

**MOLECULAR IDENTIFICATION AND CHARACTERIZATION OF
VIBRIO SPECIES ISOLATED FROM INFECTED SHRIMP:
*LITOPENAEUS VANNAMEI***

Submitted in partial fulfillment of the requirements for the award of
Bachelor of Technology degree in
BIOTECHNOLOGY

By

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Accredited with grade "A" by NAAC

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the Bonafide work of **AJITHRAN.S (Reg. No. 38230001)** who carried out the project entitled "**MOLECULAR IDENTIFICATION AND CHARACTERIZATION OF *VIBRIO* SPECIES ISOLATED FROM INFECTED SHRIMP: *LITOPENAEUS VANNAMEI***" under my supervision from December 2021 to April 2022.

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DECLARATION

AJITHRAN.S (Reg. No. 38230001) hereby declare that the Project Report entitled **“MOLECULAR IDENTIFICATION AND CHARAZERIZATION OF *VIBRIO* SPECIES ISOLATED FROM INFECTED SHRIMP: *LITOPENAUS VANNAMEI*”** done by me under the guidance of **Dr. C. VALLI NACHIYAR** (internal guide) and **Dr. R. THIRUGNANASAMBANDAM** (External guide) is submitted in partial fulfillment of the requirements for the award of Bachelor of Technology degree in Chemical Engineering.



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ABSTRACT

Shrimp farming in India is a very lucrative industry and has been growing rapidly. In the last decade, shrimp production has increased by more than 50%, and this trend is expected to continue. The vibriosis infection in Shrimp farming poses a major challenge to the industry. It is a huge threat to the food safety and people's health. The first challenge for the industry is to find an effective way to control vibriosis infection in shrimp farming ponds and farms. The second challenge is to find a way to ensure that their products are safe and free from contamination by this pathogen. The third challenge they face is how to maintain high quality standards while reducing costs and increasing efficiency. The objective of this study was to characterise *Vibrio* species of White Fecal Syndrome (WFS) infected shrimp sample collected from different aquaculture farms located at Marakkanam (Near Pondicherry province), Tamil Nadu. Five *Vibrio* isolates were obtained on TCBS (Thiosulfate-citrate-bile-salt sucrose agar and identified using biochemical tests. Isolates were further characterised based on their antibiotic susceptibility potentials using *Penicillin G*, *Chloramphenicol*, *Erythromycin* antibiotic discs. Isolates revealed high levels of resistance to *Chloramphenicol*, while lower resistance to other antimicrobials. These findings and isolates would be further taken forward by co-culturing with microalgae and their inhibitory effects against different microalgae concentrations thus, making it suitable as natural and sustainable supplements against various pathogenic strains for aquaculture farms.

Key words: Shrimp farming, Challenges, Vibriosis, White Fecal Syndrome (WFS), Antimicrobial agents, antibiotic resistance, microalgae

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CHAPTER - 1

INTRODUCTION

Modern shrimp farming is commended in late 1980's. It was mainly based on black tiger shrimp and to lesser degree the Indian white shrimp. This type of farming growth of the sector was seriously impacted a few years later when the white feces syndrome (WFS) arrived on Indian shores. It took an act of the Indian parliament to restart shrimp aquaculture and the phases of growth that followed was marked by development of independent hatcheries and farms less than 5 hectares owned or leased by small farmers. While volumes continued to rise through the mid 2000's a stagnation was released in the latter half of the decade due to disease problems that slowed the animal growth and size variability. There are more than 130 different types of vibrio species (Tanaka *et al.*, 2020). *Vibrio* is a diverse genus of bacteria that exist free-living or associated with human / animal host. *Vibrio* are gram negative bacteria; motile rods; ubiquitous marine bacteria; size: 1.4-2.6µm in length; facultative anaerobic bacteria. It is one of the fastest growing rates in all types of bacteria. *Vibriosis* is one of the most severe bacterial diseases in shrimp aquaculture and is caused by the infection of pathogenic or opportunistic *vibrio* bacteria. It can lead to mortality of up to 100% estimated to cause the shrimp sectors US \$3 billion in global losses per year. One of the recent cases of *vibrio* related diseases is Early Mortality Syndrome (EMS) which is also known as Acute Hepatopancreatic necrosis disease (AHPND). Economical losses owing to shrimp infection were 0.21 million tons worth of \$10 billion in the 2018-2019 fiscal year. Among the bacterial pathogens, the major *vibrio* species affecting shrimp aquaculture include *vibrio* *Parahaemolyticus*, *vibrio* *Vulnificus* *etc.* Pathogenic species possess unique functional traits and often occupy distinct environmental niches driven by temperature, salinity, and other biotic and abiotic factors. The worldwide economic loss due to disease outbreaks in aquaculture industries has been estimated as more than US \$60 billion per annum. Mechanism involved in causing white feces diseases appears in shrimps from approximately 2 months post culture onwards & caused by gregarines. The vermiform body formations are consisting of Aggregated Transformed Microvilli (ATM). It has originated by sloughing from epithelial cells of the shrimps hepatopancreatic tubules. They

accumulate at the hepatopancreases midgut junction before being discharged within faces via the midgut. Main features are vermiform, gregarine like bodies within hepatopancreases & midgut. Show no cellular or subculture organelles also WFS involves other protozoan or metazoan. Stripped or microbial, the originally cell undergo lysis indicate a pathological process.

May retard shrimp growth & may predispose to opportunistic pathogenic Sustainable approaches to modulate the gut microflora is farmed shrimp for preventing gut diseases. The use of selected bacteria to inoculate the gut (probiotics). Specific nutrients promoting the development of selected bacteria strain in gut. Favoring the development of beneficial bacteria & inhibiting potentially pathogenic microorganism in gut. This synergistic blend has proven to be powerful interruption of bacterial & effective modulate for the gut flora. Antibiotics agents have been used for treatment and management of bacterial infection in human and animals. The treatment of vibrio infection in human they use early treatment drug is doxycycline but for pregnant women and children azithromycin is recommended. Therefore, antibiotic resistance is a major problem for therapy and may be responsible for a significant proportion of therapeutic failures resulting in high morbidity and mortality.

CHAPTER 2

2.REVIEW OF LITERATURE

2.1THE PRESIDENT OF INDIA'S SOCIETY OF AQUACULTURE PROFESSIONALS DETAILS THE INDUSTRY'S GROWTH AMID INFRASTRUCTURE, DIEASES AND MARKETPLACE CHALLENGES

Modern shrimp farming was started in India in the late 1980s to promote seafood exports and many legal entities provided capital to build hatcheries, farms and processing plants. It was mainly based on the black tiger shrimp (*Penaeus monodon*) and to a lesser extent the Indian white shrimp (*Fenneropenaeus indicus*).

A few years later, when the White Spot Syndrome virus (WSSV) reached the coast of India, the growth of the region was severely affected, and at the request of environmental activists, the Supreme Court of India regulated shrimp farming along the coast. Water. The resumption of shrimp aquaculture in India required a law by Parliament, and marked the next stage of growth by developing independent hatcheries and farms of less than five hectares owned or rented by several small farmers. Black tiger shrimp remained an interesting species, but there was also significant production of freshwater shrimp (*Macrobrachium rosenbergii*).

In 2008, India decided to introduce the Specific Pathogen (SPF) Pacific White Shrimp (*Litopenaeus vannamei*). Farmers quickly switched to SPF *L. vannamei*, which today accounts for 90% of Indian shrimp production. In some parts of Andhra Pradesh, black tiger prawns are reared in low salinity waters, and there has been a significant increase in shrimp production in these areas over the past decade. Shrimp farming is widespread in other states, especially in the eastern coastal states of Odisha and West Bengal north of Andhra Pradesh and in the northwestern state of Gujarat.

