MOLECULAR DETECTION OF DENGUE VIRUS SEROTYPESOF RECENT OUTBREAK IN KOLKATA

Project report submitted by

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CERTIFICATE

This is to certify that ***Miss JAYITA GHOSH (Reg. No. 1081721400011, OF 2017-18)*** *studying in M.Sc. Microbiology, Bidhan Nagar College,* ***WEST BENGAL STATE UNIVERSITY,*** *satisfactorily completed her project work on "****Molecular Detection Of Dengue Virus Serotypes In Recent Outbreak In Kolkata****". She has worked at Indian Council of Medical Research Virus Laboratory, Kolkata, West Bengal under my guidance from 2nd July to 7th September, 2018. Any project similar to this should be granted as a sheer coincidence.*

Date: 07. 09. 2018

Place: Kolkata

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CONTENT

ABBREVIATION

DENV	DENGUE VIRUS
DHF	DENGUE HAEMORRHAGIC FEVER
DSS	DENGUE SHOCK SYNDROME
E	ENVELOPE
PrM	PRE-CORE MEMBRANE
NS	NON-STRUCTURAL
ORF	OPEN READING FRAME
SL	STEM LOOP
NSAID	NON-STEROIDAL ANTI-INFLAMMATORY DRUGS
ICAM 3	INTRACELLULAR ADHESION MOLECULE 3
ADE	ANTIBODY DEPENDENT ENHANCEMENT
HRP	HORSE RADISH PEROXIDASE
IG	IMMUNOGLOBULIN
µM	MICROMOLAR
ml	MILLILITRE
N	NORMALITY
TAE	TRIS ACETATE BUFFER
UTR	UNTRANSLATED REGION
WHO	WORLD HEALTH ORGANIZATION
YF	YELLOW FEVER
JF	JAPANESE FEVER
PCR	POLYMERASE CHAIN REACTION
VLP	VIRUS-LIKE PARTICLE

ABSTRACT

Dengue is emerging as the most important mosquito borne viral disease in the world expanding its habitat across Asia, Africa, Central America, South America, and the Pacific. DENV is a single stranded RNA virus of the family *Flaviviridae*, genus *Flavivirus*. This virus is transmitted to the people through the bite of the mosquitoes *Aedes aegypti* and *Aedes albopictus*, which are a day biting mosquitoes and are found especially in the tropical regions. In recent years, the dengue virus serotypes have spread throughout the tropical regions of the world. In many places, multiple strains of dengue viruses are recorded and patients with multiple dengue virus serotype infections are developing the more severe form of the disease known as Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS).DENV-specific antibodies are generated in humans are serotype- cross-reactive, weakly neutralizing, and directed against the immature pre-membrane (prM), envelope (E), and non-structural 1 (NS1) proteins. The four serotypes of dengue virus (DENV) are DENV-1, DENV-2, DENV-3, and DENV-4. It represents a global health challenge.

In this study, my aim is to find out which serotype of dengue virus is the most prevalent one in the Kolkata region. To fulfill my goal the Dengue infection is firstly diagnosed by the presence of NS1 Ag in human blood serum by the method Dengue NS1 antigen ELISA and then the serotypes of the Dengue virus were identified by the classical RT-PCR and Semi-Nested PCR process followed by visualization under gel documentation. Among a total of 285 NS1 positive samples of 2017, 51 serum samples were selected randomly and further analyzed for serotyping. 51 samples out of 51 NS1 positive samples were rechecked and all samples gave the positive result in Dengue NS1 antigen testing Experiment. Out of 51 dengue NS1 positive samples, 84% samples gave positive for DENV-2 Serotype,14% samples gave positive for DENV-4 serotype, and 6% samples are DENV-1. 4%of the samples gave positive result for DENV-3.

Based on the virological and serological investigation it can be concluded that DENV-2 was the major circulating dengue serotype in Kolkata.

KEY WORDS: *Flaviviridae, Aedes aegypti, Aedes albopictus*, Serotypes, Epidemiology, Dengue Haemorrhagic Fever, Endemic, NS1 antigen, Serological, Virologic.

INTRODUCTION

Dengue is an arthropod-borne acute viral infection with potential fatal complications. Worldwide, an estimated 2.5 billion people are at risk of this infection, approximately 975 million of whom live in urban areas in tropical and sub-tropical countries in Southeast Asia[21].Transmission also occurs in Africa and the Eastern Mediterranean, and rural communities are increasingly being affected. Dengue fever was first referred as *"water poison"* associated with flying insects. The word *"dengue"* is derived from the Swahili phrase *"Ka-dinga pepo"*, meaning *"cramp-like seizure"*. Dengue viruses (DV) belongs to the family *Flaviviridae* and genus *Flavivirus* and there are four serotypes of the virus referred to as DENV-1, DENV-2, DENV-3 and DENV-4[22]. This is a positive-stranded encapsulated RNA virus is composed of three structural protein genes, which encode the nucleocapsid or core (C) protein, a membrane-associated (M) protein, an enveloped (E) glycoprotein and seven non-structural (NS) proteins- NS1,NS2A,NS2B,NS3,NS4A,NS4B,NS5.The transmission of dengue to humans occurs through mosquitoes like *Aedes aegypti* and *Aedes albopictus*, hence these infections are also referred as arboviral infections and have become a growing public health problem in the tropical and subtropical countries. The mosquitoes are day-biting mosquitoes that prefer to feed on humans and breeds in standing water. Co-infection with Chikungunya and Zika virus complicates the matters. A vaccine_for_dengue fever has been approved and is commercially available in a number of countries. Other methods of prevention are by reducing mosquito habitat and limiting exposure to bites. This may be done by getting rid of or covering standing water and wearing clothing that covers much of the body. Treatment of acute dengue is supportive and includes giving fluid either by mouth or intravenously for mild or moderate disease respectively. Paracetamol (acetaminophen) is recommended instead of nonsteroidal anti-inflammatory drugs (NSAIDs) for fever reduction and pain relief in dengue due to an increased risk of bleeding from NSAID use.

- **Taxonomic position of the virus follows:-**

  Group: Group IV [(+) ssRNA]

  Order: Unassigned

  Family: Flaviviridae

  Genus: *Flavivirus*

  Species: Dengue virus

AIM AND OBJECTIVES

DETERMINATION OF DENGUE SEROTYPES AMONG NS1 SEROREACTIVE SAMPLES.

