4.10.1). Furthermore, plaque forming unit (PFU) was determined and was found to be 10 PFU/ mL, 15 PFU/ mL and 40 PFU/ mL respectively. The titter value determined by heat shock method was very low for phage purification and concentration which on repetition gave the same results.

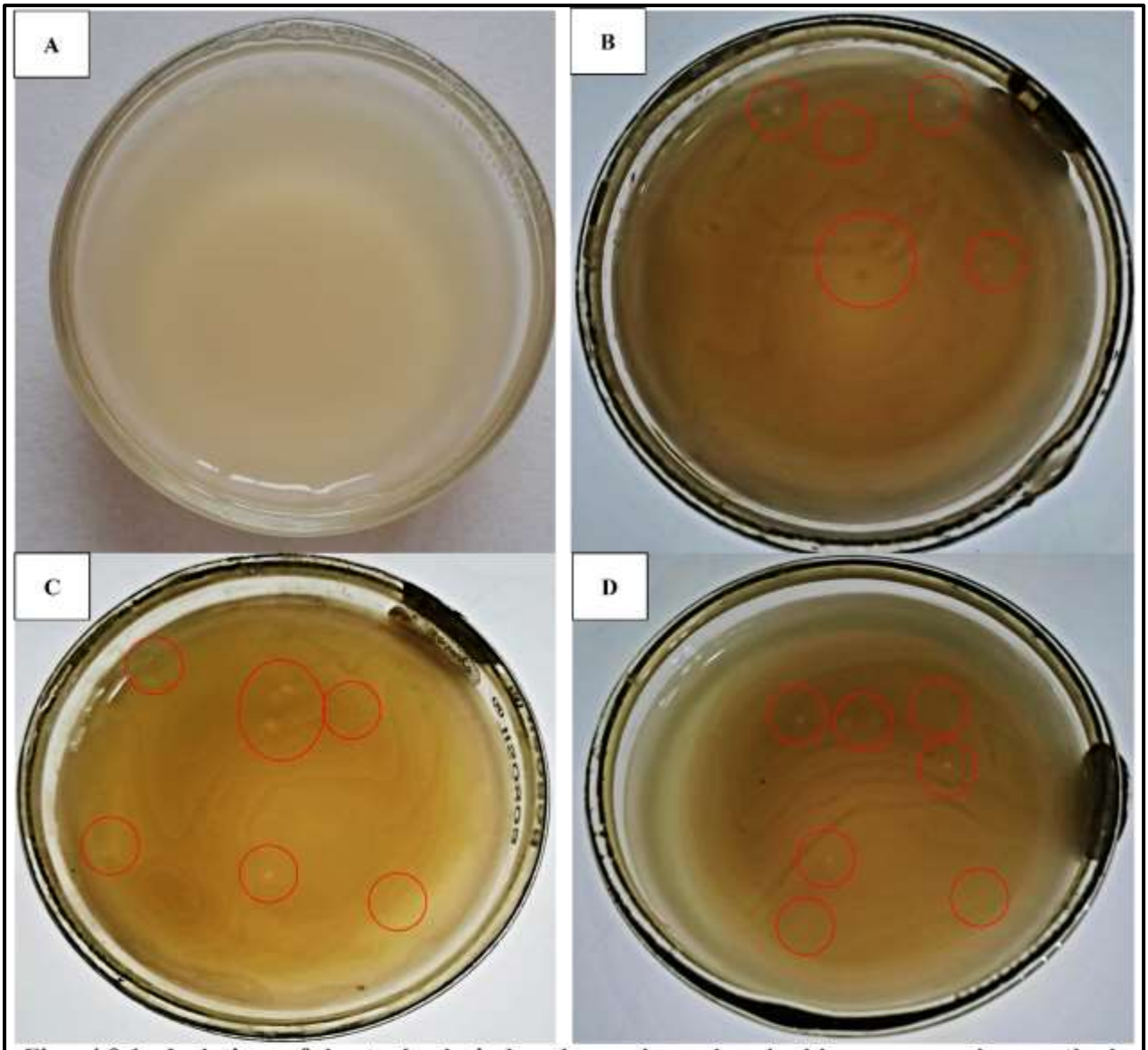

Fig- 4.9.1. Isolation of heat shock induced prophage by double agar overlay method; (A) Control plate showing only bacterial growth; (B) Clear plaques were observed of induced prophage after 1 minute of heat shock; (C) Clear plaques were observed of induced prophage after 2 minutes of heat shock; (D) Clear plaques were observed of induced prophage after 3 minutes of heat shock

Similarly, clear plaques were also observed after 4 and 6 hours of Mit C treatment as shown in (Fig- 4.10.2). Furthermore, PFU was determined and was found to be 45 PFU/ mL and 80 PFU/ mL respectively. The titter value was determined by Mit C method was also low for the purification and concentration of phages.