2.2 PREVENTION OF WHITE FECES SYNDROME, WHITE GUT DIEASES AND WHITE MUSCLE DIEASES IN SHRIMP

Acute disease associated with hepatopancreatic necrosis (AHPND) in cultured shrimp, with increasing proliferation of worm-like, Gregarine-like bodies in the middle of the shrimp hepatopancreatic (HP). A large number of these bodies cause white fecal filaments and White Fecal Syndrome (WFS). WFS appears in shrimp about 2 months after culture, and is caused by Gregarine. Vermiform formations contain aggregated transformed microvilli (ATM). ATMs are formed as a result of the rejection of epithelial cells by the shrimp hepatopancreatic tubules. They accumulate at the junction of the HP and the middle intestine and are then expelled through the feces through the middle.

Cultured Shrimp *L. vannamei* and *P. White* stool exposure has also been found in monodon, and has no association with loose shell syndrome. It has been observed that the severity of the problem caused by white feces is of great concern in shrimp farming. Interestingly, this problem has been observed in *L.vannamei* ponds with large amounts of blue-green algae. Worm-like Gregarine-like bodies in HP and midfield. No cellular or subcellular organs. WFS also includes other protozoa or metazoan. In the absence of microvilli, the nasal cells undergo lysis. Loss of microvilli and subsequent cell lysis indicate a pathological process. When the contents of the intestines or feces are examined, they contain worm-like bodies that are externally similar to Gregarine. In severely affected ponds, shrimp survival is reduced by 20 to 30 percent compared to normal ponds. There is a decrease in feed intake, growth rate, and average daily live weight gain (AWG).

Prevention [of White Feces Syndrome, White Gut Disease and White Muscle Disease in Shrimp | The Fish Site](#)

2.3 ECONOMICAL LOSSES DUE TO DISEASES INCIDENCES IN SHRIMP FARMING OF INDIA

Shrimp farming creates jobs in remote coastal villages, provides livelihood to the coastal poor and contributes valuable foreign exchange to the country's treasury. Of the 862,021 ton, 22% are shrimp and 49% are valuable of 1crore out of the total seafood exports due to financial losses due to diseases in shrimp farms in India. Over the past decade, annual shrimp production has increased from 35,500 ton in 1990-91 to approximately 216,500 ton in 2011-2012, with export value

reaching US \$ 3.5 billion. The area under cultivation has increased significantly from 65,100 ha in 1990-1991 to 121,208 ha in 2011-2012. India is the fifth largest shrimp farming country in the world. The other four largest shrimp producing countries, China, Thailand, Indonesia and Vietnam, are working on biosafe farming technologies to protect their farms from disease and to stabilize their foreign trade share. In India, the emphasis so far has been on improving shrimp farming practices to minimize environmental impact and increasing sustainability through technological alternatives. More efforts are now needed to increase shrimp production with biological safety measures to reduce production losses.

In many countries, disease has plagued shrimp production for the past two decades. In 1993, there was an explosion that reduced the production of Chinese shrimp. Diseases caused many problems for Thai shrimp production in 1996 and 1997. Serious cases of shrimp disease were reported in Ecuador in 1999 and in Brazil in 2006. A total of US \$ 1 billion has been lost due to shrimp diseases. However, in recent years, most diseases have become localized in these countries. India should definitely develop innovative technologies in shrimp farming, focusing more on minimizing production losses due to disease, to revitalize the sector.

The economic implications of the disease are more obvious to farmers, and the fight against them is linked to the cost of balancing profits. A comprehensive understanding of the impact of disease on shrimp productivity and complex economic losses is crucial to the formulation of various health management measures. The financial cost of the disease is directly related to the loss of livestock and the cost of infection control. Indirect costs may include job losses in upstream (hatcheries, feed mills), downstream (export processing, etc.) businesses and consequent losses to the Treasury. Product loss in shrimp aquaculture nationally in Thailand (1996–97, 2013), China (1993) and Brazil (2006) contributed to the spread of infectious diseases.

N. Kalaimani , T. Ravisankar*, N. Chakravarthy, S. Raja, T. C. Santiago and A. G. Ponniah.

[Economic Losses due to Disease Incidences in Shrimp Farms of India \(researchgate.net\)](#)

2.4 MANAGEMENT OF WHITE FETAL SYNDROME(WFS) IN P.VANNAMIE FARM

During WFS exposure, the amount of feed in the shrimp ponds may decrease. White fecal filaments containing dense EGP sperm should be removed daily from affected reservoirs. Because shrimp have the ability to regenerate hepatopancrease drugs / supplements (eg taurine) may be used to increase epithelial cell proliferation. Because EHP is prevalent in WFS-infected shrimp, the recommended effective measures against EHP can be applied. The pond should be dried and disinfected after each harvest and the pond should be prepared according to the best management practices (BMP) to ensure that the EHP spores are destroyed along with the vectors. It is recommended to treat the pond residue by applying CaO (Quicklime) at the rate of 6 tons per hectare. It is necessary to use high doses of lime as the seeds will be destroyed only if the pH of the soil is raised to 12 or higher. It is recommended to pour CaO into the dry pond residue (10-12 cm) and irrigate the residue to activate the lime. NACA prescribes 15 ppm KMnO₄ or > 40 ppm chlorine to inactivate soil sperm. The pool should be left to dry for a week before filling. Farmers are advised to keep only EHP-free PCR seeds in well-drained / flowering ponds. In hatcheries, PCR should test all live feeds to ensure the absence of EHP.

[Management-of-white-feces.pdf \(ciba.res.in\)](http://ciba.res.in/Management-of-white-feces.pdf)

CHAPTER 3

AIM AND OBJECTIVE

3.1 AIM

The aim of this work will determine the isolation and characterization of *Vibrio* species isolated from infected shrimp.

3.2 OBJECTIVE

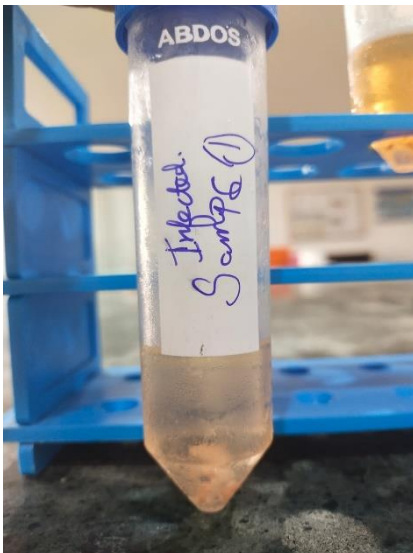
- * To isolate and identify the *Vibrio* species by using TCBS agar and biochemical techniques.
- *To determine the molecular characterization of isolation *Vibrio* species from polymerase chain reaction, Agarose gel electrophoresis , 16s rRNA sequences .
- * To assess the antibiotic susceptibility assay in the *Vibrio* species.

CHAPTER 4

MATERIALS AND METHRODS

4.1 ISOLATION AND PHENOTYPIC IDENTIFICATION OF BACTERIA FROM INFECTED SHRIMP

Shrimp was suffering from white faces diseases were collected from different pond located at Marakonam (near to Pondicherry). shrimp were kept in individually by sterilized centrifuge tube and collect water sample also kept in sterilized water bottle at different time interval of temperature (max 30°C – min 28°C) at different time interval and transport to laboratory of Jeppiaar research park in Sathyabama institute of science and technology, Chennai maintaining in aseptic condition. The infected shrimp was dissected cut the individual part like gut, indigestion tract and hepatopancreas was crushed by motor and pistil add small amount of saline water in it and stored in 1.5 ml of centrifuge tube and sample were serially diluted (10^{-5} - 10^{-9}) sample were spread through TCBS agar (Thiosulfate citrate bile salt) to isolated vibrio species. The plate was incubated 32°C for 24-48hrs and see the different type of vibrio. Several colonies were randomly selected and streak through the Luria Bertani agar (LB agar) to obtain the pure culture. Culture was maintained in Luria Bertani broth (LB broth) for further characterization. Individual colonies were grown on TCBS agar plate were observed and colonies characterization such as colony size , shape, color was observed. To identified the Gram straining bacteria, motility, catalase , oxidase test, indole production , Voges- Proskauer test, urease test , sugar test – glucose and sucrose , MacConkey agar , hydrogen sulfide (H_2S) production and acid production from glucose , mannitol , sucrose . Growth of the bacteria was isolate from different NaCl condition of 2,4,6,8% and incubate 28°C for 24 hours and study the growth of bacteria.