REVIEW

ON

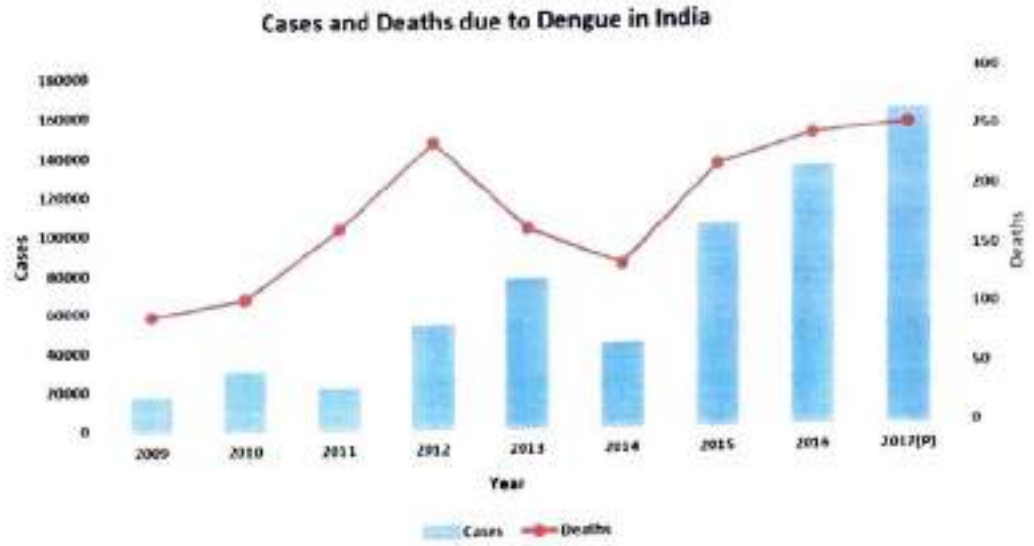
LITERATURE

EPIDEMIOLOGICAL INFORMATION

- The first reported epidemics of Dengue-like disease occurred on three separate continents almost simultaneously in 1779 in Batavia (present- day Jakarta) and in 1780 in Philadelphia. Although there is some disagreement as to whether all of these epidemics were caused by Dengue virus. It is clear that Dengue and other arboviruses with similar ecology had widespread distribution in the tropics as long as 200 years ago.
- For the next 175 years major pandemics of Dengue-like illness, occurred in Asia and the Americas at variable intervals ranging from 10 to 30 years. Since then, epidemics have been reported in Calcutta (1824, 1853, 1871 and 1905), the West Indies (1827), Hongkong (1901), Greece (1927-1928), Australia (1925-1926, 1942), the United States (1922), and identification of the four Dengue virus serotypes, their distribution became better known.
- Asia historically has been the area of highest endemicity, with all four Dengue serotypes circulation in the large urban centre of most countries. During and shortly after World War II , Aedes aegypti became more widespread in Asia, and with the subsequent urbanization that occurred in most countries, the incidence of Dengue infection increased dramatically.
- This increase coincided with the emergence of epidemic DHF in the 1950s (Gubler, 1988). The factors responsible for this global resurgence of DF and the emergence of the DHF include unprecedented population growth, unplanned and uncontrolled urbanization, increased air travel, the lack of effective mosquito control, and the detoriation during the past 30 years, of public health infrastructure (Rigau-Perez et. al., 1998).
- Dengue is predominant in tropical areas mostly in South-East Asia, Africa and Southern parts of the United States. The first large epidemic of DHF (Dengue Haemorrhagic Fever) occurred in Cuba in 1981 with 24,000 cases of DHF and 10,000 cases of DSS (Dengue Shock Syndrome). In the 1980s, DHF began a second expansion into Asia when Sri Lanka, India, and the Maldives had their first major DHF epidemics : Pakistan first reported an epidemic of Dengue fever in 1994.

The epidemics in Sri Lanka and India were associated with multiple Dengue virus serotypes, but DEN-3 was predominant and was genetically distinct from DEN-3 virus previously isolated from infected persons in those countries.

- After an absence of 35 years, epidemic Dengue fever reemerged in both Taiwan and the People's Republic of China in the 1980s. In 1986 and 1987 massive outbreaks of Dengue were reported in Brazil. In 1988, an epidemic of DF was reported at 1700m above sea level in Guerrero State, Mexico 3 and in 1990 almost one-fourth of 3,00,000 people living in Iquitos, Peru contracted DF.
- The average number of DF / DHF cases reported to WHO per year has risen from 908 between 1950 and 1959 to 514,139 between 1990 and 1999. The real figure is estimated to be closer to 50 million cases a year causing 24,000 deaths. Of an estimated 500,000 cases of DHF /DSS requiring hospitalization each year, roughly 5% die according to WHO statistics (Guha-Sapir & Schimmer, 2005).
- In the recent outbreaks, during the first months of 2007 over 16,000 cases have been reported in Paraguay, maximum of which have been detected as DHF cases. This new epidemic is expected to continue in Paraguay for several months, given the forecast of continuous rain all through the summer. Ten deaths have also been reported, including recently a high-ranking member of the Ministry of Health. The epidemic has been root of a scandal in the Paraguyan Department of Health, where one official has resigned because he had approved the use expired batches of insecticide to control the mosquito vectors of Dengue. The disease has propagated to Argentina (where it is not considered endemic), in almost all cases by people who recently arrived from Paraguay. In the Brazilian state of MatoGroso do Sul, which borders on Paraguay, the number of cases in March 2007 is estimated to be more than 45,000. Epidemics in the states of Ceara, Para, Sao Paulo and Rio de Jaeiro have taken the Brazilian National tally of cases this year to over 70,000, with upwards of 20 deaths. The proportion of case registered as DHF is reported to be higher than in previous years.



Source: Directorate of National Vector Borne Disease Control Programme, Dte.GHS, Ministry of Health & Family Welfare

Fig 1: Number of reported cases and fatality rates in western India, 2009 to 2017.

Distribution of dengue, worldwide, 2016

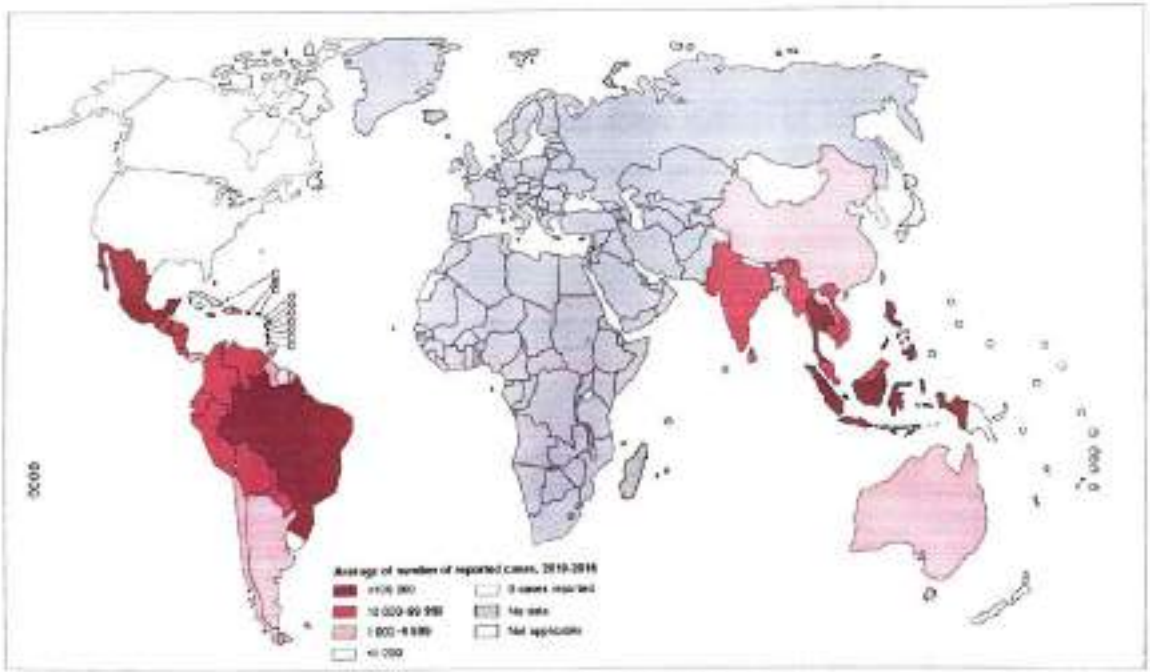


Fig 2: A map showing dengue outbreak in 2016.