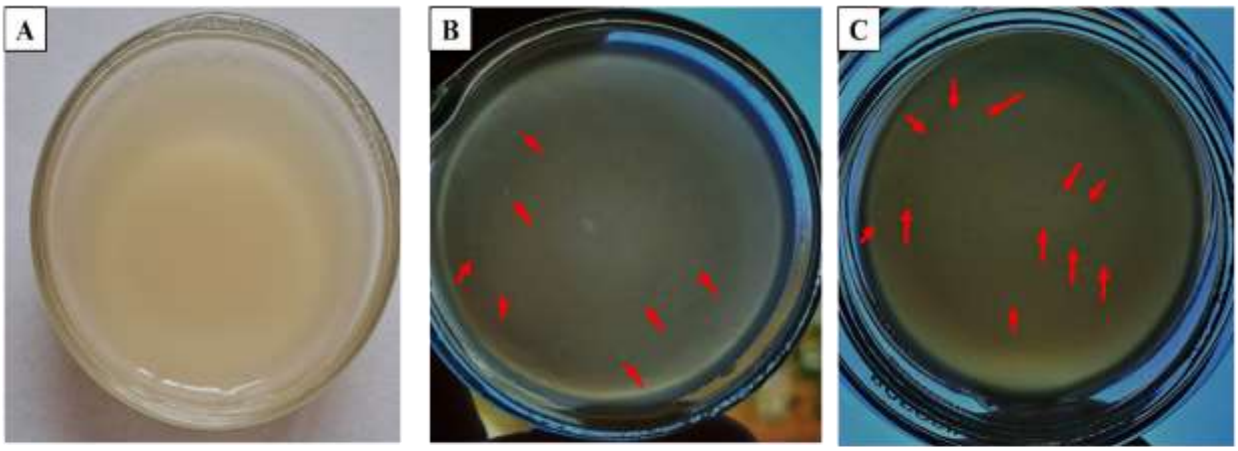

Fig- 4.9.2. Prophage induction by Mit C treatment method; (A) Control plate showing only bacterial growth (B) Clear plaques were obtained after 4 hours of Mit C treatment; (C) Clear plaques were obtained after 6 hours of Mit C treatment

In this study bacterial strain DP203 was studied for its CP degrading ability, all the while two methods of prophage induction were performed to check the prophage production by CP degrading strain DP203 by heat shock method and Mit C treatment method. As described earlier the titter value determined by both the methods were very low for phage purification, concentration and its genome analysis. However, new induction methods should be determined for the induction of prophage from strain DP203 which could result in higher titter value, as the above mentioned two methods were insufficient in induction of prophages.

FUTURE PROSPECTS

- Whole genome sequence analysis of host bacteria.
- Concentration and purification of phage.
- Host range specificity of phage.
- Viral genome sequencing analysis.

  Atomic force microscopy and transmission electron microscopy can be explored in order to study the lytic cycle of phage and the morphological changes that the phage causes in host bacteria.

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Appendix- I

Chemicals and Media Used

S. No.	Name of the Chemicals and media used	Company
1.	Tris Base (Trizma)	HiMedia
2.	Magnesium Sulphate heptahydrate	HiMedia
3.	Potassium Chloride	HiMedia
4.	Diammonium Phosphate	HiMedia
5.	Sodium Chloride	HiMedia
6.	Calcium Chloride	HiMedia
7.	Hydrochloric Acid	HiMedia
8.	Lugol's Iodine	HiMedia
9.	Mitomycin C	HiMedia
10.	Glycerol	HiMedia
11.	Glutaraldehyde	HiMedia
12.	Ethanol	HiMedia
13.	Agar	HiMedia
14.	Zobell Marine Broth (ZMB)	HiMedia
15.	Zobell Marine Agar (ZMA)	HiMedia
16.	Agarose	HiMedia
17.	Sodium Alginate	HiMedia
18.	Carrageenan	HiMedia
19.	Gel rite	HiMedia
20.	Phenol Red dye	HiMedia

Appendix- II

Preparation of Chemicals and Media

1. Artificial Sea Water (ASW) media:

Media Composition	g/L
Tris Base	6.05
$MgSO_4$	12.32
KCl	0.74
$(NH)_2\ HPO_4$	0.13
NaCl	17.52
$CaCl_2$	0.14

pH should be 7.0

Preparation: In a conical flask take 800 mL of distilled water and dissolve all the chemicals one by one completely, adjust the pH immediately with Concentrated HCl and make up the volume to 1000 mL with distilled water

2. Lugol's iodine:

Composition	g/ 100 mL
Potassium Iodide (0.1 M)	1.66
Iodine (0.05M)	1.26

Preparation: Weigh the above-mentioned compositions and add to 100 mL of distilled water in an ambered color reagent bottle and stare at room temperature vigorously till iodine crystals dissolved.

- Note: store Lugol's iodine in ambered color reagent bottle at room temperature.

3. Phenol Red Dye

Composition	g/ 100 mL
Phenol red	0.02

Add in a clean 100 mL beaker containing 75 mL distilled water, mix well and dilute to a final volume 100mL with distilled water

4. Mitomycin C stock solution:

To prepare 1mg / mL Mit C stock dissolve 2 mg of Mit C in 2 mL of Di- methyl sulfoxide and store at -20°C in dark.

Appendix- III

List of the kits used

S. No.	Name of the kit	Company
1.	Gram Stains- kit K001- KT	HiMedia
2.	HipurA® Bacterial Genomic DNA Purification Kit MB505	HiMedia- HiGenoMB
3.	KB002 HiAssorted™ Biochemical Test Kit (for Gram- negative rods)	HiMedia
4.	KB009 HiCarbo™ Kit (KB009A / KB009B1 / KB009C)	HiMedia

Appendix- IV

List of Instruments Used

S. No.	List of instruments
1.	Light table
2.	pH meter
3.	Laminar Air Flow
4.	Compound Microscope
5.	Shaker incubator 30°C
6.	Water bath
7.	Weighing balance
8.	Autoclave
9.	Spectrophotometer (UV vis spec mini-1240)
10.	Thermal cycler
11.	Carl Zeiss Scanning Electron Microscope