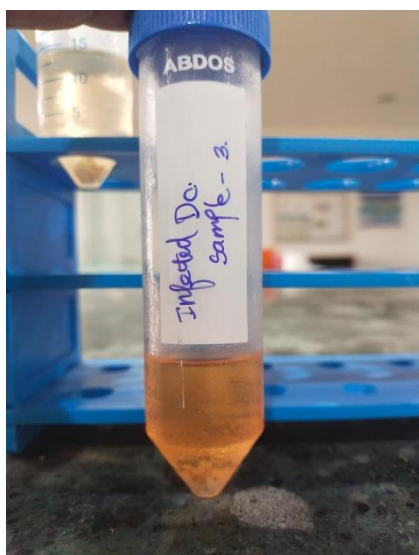


FIG 4.1

Infected shrimp and water sample collect from different pond

Sample no.	Symptoms	Site of isolation
01	Infected blackish water	Water source
02	Fresh water pond	Water source
03-04	Infected live shrimp	Hepatopancreases
05	Infected Dc shrimp	Hepatopancreases

Table 4.1

4.2 PREPARATION OF TCBS AGAR

TCBS (Thiosulfate citrate bile salt) is used for selective media for isolation of all type vibrio species except *vibrio* *Hollie*. TCBS agar is composed of yeast extract, peptone, sodium thiosulfate, sodium citrate, iron (III) citrate, bromothymol blue, and distilled water or deionized water.

- 18 grams of TCBS agar is dissolved in 200ml of sterilized sea water. To this added 2 percent agar (4 grams of 200ml).

- The solution was well heated using hot plate for 10 mins until the media component were clearly dissolved in 10 mins.
- The media was kept undisturbed until it reaches room temperature to avoid bubbles.
- The media was poured in sterilized petri plates.



FIG 4.2

4.3 PREPARATION OF NUTRIENT AGAR

Nutrient agar is used to cultivation of microbes supporting growth in non-fastidious animal. It is an ideal nutrient medium for bacterial growth used for demonstration and teaching purpose. It is mainly used for isolation of purification of colonies. It is also used for antibiotics sensitivity test. Nutrient agar is made up of petri dish of animal tissue, sodium chloride, beef extract, yeast extract, agar.

The following procedure is used for the preparation of nutrient agar

- 2.8 grams of Nutrient agar was dissolved in 100 ml of distilled water and add 2percentage of agar (2 gram) and 2 grams of NaCl in it.
- Dissolved all chemical in conical flask and tightly covered by cotton plug.
- Place the conical flask inside the autoclave and sterilize at 121°C for 30mins
- When the temperature cools down and transfer to sterilized petri plate and allow to solidify the nutrient agar medium.
- Use the plate in laminar air flow chamber for further use.

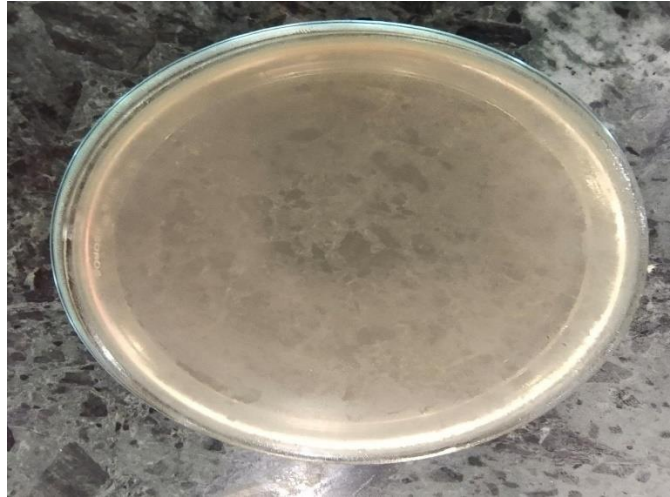


FIG4.3

4.4 PREPARATION OF LURIA BERTANI AGAR

Luria Bertani agar is a high medium for culturing the growth of most microorganism. It lacks antibiotics. It is made up of 1.0% bacto tryptone, 0.5% yeast extract, 1.0% NaCl, 0.1% glucose.

Procedure for preparation for Luria Bertani agar

- Take 2.5 grams of Luria Bertani agar in 100 ml of distilled water additionally 3 gram of NaCl and 1.25 gram of agar is added to it.
- Mix well until the powdered completely dissolved and tightly covered the conical flask by cotton plug.
- Place the conical flask inside the autoclave for sterilization at 121°C for 30 mins.
- When the temperature cools down media was poured in to sterilized petri plate.
- Leave plate in laminar hood until agar set.

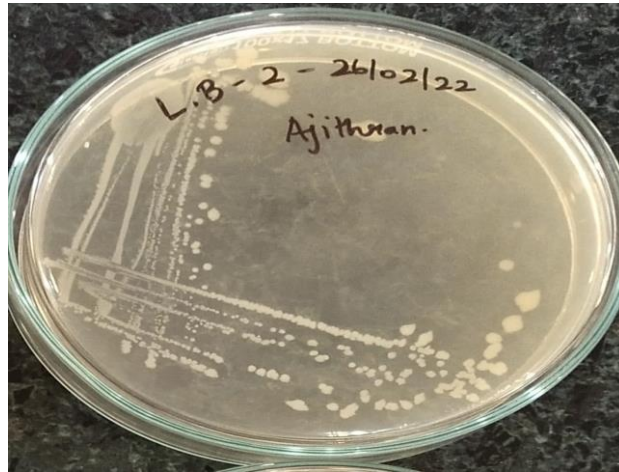


FIG 4.4

4.5 PREPARATION OF LURIA BERTANI BROTH

Luria Bertani broth is introduced by Sambrook J.& Russel, D W. It is nutrient rich liquid broth for culturing bacteria. It is commonly used during cloning experiment to amplify competent bacteria. It is used as the growth surface to analyzed bacterial colony morphology. LB contain Antibiotics. It is the combination of 10 gram of tryptone, 5 gram of yeast extract, 10gram NaCl.

Protocol of Luria Bertani broth:

- Add 2.5 gram of LB broth addition of 2 gram of NaCl in 100 of sterilized distilled water.
- Seal the cap of the media bottle and mixed well until media was completely dissolved.
- Autoclaved on liquids settings for approximately 30 mins at 121°C.
- Allow the media cool down for the room temperature before handing and stored in sterilized borosilicate glass reagent bottle.
- It should for good for 3-4 month and maintain in -4°C freezer.



Fig 4.5

4.6 Biochemical characterization

Bacterial colonies are different in morphological, so we identify the different species of bacteria. They are different in cellular morphology and staining properties.

4.6.1 Gram straining

This technic is used straining the bacterial colony to observe the morphological and arrangement of bacterial cells. Gram positive represent purple color and gram negative shows pink in color. They are using four different type of dyes (methylene blue, grams iodine, decolorize, safranin pink).