VIRAL GENOME

The first structure of a flavivirus has been determined by using a combination of cryoelectron microscopy and fitting of the known structure of glycoprotein E into the electron density map. The virus core, within a lipid bilayer, has a less-ordered structure than the external, icosahedral scaffold of 90 glycoprotein E dimers. The three E monomers per icosahedral asymmetric unit do not have quasi-equivalent symmetric environments. Difference maps indicate the location of the small membrane protein M relative to the overlaying scaffold of E dimers. Electron micrographs showed that dengue virions are characterized by a relatively smooth surface, with a diameter of approximately 500 Å, and an electron-dense core surrounded by a lipid bilayer. In addition to the plus-sense RNA genome of ~10,700 nucleotides, there are three structural proteins that occur in stoichiometric amounts in the particle: the nucleo-capsid or core protein (C, 100 amino acids), a membrane-associated protein (M, 75 amino acids), and an enveloped glycoprotein (E, 495 amino acids). In addition there are also seven non-structural (NS) proteins - NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. The structural proteins are components of the mature virus particle whereas the NS proteins are expressed only in the infected cell and are not packaged to detectable levels into mature particles. The structural proteins are not involved in replication of the viral genome. The genome contains a single open reading frame (ORF) of about 10,000 nucleotides encoding three structural and seven nonstructural proteins.

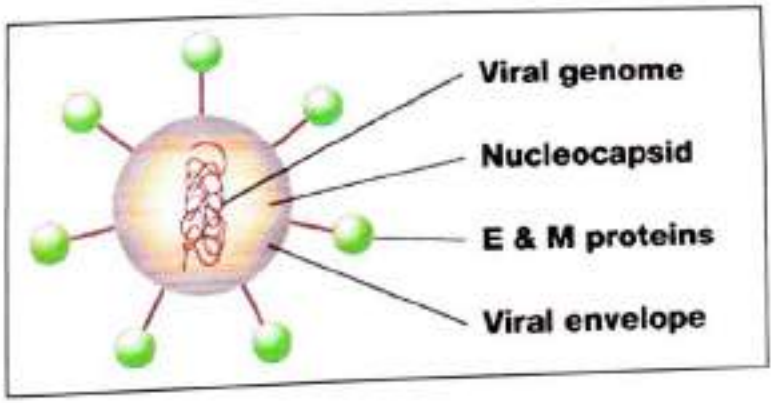


Fig 3: Cross section of Dengue virus showing structural components (© 2011 Nature Education)

❖ Some structural features of Dengue virual genome:

• The 5'end of the RNA has a type-I cap structure but lacks a poly-A tail at the 3'end[23] but ends in a conserved stem loop (SL) structure.

• The genes that encode the Dengue virus structural proteins are located at the 5'end of the genome & comprise slightly more than one-fourth of the coding capacity of the viral RNA.

• They are synthesized as a polyprotein of about 3000 amino acids that is processed co-translationally and post-translationally by viral and host proteases.

• The structural proteins include a capsid protein rich in arginine and lysine residues and a non-glycosylated prM protein.The **DENV M** (membrane) protein, which is important in the formation and maturation of the viral particle, consists of seven antiparallel β-strands stabilized by three disulfide bonds.

• The major **DENV E** (structural envelope) protein, found as a dimer on the surface of the mature viral particle. It has three domain- domain I functions as the hinge region, domain-II contains the fusion protein at its distal tip portion, & domain III is responsible for receptor binding activity.It is involved in the main biologic functions of the virus particle such as cell tropism, acid catalyzed membrane fusion and the induction of haemagglutination-inhibiting, neutralizing and protective antibodies.Several molecules which interact with the viral E protein are ICAM3-grabbing non-integrin, CD209, Rab 5,GRP 78, and the mannose receptor have been shown to be important factors mediating attachment and viral entry.

➢ **The non-structural proteins are considered as NS1-7,which are as follows-**

•**NS1**, a glycoproteinwith two conserved glycosylated sites. They are synthesized in ER as a hydrophilic monomer but mainly remained as hydrophobic homodimer during the transportation to golgi apparatus where it undergoes carbohydrate trimming. is detected in high titers in patients with secondary dengue infections. Though its function is unknown, it is believed to facilitate viral infection & pathogenesis.

•**NS2** region, which codes for two proteins (NS2A and NS2B), are thought to be implicated in polyprotein processing. NS2A is a small hydrophobic protein.

• **NS3** is the viral serine proteases that function in the cytosolic polyprotein processing by its N terminal end & require NS2B as its cofactor.

• **NS4** region codes for two small hydrophobic proteins that seem to be involved in the establishment of the membrane bound RNA replication complex& believed to play an inhibitory role in IFN mediated signal transduction.

• **NS5** codes for a protein with a molecular weight of 105,000 and is the most conserved flavivirus protein. NS5 protein has three major functional domains: the N-terminal S-adenosyl methionine methyltransferase (SAMTase), the nuclear localization sequences (NLS), and the RNA dependent RNA polymerase (RdRp) activity in its C-terminal domain. This protein is believed to play the role in viral genome synthesis.

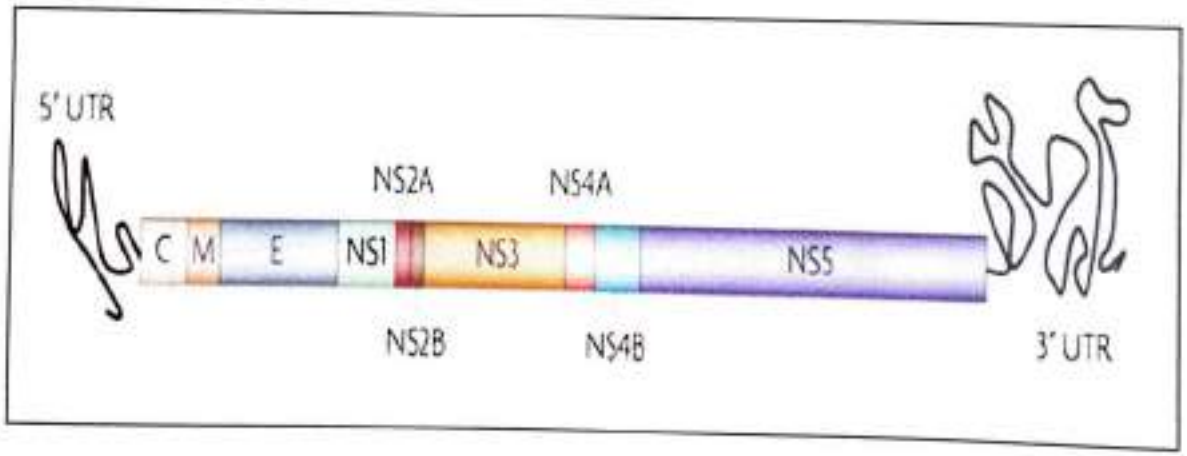


Fig 4: Schematic of DENV RNA genome showing the genomic organization [24]

REPLICATION CYCLE OF DENV

The various steps in the DENV life cycle include -

- Virions are bound to cell-surface attachment molecules and receptors, and are internalized through endocytosis.
- Due to the low pH of the endosome, viral glycoproteins mediate fusion of viral and cellular membranes, allowing disassembly of the virion and release of viral RNA into the cytoplasm.
- Viral RNA is translated into a polyprotein that is processed by viral and cellular proteases.
- The viral NS proteins replicate the genome RNA.
- Virus assembly occurs at the endoplasmic reticulum (ER) membrane, where C protein and vRNA are enveloped by the ER membrane and glycoproteins to form immature virus particles.
- Immature virus particles are transported through the secretory pathway, and in the acidic environment of the trans-Golgi network (TGN), furin-mediated cleavage of M protein drives maturation of the virus.
- Mature virus is released from the cell.