Protocol for gram straining

- smear the sample in glass slide and show in the spirt lamp.
- Cover the smear with primary strain (methyl violet, crystal violet) and wash off the strain after 60 second.
- Cover the smear with mordant (grams iodine) wait it for 3mins and wash of the strain with tap water.
- decolorize the smear with spirt or acetone alcohol to reduce the dye in the slide and wash with clean tap water.
- Pour counter strain (safranin pink or neutral red) and wait it 60 sec then wash the strain.
- Examine the smear under microscope (100x oil immersion optative lens).

4.6.2 oxidase test

To test to production of oxidase reaction. It differentiates the families of Pseudomonadaceae(ox+) and Enterobacteriaceae (ox-) use for identifying the may bacteria that have to use oxygen as the final electron acceptor in aerobic respiration.

Procedure of oxidase test

- The test microorganisms were rubbed onto a reagent-soaked filter paper disk using a sterile applicator.
- Controls were stored along with the test and the response was monitored within 10 seconds.

4.6.3 MacConkey agar

MacConkey agar is a selective media for bacteria culture for isolate of gram negative bacteria and enteric bacteria when pink or red formation show lactose fermentation and no color shows non lactose fermentation.

Procedure of MacConkey agar

- Suspend 4.95-gram MacConkey agar and 1 gram of NaCl of dehydrated medium in 100 ml of distilled water.
- Sterilize by autoclave at 121°C at 30 mins.
- Pour in to sterilize Petri plate and allow to solidify.
- Streak the respective culture and incubate overnight at 37°C

4.6.4 catalase test

Catalase test is shows the whether the organism having catalase is present or not. Catalase is an enzyme which produce microorganism that live in oxygen environment. Catalase positive bacteria include strict aerobes and facultative anaerobes they are ability to respire using oxygen as terminal electron acceptor.

Catalase negative bacteria may be aerobes or facultative anaerobes they do not respire using oxygen as a terminal electron acceptor.

Protocol of catalyze test

- Transfer a small amount of the bacterial colony to the surface of the clean and dry slide using a loop or sterile slide.
- Place a drop of 30% H₂O₂ on a glass slide and mix.
- A positive result is the rapid release of oxygen (within 5 sec), bubbling proves.
- Negative - No bubbles or only a few scattered bubbles.

4.6.5 indole production test

Indole production test is ability of microorganisms to decomposes the amino tryptophan to indole. Tryptophan is an essential amino acid that is oxidized by certain bacteria. Tryptophanase enzyme, leading to indole formation contain the tryptophan underwear used microorganism.

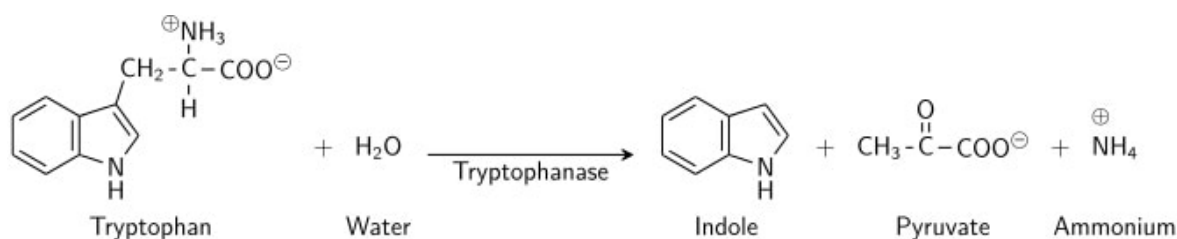


FIG 4.6.5 Enzymatic degradation of tryptophan

Procedure of indole production test

- Peptone water tubes were injected using a bacterial broth culture using sterile needle technology.
- An uninoculated tube was used as a control.
- Both tubes were incubated at 37 C for 24–48 h.
- After proper incubation, 1 ml of Kovac reagent was added to both tubes, including the control tube.
- After 10-15 minutes shake the tubes gently.
- Tube was observed colour changes in top of the reagent.

4.6.6 Voges – Proskauer test

Voges Proskauer test is ability of microorganism to produce acetone during fermentation of glucose. Pyruvic acid is product of glucose metabolism metabolized. When Barrit's reagent A (40%KOH) and Barrit's reagent B (5% alpha naphthol) is added presence of acetoin. Acetoin is presence of oxygen and Barrit's reagent A, oxidized to diacetyl, when alpha naphthol act as catalyst. Diacetyl react with component of peptone to produce a cherry red color.

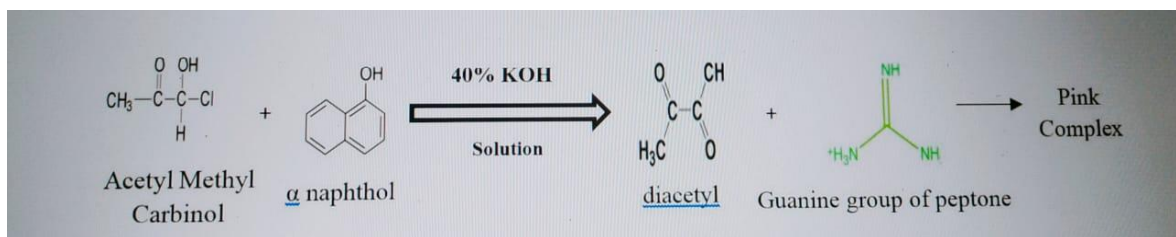


FIG 4.6.6

Procedure of Voges -Proskauer test

- It is an experimental organism, using sterile technology. The VP is injected into the char through loop inoculation. A tube remains uninoculated as a control.
- The tube is incubated at 37 C for 24-48 hours.
- After proper incubation, reagent of about 3 ml barrettes A and Regent B of 1 ml barrette were added to both solutions. Tubes including control.
- Shake the tubes gently for 30 seconds using covers to expose the carrier to oxygen.
- Responses allowed 15-30 minutes to complete. Tubes have also been seen.

4.6.7 H₂S production

H₂S production is detect the fermentation process in bacteria. The Sulphur is used as final hydrogen acceptor during anaerobic respiration leading to the formation H₂S.

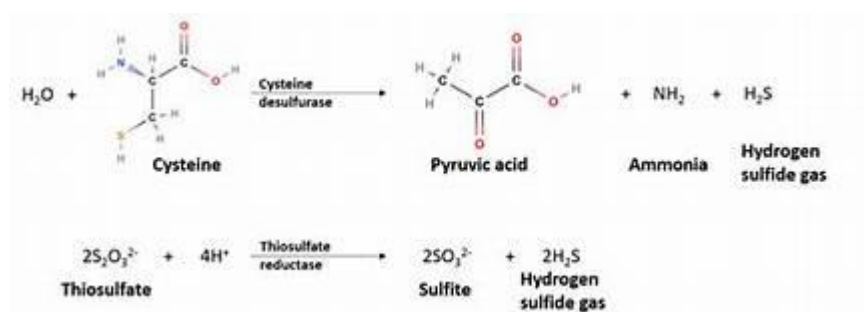


FIG 4.6.7

Procedure of H₂S production test

- Prepare and sterilize TSI agar medium and make it as slant.
- Incubate the slant with single colonies of bacteria selected from plate by stabbing down the centre of agar butt.
- Incubate needle carefully and streak the slant surface.
- Incubate the tube at 37°C.
- Positive result blackening on the medium.
- Negative result no blackening on the medium.