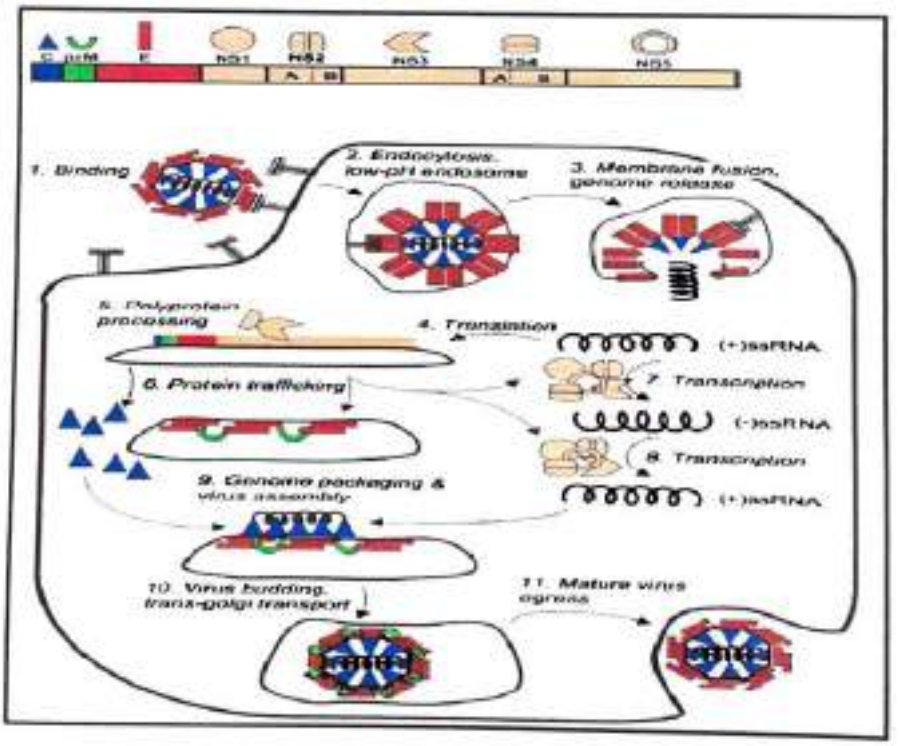


Fig 5: Schematic diagram of dengue proteins likely involved in the various phases of the dengue virus replication cycle

IMMUNOLOGICAL RESPONSE

In the skin, dengue viruses infect immature dendritic cells through the non-specific receptor dendritic cell-specific ICAM3-grabbing non-integrin. Infected dendritic cells mature and migrate to local or regional lymph nodes where they present viral antigens to T cells, initiating the cellular and humoral immune responses. There is also evidence of abundant replication of DENVs in liver parenchymal cells and in macrophages in lymph nodes, liver and spleen, as well as, in peripheral blood monocytes.

- **The humoral immune response:**

The humoral immune response is hypothesized to be vital for controlling DENV infection and dissemination, and infection with one serotype provides long-lasting protection to that specific serotype (homotypic immunity). Subsequent infection by another serotype results in short-lived protection (heterotypic immunity), and may eventually be harmful and increase the risk of severe dengue disease. The transient nature of heterotypic immunity is believed to be due to cross-reactive viral E protein–specific antibodies which are protective above a certain concentration threshold.

The principal targets of the antibody response to DENV infection in humans are the prM, the E structural proteins, and the NS1 protein. Weak antibody responses to other NS proteins, for example, NS3 and NS5, have also been detected. Neutralizing antibodies are directed against the viral E protein and inhibit viral attachment, internalization, and replication within cells. There are multiple epitopes residing within each of the three E domains, but not all are equally accessible for antibody binding due to the dimeric conformation of the E protein on the virion surface, and its tight packing in the mature form.

Domain III of the E protein, which contains the putative host receptor-binding site, is the most variable in amino acid sequence between serotypes. As a result, antibodies specific for this domain show the greatest degree of serotype specificity. However, mutations in domain III of the E protein are common for escaping neutralizing antibody. Loss of an effective neutralizing antibody response due to sequence variation has also been detected for the C and NS2B proteins.

Antibodies against DENV may also bind to complement proteins and promote their activation. Anti-prM and/or E protein antibody–mediated complement fixation to virions can inhibit viral infection. As for other host immune responses to dengue, complement

involvement may also be pathological. Complement activation is a feature of severe dengue and is temporally related to plasma leakage. This suggests that complement activation constitutes a major factor in the pathogenesis of dengue hemorrhagic shock. Increased complement activation at endothelial cell surfaces could contribute to the vascular leakage, and the viral protein NS1 is proposed to be a modulator of the complement pathway. By promoting efficient degradation of C4 to C4b, NS1 may protect DENV from complement-dependent neutralization in solution.

Both in vitro and in vivo, macrophages and monocytes participate in antibody-dependent enhancement (ADE). ADE occurs when mononuclear phagocytes are infected through their F_c receptors by immune complexes that form between DENVs and non-neutralizing antibodies. These non-neutralizing antibodies result from previous heterotypic dengue infections or from low concentrations of dengue antibodies of maternal origin in infant sera. The co-circulation of four DENV serotypes in a given population might be augmented by the ADE phenomenon. The acquired immune response to dengue infection consists of the production of antibodies that are primarily directed against the virus envelope proteins. The response varies depending on whether it is a primary or secondary infection. A primary antibody response is seen in individuals who are not immune to dengue and a secondary immune response is observed in patients who have had a previous dengue infection. A primary infection is characterized by a slow and low-titre antibody response. Immunoglobulin (Ig) M antibodies are the first isotype to appear, by day 3–5 of illness in 50% of hospitalized patients and by day 6–10 of illness in 93–99% of cases. The IgM levels peak ~2 weeks after the onset of fever and then generally decline to undetectable levels over the next 2–3 months. Dengue-specific IgG is detectable at low titre at the end of the first week of illness and slowly increases. By contrast, during a secondary infection, high levels of IgG antibodies that cross-react with many flaviviruses are detectable even in the acute phase and rise dramatically over the following 2 weeks.

- **The cellular immune response:**

In addition to the humoral immune response, cellular immune responses are also crucial in dengue pathogenesis. The DENV can infect both CD4+ T-cells and CD8+ T-cells, and similar to DENV-specific antibodies, the cellular immune responses can be either protective or harmfully reactive. DENV-specific T-cells respond with a diverse set of effectors' functions, including proliferation, target cell lysis, and the production of a range of cytokines. CD4+ T-cells produce IFNγ, TNFα, TNFβ, interleukin (IL)-2, and CC-chemokine ligand 4 (CCL4; also known as MIP1β) which may contribute to

pathogenesis. The production of T helper type-2 cytokines, such as IL-4, is less common. In uncomplicated DENV infections, relatively more CD8+ T-cells are present resulting in lower levels of IFNγ and TNFα. CD8+ T-cell clones specific for DENV partially protect mice from lethal DENV challenge. The role of T-regulatory cells is unclear in dengue, but there is a study suggesting they are functional and expand in acute DENV infection.