4.7 Bacterial growth studies

Bacteria are unicellular organism. They reach the certain size and divide into binary fissions means one cell divided into two and two into four that make a geometric matter. Bacterium is known as active growing phase. The viable cells of the bacterium should be incubated onto sterile broth and incubated under optimal growth. The bacterium start utilizing the component of media and it will increase in it size and cellular mass. Dynamics of the bacterial growth can be studying the plotting the cell growth the incubation time or log of cell number. Sigmoid is also known as standard growth curve. The increase in cell mass organism measured by

using the spectrophotometer. Spectrophotometer measure the optical density which measure the light of absorbed by a bacteria suspension. The degree of turbidity in the broth culture is directly related number of microorganisms viable or dead cell, convenient and rapid method of measuring cell growth rate of organism. Increase the turbidity of broth medium indicate an increase of microbial cell mass.

The four different stages:

- lag phase
- logarithmic phase
- stationary phase
- death phase

4.7.1 Lag phase

- The lag phase is also known as adaptation period during the phase bacteria cell adjust in their new conditions.
- The length of the lag phase depends directly on growth condition of organism.
- When the medium is poor in growth whether the organism will synthesized their necessary protein co-enzyme vitamins and they will increase in lag phase.
- When the medium is rich in nutrient cell division start immediately without any delay.
- At the end of this phase cell divides begins to increase the population.

4.7.2 Logarithmic phase (log phase)

- Microorganism are rapidly growing and dividing stage.
- Metabolic activity increase and divide by binary fission at a constant rate.
- The growth medium is exploited at maximal rate.
- The culture reaches the maximum growth rate and number of bacteria increase exponential.

- The time taken by bacteria to double in number during a specific time period is known as generation time.

4.7.3 stationary phase

- The bacteria population grow in steady state as the result in the accumulation of waste material, toxic metabolism, and inhibitory compounds such as antibiotics in the medium.
- The change in pH and temperature of the medium thereby creating unfavourable condition for the bacterial growth.
- So bacterial rate get slow and division its stop completely
- The cell number is not increase and growth rate is stability.

4.7.4 Decline or Death phase

- After the stationary phase bacteria grow will decrease and enter into the death phase.
- The depletion of nutrient of metabolic waste product and toxic material in the media makes the bacterium loss to reproduce.
- Individual bacteria begin to death due to unfavourable condition and death is rapid a uniform rate.
- Number of cell death is exceed to number of live cell.

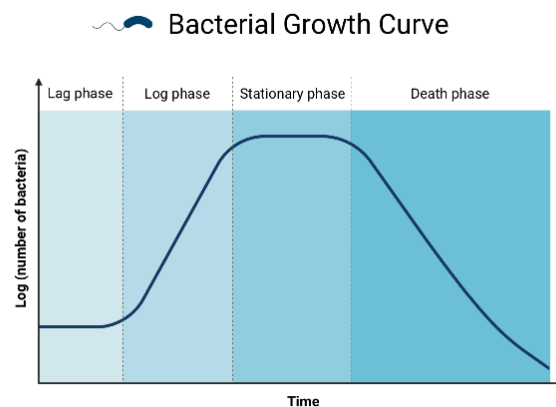


FIG 4.7 GROWTH KINETIC

4.8 Molecular identification of vibrio isolates

There are isolated different type of vibrio species was extracted by commercial DNA extraction kit and Genomic DNA purification kit K0721. Quality of DNA was checked by agarose gel electrophoresis on 1% in 50ml 8 well cast tray, 1 gram in 100 ml of TAE Buffer and 3 μ l of ETBr and compared with a lambda DNA marker. DNA was stored as -20 $^{\circ}$ c for further use. Polymerase chain reaction (PCR) for amplification of targeted in 16Sr RNA gene sequence of the isolate was performed with universal primer sets 27F(5' -AGAGTTGATCCTGGCTCAG-3') and 1492R(5'-GGTTACCTTGTTACGACTT-3'). Each PCR master mixer 5 μ l, primer (FP& RP) 1 μ L,DNA 1 μ l, M.Q water 3 μ l of a 10 μ M solution of a each primer in small scale and see 100 base pair DNA band in agarose gel electrophoresis . large scale of PCR master mixer 30 μ l, primer(FP&RP) 6 μ l , water 18 μ l ,DNA sample 6 μ l of a 60 μ M in large scale of same 100 base pair DNA run in agarose gel electrophoresis. The PCR thermocycler. The optimal condition for PCR was set of initial denaturing 94 $^{\circ}$ c for 1 mins, annealing at 52 $^{\circ}$ c for 45 sec, extension at 72 $^{\circ}$ c for 90 secs for 35 cycles after that maintain in -4 $^{\circ}$ c. A small portion of the PCR amplicons was mixed with 2 μ l of 5 sample 1 control of loading dye and loaded in 1 % agarose gel with 1Kb ladder marker. Electrophoresis was performed in 1% TAE buffer for 40mins at 50 volts. Amplificons were visualized by UV light in gel documentation system to see that band of DNA. The PCR product was purified using DNA purification kit. The sequence was extracted by using FASTA format. DNA sequence was isolate were analyzed using web-based Basic Local Alignment Search Tool (BLAST) of National Center for Biotechnology Information (NCBI).



FIG 4.8.1 PCR

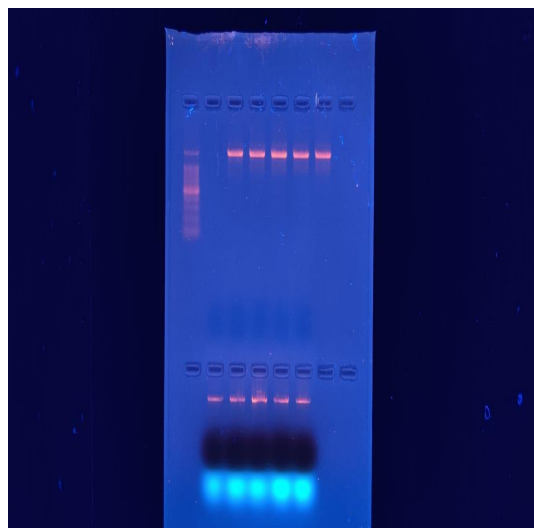


FIG 4.8.2 Gel band

4.9 Antimicrobial susceptibility of vibrio species isolates

Yellow and Green colonies on the agar plate were streak into TCBS agar plate for identify the pure colonies of vibrio species was observed. Bacterial susceptibility to antimicrobial agents was performed by the disk diffusion method using guidelines established by Bauer *et al.* (1996). Total 3 main antibiotic disk was taken Penicillin-G, Chloramphenicol C30, Erythromycin E15 were taken. Place the disk in Muller-Hinton agar plate spread by the sample by using spread plate method in 3 Quadrant place that disk and leave it 15 mins in blower and kept it in incubated at 37°C for 15-18 hours. After that incubation period over plate were examination and diameter of the zones of complete inhibition to the nearest whole millimeter were measured. The zone diameter for individual antimicrobial agents was translate to susceptible, intermediate and resistance category.