Following primary infection, both serotype-specific and serotype cross-reactive memory T-cells are generated. Upon secondary exposure, both the protective and cross-reactive memory T-lymphocytes are activated and the non-protective memory T-cells will augment infection. Activated memory T-cells recognizes both conserved and altered peptide ligand epitopes. The antigen sequence differences depend on the specific DENV epitope but will nevertheless affect the quality of the effector T-lymphocyte response. This in turn modifies the immunological repertoire and is suggested to be involved in the development of plasma leakage. A full agonist peptide will induce a full range of T-cell responses including production of multiple cytokines (e.g. IFNγ, TNF, and CCL4) and lysis of the infected cell. A partial agonist peptide, which varies at one residue, will cause cross-reactivity in memory T-cells and induce a skewed functional response, involving production of some cytokines but little of other cytokines and inefficient cell lysis. Thus, because of sequence diversity between DENV serotypes, the memory T-cells (and B cells) that are re-activated during a secondary DENV infection may not have optimal avidity for the epitopes of the new infecting virus. The 'memory' of the primary DENV infection alters the immune response to the secondary infection influencing the clinical outcome. There is a correlation between the level of T-cell responses and disease severity. The phenomenon of low affinity for the current infecting serotype but a high affinity for a past infection with a different serotype is referred to as "*Original Antigenic Sin*", and is the net effect of an altered balance between a protective and pathological outcome. The pattern of antibody/T-cell responses in secondary DENV infections is also influenced by the sequence and interval between DENV infections. As for the ADE scenario, memory T-cell responses exhibiting serotype cross-reactive proliferative activity decades after the primary infection could potentially alter the balance from a protective immune response toward an improper and non-protective immune response. Interestingly, most of the identified CD4+ and CD8+ T-cell epitopes reside in the NS3 protein, which represents only ~20% of the DENV amino acid coding sequence.

- **Autoimmune responses in dengue virus infection:**

Anti-DENV antibodies can cross-react to host proteins and endothelial cells, and this could enhance the endothelial dysfunction observed in DHF/DSS. Antibodies against the viral surface E protein cross-react with plasminogen and have been associated with bleeding in acute DENV infection, and anti-DENV NS1 antibodies cross-react with host proteins and endothelial cells. In addition, immune activation markers (e.g. IL-6, IL-8, TNFα, IFNγ, and complement components 3A and 5A) together with altered platelet, DC, monocyte, and T-cell functions suggest that immune responses to various DENV components could contribute to autoimmune processes resulting in DHF/DSS.

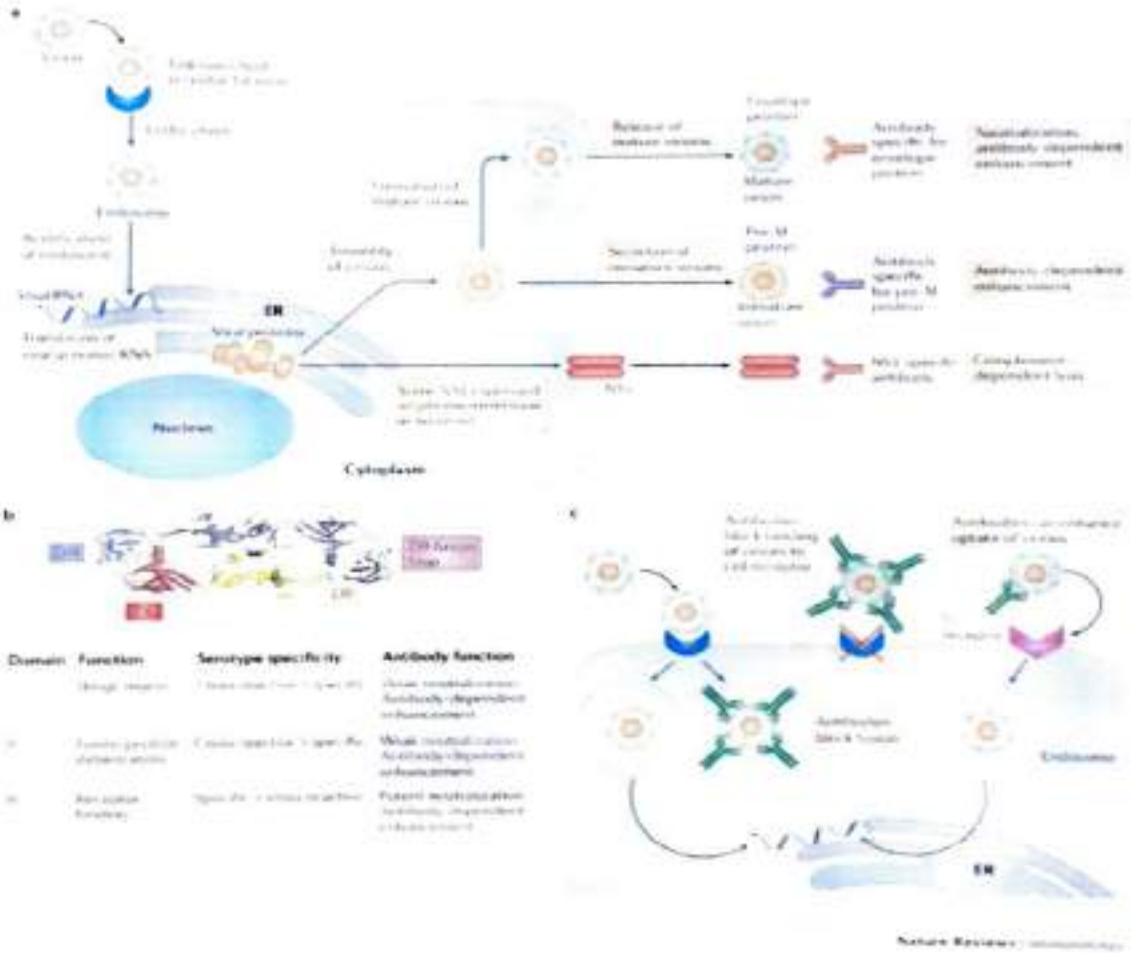


Fig 6: An illustration of Antibody responses to dengue virus protein targets and its functions. [25]

Cytokine	Levels in DF	Levels in DHF	References
IL-1β	-	-	173
IL-2	↑↑	↑	139
IL-4	↓	↑↑	139, 174, 175
IL-6	↑	↑↑	139, 173, 176
IL-8	↑↑	↑↑	173, 176, 177
IL-10	↓	↑↑	139, 173
IL-12	↑↑	↓	173, 178
IL-13	↓	↑↑	179
IL-18	↑	↑↑	179
TNF-α	↑↑	↑↑	139, 173, 175, 176, 180
IFN-γ	↑↑	↑	139, 174, 175, 176, 180
TGF-β	↓	↑↑	181
Human CF	↑	↑↑	136, 139

IL, interleukin; TNF, tumour necrosis factor; IFN, interferon; TGF, transforming growth factor

Table1: Serum cytokine levels in the pathogenesis of dengue [2]