FIG 4.9 antibiotic disk

CHAPTER 5

RESULT AND DISCUSION

5.1 Thiosulfate citrate bile salts sucrose agar

- We were done a serial dilution results of 10^{-5} - 10^{-9} getting in spread plate method 5 different type of *Vibrio* species were identified

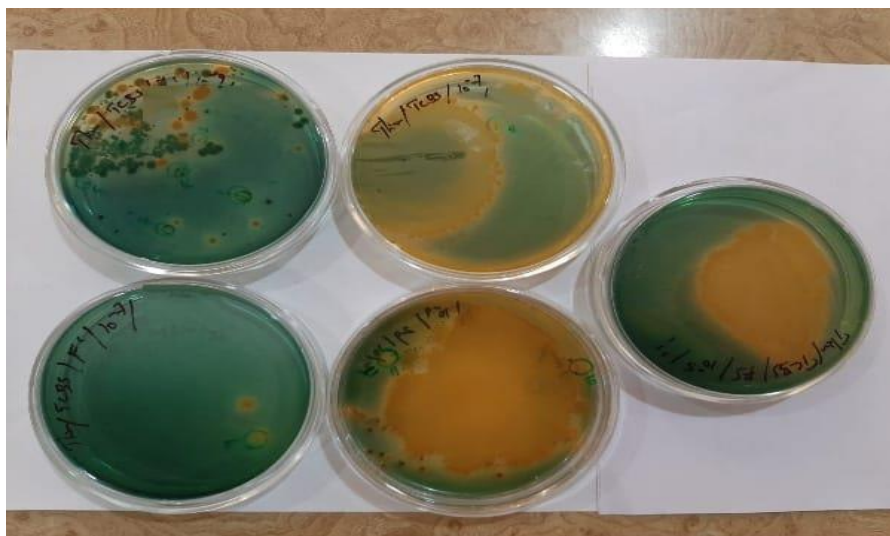


FIG 5.1 SPREAD PLATE

- Further we can streak that plate to isolate specific colonies in vibrio

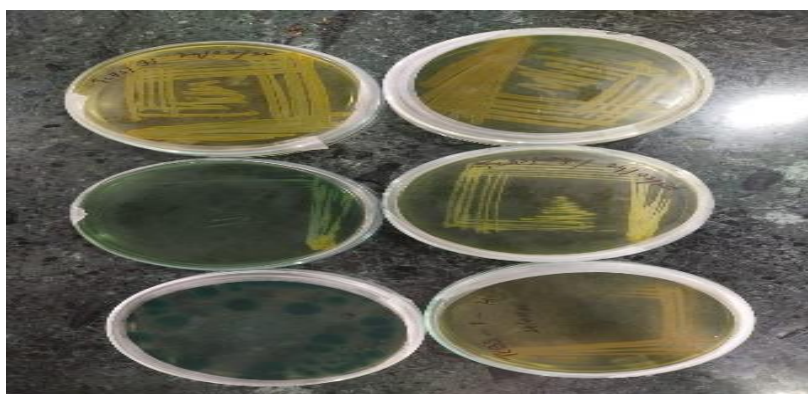


FIG5.2 STREAK PLATE

- We are getting 5 different colonies that are 4yellow and 1 green in colour colonies were observed in streak method to identify vibrio species.
- TCBS is specifically identify for vibrio species.

5.2 Nutrient agar

We were using different media for isolate vibrio species. Nutrient agar is used different growth ratio NaCl compounds of 2%,4%,5% and 7% are streak get a result of 2% and 7% little growth is happens and 4% and 5% growth is record.

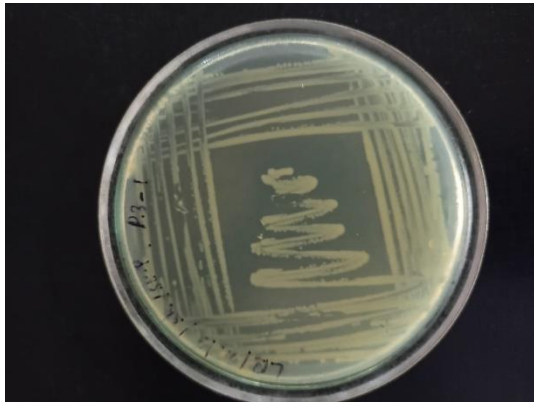


FIG 5.2.A 5% Growth

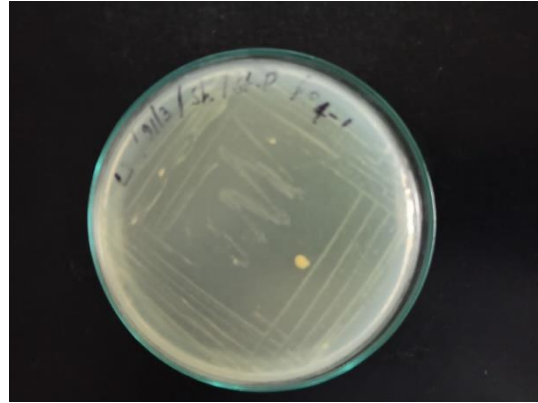


FIG 5.2.B 2 % GROWTH

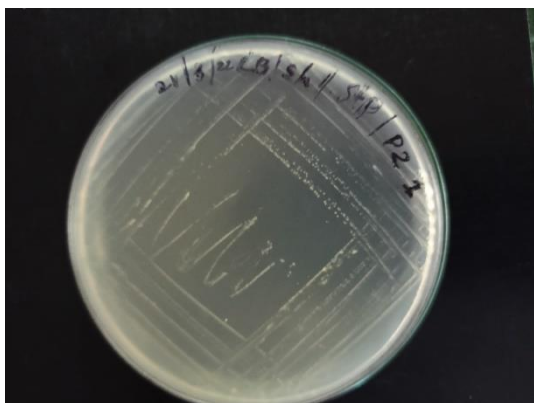


FIG 5.2.C 7% Growth

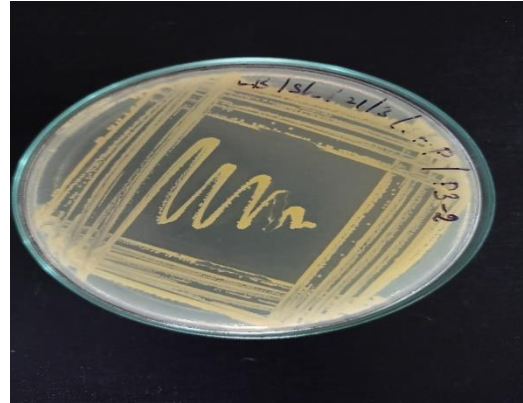


FIG 5.2.D 4 % Growth

5.3 Luria Bertani agar plate

- Further streak in luria bertani agar for purify the pure colonies of *Vibrio* species.
- Using different media for isolating the *vibrio* for making puriest form in final stage

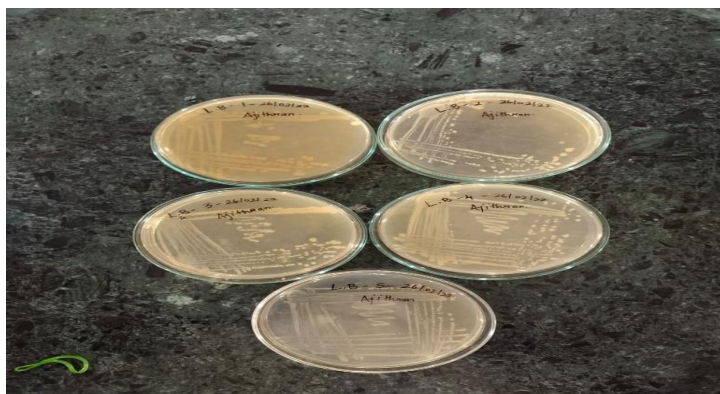


FIG 5.3 Luria Bertani agar

5.4 Luria Bertani Broth

- Luria Bertani broth for isolation of DNA , some Biochemical characterization antibiotics susceptibility , growth kinetics.

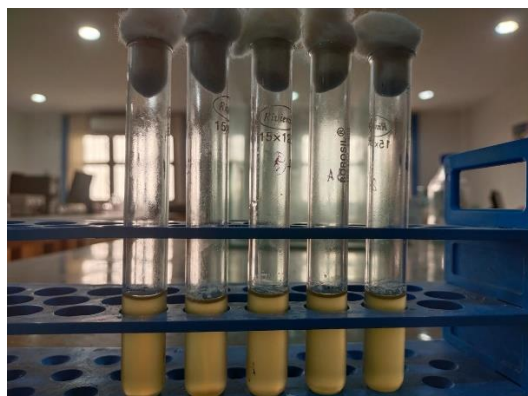


FIG 5.4 LURIA BERTANI BROTH

5.5 Biochemical characterization

Morphological and Biochemical characterization of vibrio species

Test Type	Test	Characterization
Morphological characterization	Shape	Comma or rod
	Motility	+
	Growth in 0% NaCl	-

	Growth in 2,4,8 % NaCl containing media	+
	Growth at 4°C	-
	Growth at 40°C	+
Biochemical characterization	Gram staining	-
	Oxidase	+
	Catalase	+
	MacConkey agar	+
	Indole Production	+
	Voges – proskauer	-
	H ₂ S production	-

Table 5.5

5.6 BACTERIAL GROWTH CURVE

Growth Kinetics was to form of optical density measured using multimode reader. Values obtained are plotted in from graph. It can be seen that bacterial growth curve has been formed well over the period on time.

5.6.1 Growth kinetic for sample 1 OD value

Time (mins)	9.15	10.15	11.15	12.15	1.15	2.15	3.15	4.15
Growth Distance	0.338	0.396	0.562	0.634	0.611	0.611	0.513	0.5

Table 4.6.1

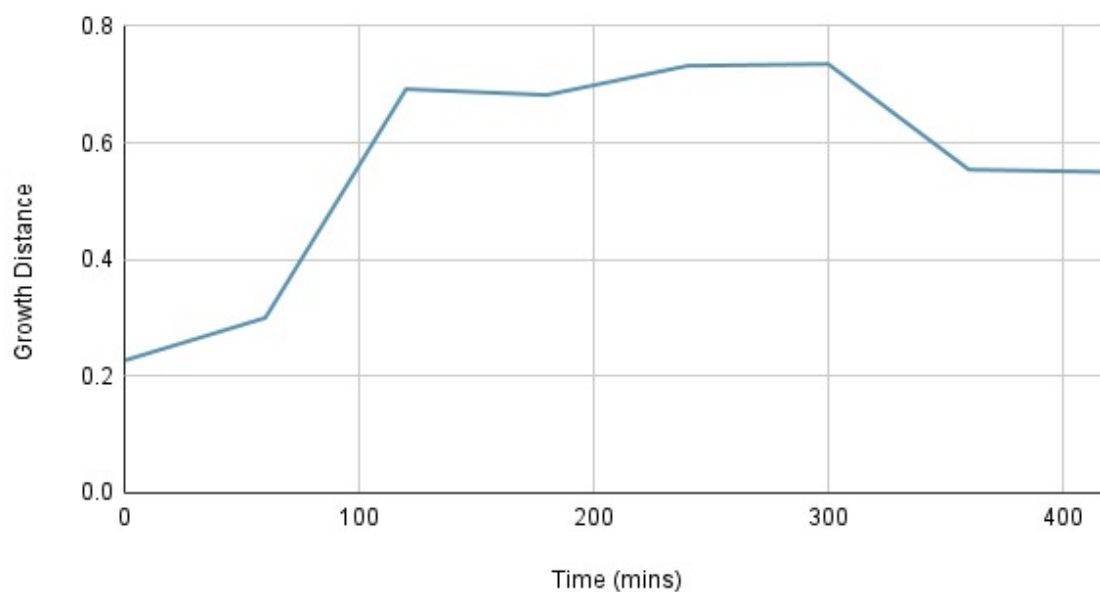


FIG 5.6.1

5.5.2 Growth kinetics for sample 2 OD value

Time (mins)	9.15	10.15	11.15	12.15	1.15	2.15	3.15	4.15
Growth distance	0.338	0.396	0.562	0.634	0.611	0.611	0.513	0.5

Table 5.5.2

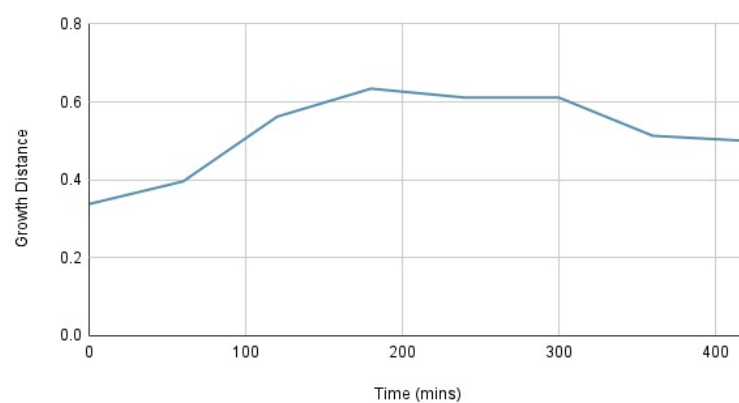


Fig 5.6.2

4.6.3 Growth kinetic in sample 3 OD value

Time (mins)	9.15	10.15	11.15	12.15	1.15	2.15	3.15	4.15
Growth Distance	0.346	0.586	0.929	1.003	0.989	0.921	0.9	0.85

Table 4.6.3

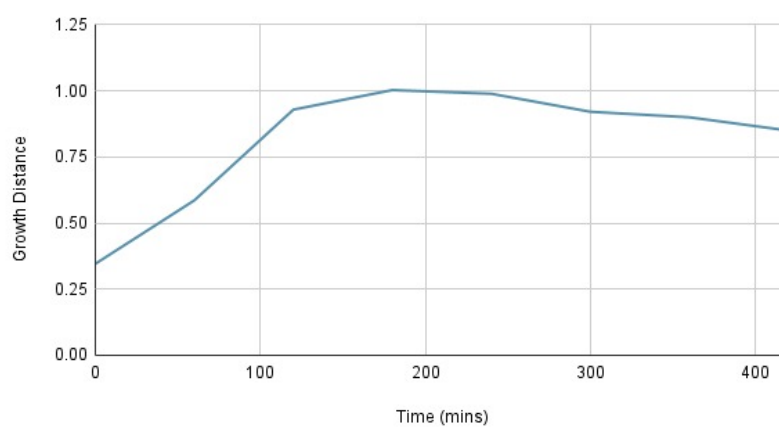


Fig 5.6.3

5.6.4 Growth kinetics for 4 OD value

Time (mins)	9.15	10.15	11.15	12.15	1.15	2.15	3.15	4.15
Growth Distance	0.257	0.332	0.632	0.863	0.993	0.969	0.834	0.75

Table 5.6.4

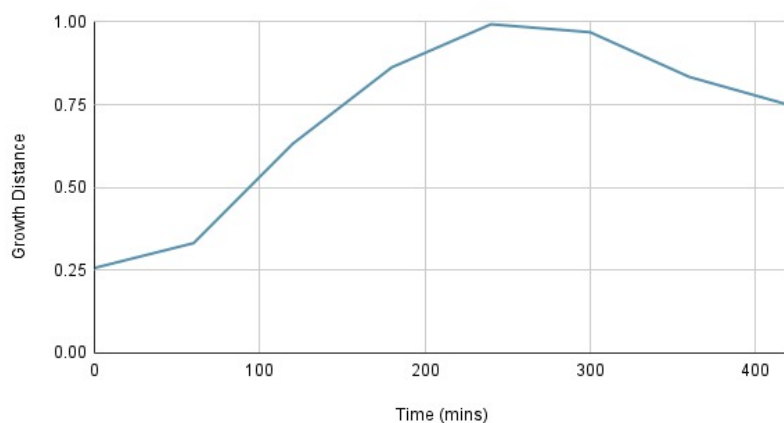


Fig 5.6.4

5.6.5 Growth kinetic for sample 5 OD value

Time (mins)	9.15	10.15	11.15	12.15	1.15	2.15	3.15	4.15
Growth Distance	0.155	0.158	0.492	0.5	0.502	0.42	0.412	0.4

Table 5.6.5

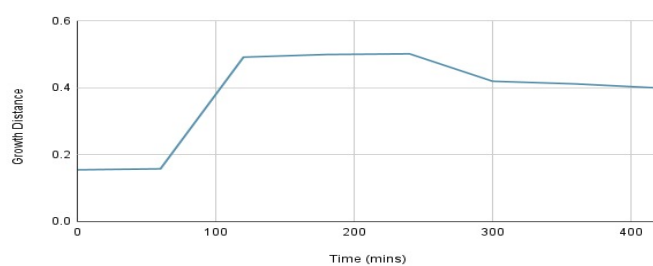


Fig 5.6.5

5.7 Molecular identification of vibrio species

5.7.1 Gel electrophoresis

- Gel electrophoresis is used to separate DNA fragment according to their size
- DNA sample are loaded on 8 well tray.
- One end gel and electric current is applied.
- DNA fragment are negative charged and they moving towards positive electrode.