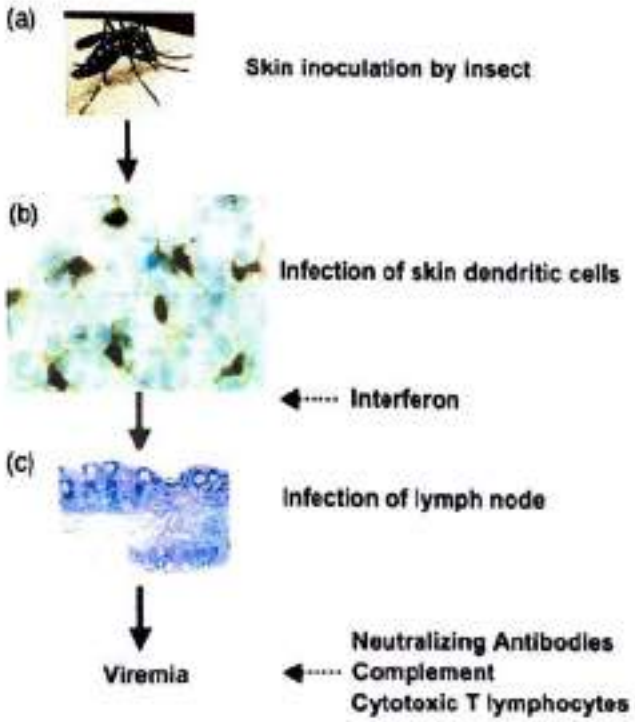


Fig 7: Dengue viral infection. [26]

DISEASE INFORMATION

The WHO 2009 classification divides dengue fever into two groups: uncomplicated and severe, though the 1997 WHO classification is still widely used. The 1997 classification divided dengue into undifferentiated fever, dengue fever (DF), and dengue haemorrhagic fever (DHF).

Dengue virus presents as three clinical syndromes & their symptoms:

❖ Undifferentiated Fever:

The first infection with dengue virus presents with an undifferentiated viral like illness. Maculopapular rashes may appear during the fever or during defervescence. Fever may be associated with nausea, vomiting, retro-orbital pain, asthenia and myalgia.

❖ Dengue Fever:

The clinical features of Dengue fever (DF) frequently depend on the age of the patient. Infants and young children may have an undifferentiated febrile disease, often with a maculopapular rash. Older children and adults may have either a mild febrile syndrome or the classic incapacitating disease with high fever of abrupt onset, sometimes with 2 peaks (saddle-backed), severe headache, pain behind the eyes, muscle and bone or joint pains, nausea and vomiting, and rash. Skin hemorrhages (petechiae) are not uncommon. Thrombocytopenia may be observed. Recovery may be associated with prolonged fatigue and depression, especially in adults. In some epidemics, DF may be accompanied by bleeding complications, such as epistaxis, gingival bleeding, gastrointestinal bleeding, haematuria, and menorrhagia.

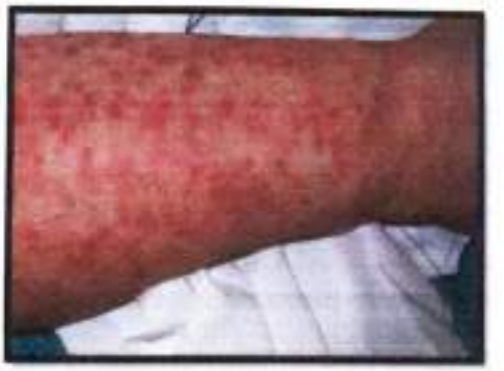


Fig 8: Petechiae & maculopapular rashes on lower limb

❖ Dengue Hemorrhagic Fever (DHF):

Typical cases of DHF are characterized by four major clinical manifestations: high fever, hemorrhagic phenomena, and often, hepatomegaly and circulatory failure. Moderate to marked thrombocytopenia with concurrent haemoconcentration is a distinctive clinical laboratory finding of DHF. The major path physiological change that determines the severity of disease in DHF, and differentiates it from DF, is the leakage of plasma, as manifested by an elevated haematocrit (i.e. haemoconcentration), a serous effusion or hypoproteinaemia.

Children with DHF commonly present with a sudden rise in temperature accompanied by facial flush and other non-specific constitutional symptoms resembling DF, such as anorexia, vomiting, headache, and muscle or bone and joint pain. Some patients complain of sore throat, and an injected pharynx is frequently evident on examination, but rhinitis and cough are infrequent. Mild conjunctival injection may be observed (see Table 2.2). Epigastria discomfort, tenderness at the right costal margin, and generalized abdominal pain are common. The temperature is usually high (39°C) and remains so for 2–7days. Occasionally, temperature may be as high as 40–41°C; febrile convulsions may occur, particularly in infants.

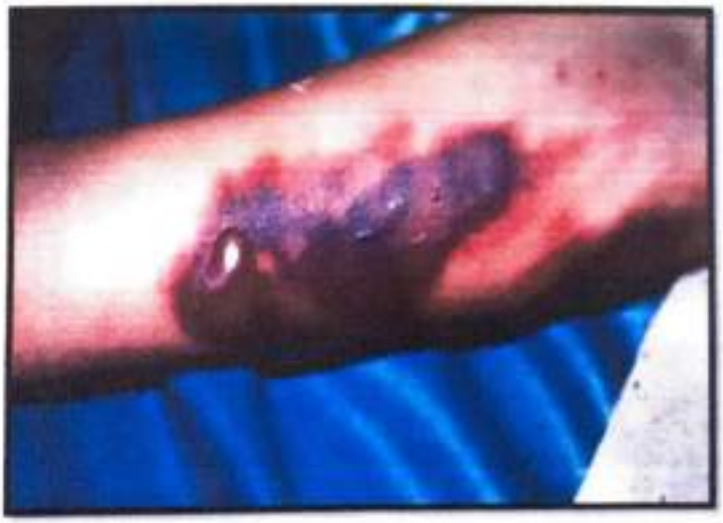


Fig 9: Patient with dengue hemorrhagic fever has a large subcutaneous hemorrhage on upper arm.

❖ Dengue Shock Syndrome (DSS):

The condition of patients who progress to shock suddenly deteriorates after a fever of 2–7 days' duration. This deterioration occurs at the time of, or shortly after, the fall in temperature—between the third and the seventh day of the disease. There are the typical signs of circulatory failure: the skin becomes cool, blotchy, and congested; circumpolar cyanosis is frequently observed; the pulse becomes rapid. Patients may initially be lethargic, then become restless and rapidly enter a critical stage of shock. Acute abdominal pain is a frequent complaint shortly before the onset of shock. DSS is usually characterized by a rapid, weak pulse with narrowing of the pulse pressure (20mmHg, 2.7kPa), regardless of pressure **levels**, e.g. 100/90mmHg (13.3/12.0kPa) or hypotension with cold, clammy skin and restlessness. Patients in shock are in danger of dying if appropriate treatment is not promptly administered. Patients may pass into a stage of profound shock, with the blood pressure or pulse becoming imperceptible. However, most patients remain conscious almost to the terminal stage. The duration of shock is short, typically the patient dies within 12–24 hours.

- **INCUBATION PERIOD:** The incubation period for Dengue is four to six days.

- **VECTOR INTERACTION:** Transmission of DENVs is dependent on the vector mosquito *Aedes aegypti*, and to a lesser extent *Aedes albopictus*. The spread of DENVs mirrors the vectors' geographical distribution underlining why mosquito density is an important parameter for predicting DENV epidemics. The female mosquitoes lay their eggs in artificial water containers such as tires, cans, and jars. Due to water requirements for breeding, mosquito densities peak during wet season, with the direct consequence of rising numbers of dengue cases. The *A.aegypti* mosquito is well adapted to an urban environment and is a highly competitive vector due to its anthropophilic nature. The female mosquito can infect multiple persons in order to complete a single blood meal. Protective clothing and mosquito-repellent sprays are essential to avoid DENV transmission since the *Aedes* mosquitoes are active during the day, minimizing the use of bed nets.