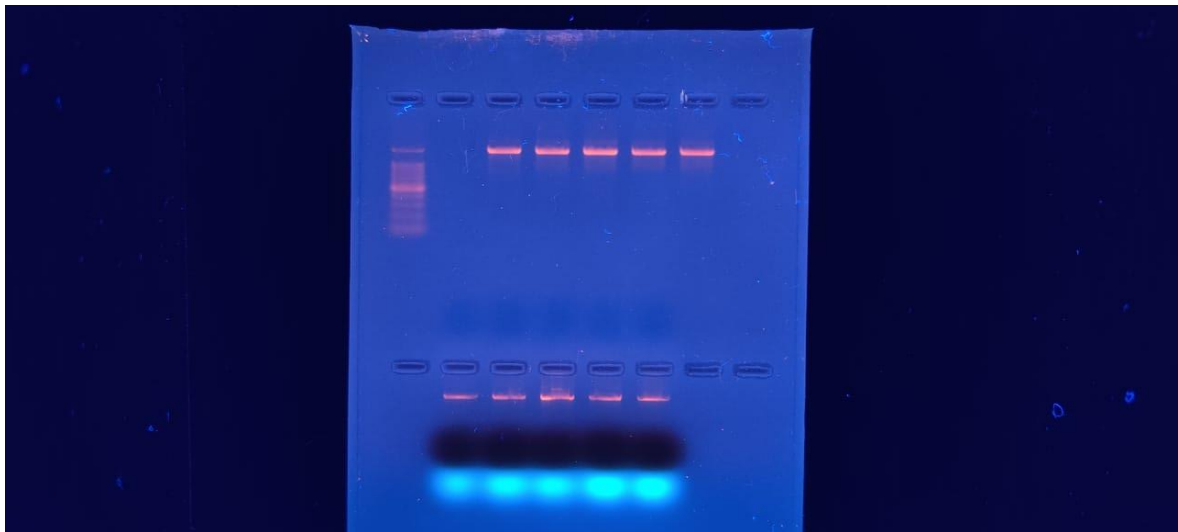


FIG 5.7.1

5.7.2 16s rRNA Sequences

We were getting 6 different type *Vibrio harveyi*, *Vibrio campbellii*, *Vibrio parahaemolyticus*, *Vibrio proteolyticus*, *Vibrio rotiferianus*, *Vibrio alginolyticus*, *Vibrio owensii*.

5.8 Antimicrobial susceptibility

In the result of antimicrobial susceptibility we will discuss about zone of inhibition in *Vibrio* species . we used three antibiotic disk that are Penicillin ,Chloramphenicol, Erythromycin. In figure 5.8.1 Chloramphenicol shows more zone of inhibition nearly 1.5cm and Erythromycin show nearly 0.8 cm . In figure 5.8.2 chloramphenicol resist more zone in it and Erythromycin show less zone. In figure 5.8.3 chloramphenicol show less than 1 cm zone of inhibition. All of three plate chloramphenicol resist more of zone of inhibition in *Vibrio* species. But penicillin does not react with *vibrio* that is no zone of inhibition

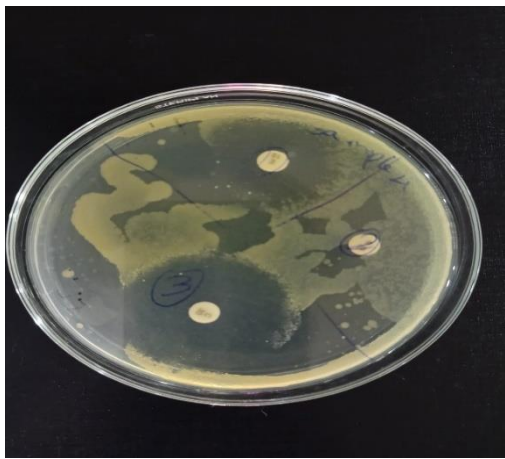


FIG 5.8.1

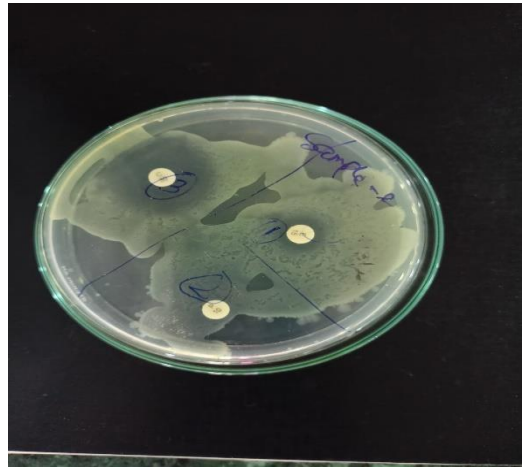


FIG 5.8.2

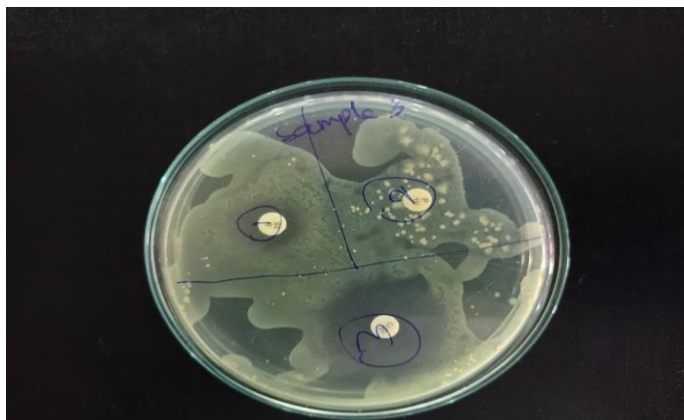


FIG 5.8.3

CHAPTER – 6

CONCLUSION AND SUMMARY

Vibrios are the most common genera associated with shrimp aquaculture practices. These practices often cause significant economic losses. It can also cause change in the ecosystem and their external factors. The infected shrimp *Litopenaeus vannamei* was collected from aquaculture ponds. The isolate was screened on thiosulphate citrate bile salt sucrose (TCBS) agar plates for the selection of vibrio species. Biochemical tests are done for the conformation of chemical groups that are present in the isolates. Water parameters are done for physical and chemical properties and microbiological characteristics of water. PH and salinity is considered as one of the most important factors. Water should be rich in microorganisms. Vibrionaceae were reported. Of those, 6 isolates are related to vibrio species which are responsible for vibriosis in shrimps. Those were vibrio harveyi, vibrio campbellii, vibrio parahaemolyticus, vibrio proteolyticus, vibrio rotiferianus, vibrio alginolyticus. Vibrio owensii. Chloramphenicol the antibiotic disk the resist more zone of inhibition. Growth kinetics also good result of lag, log , stationary phase , death phase bacteria are mention in above result.

CHAPTER 7

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