  In general, *A.aegypti* is less susceptible to infection by DENV than *A albopictus*, which could act as a selection mechanism for more virulent strains of DENV; the lower susceptibility would require a higher viral load in the human host in order to infect the

mosquito. High viral titers in humans have been seen to be correlated to severe DHF or DSS. On the contrary, the secondary vector *Ae. albopictus* could transmit DENV strains that do not replicate to such high titers resulting in less clinically overt or severe disease.

Once ingested by the mosquito, the DENV establishes a productive infection in the mosquito midgut, wherefrom the virus disseminates and replicates in other tissues. In order to be transmitted to a human (or non-human primate) host during the next blood meal, the DENV must ultimately infect the salivary glands and be shed in the saliva. Rapid, unplanned growth of urban centers in South-East Asian and South American countries combined with inadequate water supply and sewerage systems have dramatic consequences on the transmission of DENV.

Fig 10: Aedes *aegypti*

- **TRANSMISSION CYCLE:**
  - The transmission cycle of Dengue virus by the mosquito *Aedes aegypti* begins with a Dengue infected person, who have virus circulating in the blood, called viremia that lasts about 5 days.
  - During the viremic period, an uninfected female *Aedes aegypti* mosquito bites the infected person and ingests blood that contains Dengue virus. Although there is some evidence of trans-ovarial transmission of Dengue virus in *Aedes aegypti*, but usually mosquitoes are only infected by biting a viremic person.
  - Then, the virus replicates in mosquito midgut and other organs and infects the salivary glands during an extrinsic incubation period of 8 to 12 days.

- The mosquito then bites a susceptible individual and transmits the virus to him or her, as well as , to every other susceptible person the mosquito bites, for the rest of its lifetime.
- The virus then replicates in the second person and produces symptoms. The symptoms begin to appear an average of 4 to 7 days after the mosquito bites. This is the intrinsic incubation period.
- While the intrinsic period averages from 4 to 7 days, it can run from 3 to 14 days. The viremia begins slightly before the onset of symptoms.
- Symptoms caused by Dengue infection may last 3 to 10 days, with an average of 5 days, after the onset of symptoms. So the illness persists several days after the viremia has ended.

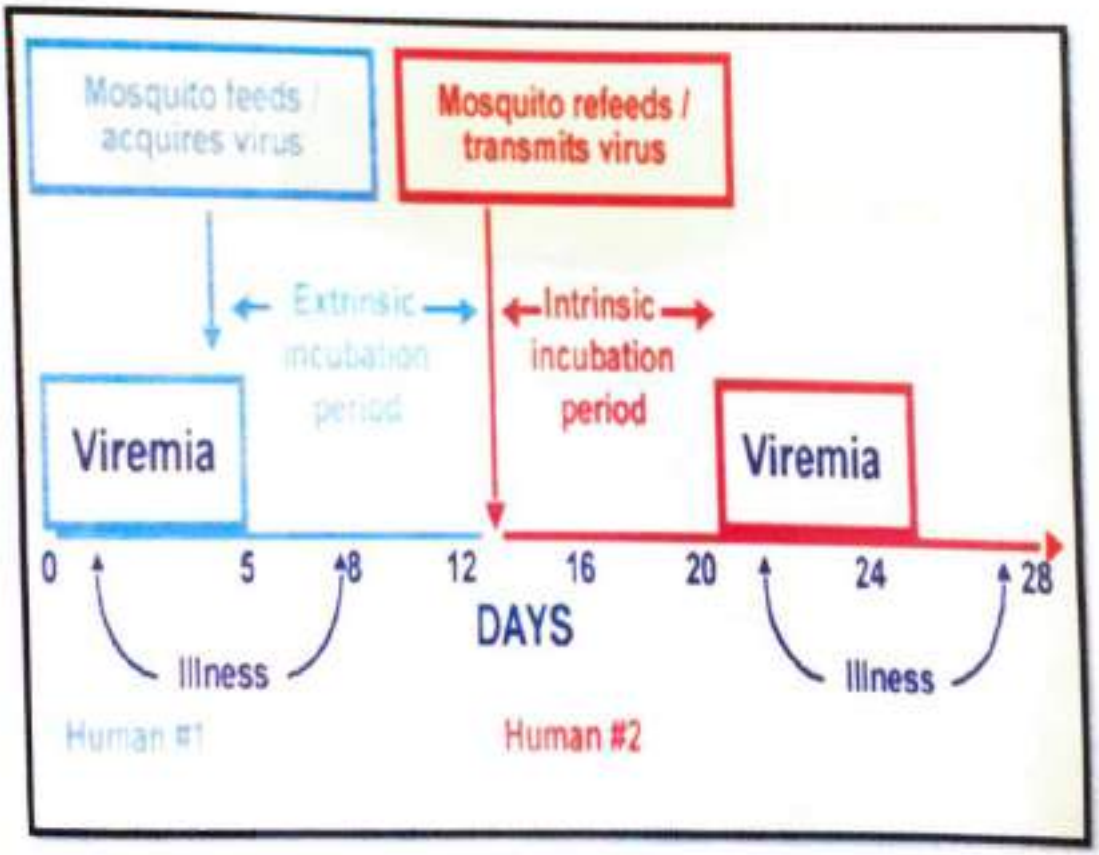


Fig 11: Transmission cycle of Dengue virus.

CLINICAL SYMPTOMS

Dengue is an acute infectious disease of viral etiology characterized by fever, headache, and rash, pain in various parts of the body, and leucopenia & decreasing amount of platelets count. Four main characteristic manifestations of dengue illness are -

(i) continuous high fever lasting 2-7 days;

(ii) haemorrhagic tendency as shown by a positive tourniquet test, petechiae or epistaxis;

(iii) thrombocytopenia (platelet count <100x109/l); and

(iv) Evidence of plasma leakage manifested by haemoconcentration (an increase in haematocrit 20% above average for age, sex and population), pleural effusion and ascites.

Dengue virus presents as the following clinical syndromes & their symptoms -

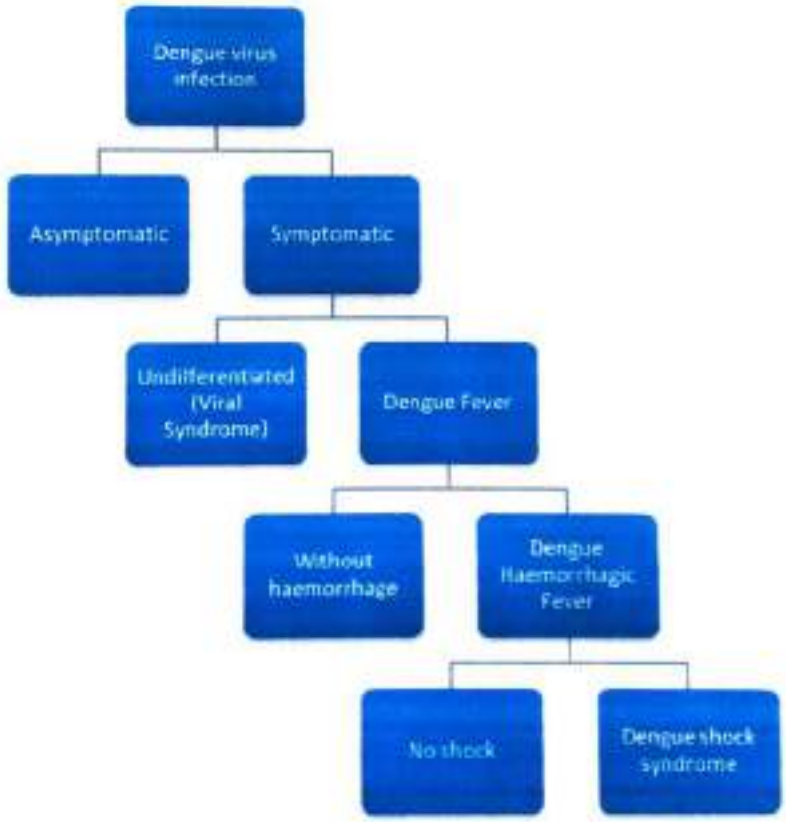


Fig 12: Clinical spectrum of dengue virus infection

✓ SYMPTOM INFORMATION :

The characteristic symptoms of dengue are sudden-onset fever, headache (typically located behind the eyes), muscle and joint pains, and a rash. The alternative name for dengue, "break- bone fever", comes from the associated muscle and joint pains. The course of infection is divided into three phases: febrile, critical, and recovery.

The febrile phase involves high fever, often over 40 °C (104 °F), and is associated with generalized pain and a headache; this usually lasts two to seven days. At this stage, a rash occurs in 50–80% of those with symptoms. It occurs in the first or second day of symptoms as flushed skin, or later in the course of illness (days 4–7), as a measles-like rash. Some petechiae (small red spots that do not disappear when the skin is pressed, which are caused by broken capillaries) can appear at this point, as may some mild bleeding from the mucous membranes of the mouth and nose. The fever itself is classically biphasic in nature, breaking and then returning for one or two days, although there is wide variation in how often this pattern actually happens.

In some people, the disease proceeds to a critical phase, which follows the resolution of the high fever and typically lasts one to two days. During this phase there may be significant fluid accumulation in the chest and abdominal cavity due to increased capillary permeability and leakage. This leads to depletion of fluid from the circulation and decreased blood supply to vital organs.[9] During this phase, organ dysfunction and severe bleeding, typically from the gastrointestinal tract, may occur. DSS and DHF occur in less than 5% of all cases of dengue; however those who have previously been infected with other serotypes of dengue virus ("secondary infection") are at an increased risk.

The recovery phase occurs next, with resorption of the leaked fluid into the bloodstream. This usually lasts two to three days. The improvement is often striking, but there may be severe itching and a slow heart rate. Another rash may occur with either a maculopapular or a vasculitic appearance, which is followed by peeling of the skin. During this stage, a fluid overload state may occur; if it affects the brain, it may cause a reduced level of consciousness or seizures. A feeling of fatigue may last for weeks afterwards.

✓ Associated problems :

Dengue can occasionally affect several other body systems, either in isolation or along with the classic dengue symptoms. A decreased level of consciousness occurs in 0.5–6% of severe cases, which is attributable either to infection of the brain by the

virus or indirectly as a result of impairment of vital organs, for example, the liver. Other neurological disorders have been reported in the context of dengue, such as transverse myelitis and Guillain-Barré syndrome [World Health Organization (WHO), 2009]. Infection of the heart and acute liver failure are among the rarer complications.

❖ **Symptoms Shown in the Syndrome [61] -**

SYMPTOMS	DESCRIPTION	OBSEVATION
Abdominal pain	---------	9.1% in patients with Dengue fever.
Bleeding manifestations	---------	15.2% in patients with Dengue fever.
Fever	Usually seen in older children and are characterized by a rapidly rising temperature that lasts 5-6 days.	76-100% in adults with classical Dengue fever.
Flushed appearance	---------	36.4% in patients with Dengue fever.
Diarrhoea	---------	21.2% in patients with Dengue.
Gum bleeding	Minorhaemorrhagicmanifestations like petechiae, epistaxis, and gingival bleeding do occur.	3% in patients with Dengue fever had gum bleeding.
Haedaache	The febrile period is accompanied by severe headache, retro-orbital pain, myalgia, and nausea and vomiting.	78.8% in patients with Dengue fever had headache.

SYMPTOMS	DESCRIPTION	OBSERVATION
Hepatomegaly	---------	30.3% in patients with Dengue fever had an enlarged liver.
Leukopenia	---------	76-100% in adults with classical Dengue fever.
Lymphadenopathy	---------	26-50% in adults with classical Dengue fever.
Maculopapular rash	Over half of infected people report a rash during the febrile period that is initially maculopapular and becomes diffusely erythromatous, sparing small areas of normal skin	26-50% in adults with classical Dengue fever.
Myalgia / Arthralgia	The febrile period is accompanied by severe headache, retro-orbital pain, myalgia, nausea, and vomiting.	51-75% in adults with classical Dengue fever & 78.8% in patients with Dengue fever had myalgia. 57.6% in patients with Dengue fever had arthralgia.
Petechiae / Ecchymosis	Minor haemorrhagic manifestations like petechial, epistaxis, and gingival bleeding do occur.	---------
Vomiting	Generalized abdominal pain, persistent vomiting, a sudden drop in the platelets count, and a rapid rise in the hematocrit.	62% of Puerto Rican cases confirmed with DHF in the laboratory had vomiting.

DIAGNOSIS

The definitive diagnosis of Dengue virus infection can only be made in the laboratory and it depends on the isolation of these viruses , the detection of viral antigens or RNA in serum or tissues, or the detection of specific antibodies in the patient's serum [27]. Five serological tests have been used for the diagnosis of Dengue infection: Hemagglutination inhibitor (HI), Complement fixation (CF), Neutralization test (NT), IgM capture enzyme linked immunosorbent assay (MAC-ELISA) and Indirect IgG ELISA. The limitations of these tests are the high cross reactivity observed with these tests, requiring a comprehensive pool of antigens, including all 4 serotypes, another flavivirus (Yellow fever virus, Japanese encephalitis virus or St. Louis encephalitis virus) and in some areas another virus that causes similar clinical manifestations but that is not *lavivirus*, such as Oropouche, Mayaro or Chikungunya viruses. Furthermore the Dengue antibodies are better detected around the fifth day of disease onset, making this technique unfeasible for rapid diagnosis[27]..Dengue antigens can be detected in tissues such as liver, spleen and lymph nodes as well as tissues from fatal cases (slides from paraffin-embedded, fresh or frozen tissues) using an enzyme and a colorimetric substrate with antibodies that target dengue-specific antigens.

NS1 is a glycoprotein produced by all flaviviruses and is essential for viral replication and viability. Because this protein is secreted into the bloodstream, many tests have been developed to diagnose DENV infections using NS1. These tests include antigen-capture ELISA, lateral flow antigen detection and measurement of NS1-specific IgM and IgG responses. NS1 antigen detection kits are now commercially available. As yet, these kits do not differentiate between the different DENV serotypes. Additional independent studies are needed to confirm the performance of these kits and to further validate the diagnostic and prognostic significance of NS1 and NS1-specific antibody detection.

Many nucleic acid amplification tests (NAATs) have been developed for the diagnosis of dengue infection. Some techniques are quantitative and others can be used for serotyping. The most commonly used NAATs are based on nested RT-PCR assay. The nested PCR reaction involves an initial reverse transcription and amplification step using dengue primers that target the conserved region, prM of the virus genome followed by a second amplification step that is serotype specific. The products of these reactions are separated by electrophoresis on an agarose gel, which allows the dengue serotypes to be differentiated on the basis of size of genome.

TREATMENT

The management of dengue virus infection is essentially supportive and symptomatic. No specific treatment is available. However, there are Indian studies which have contributed in terms of better management of DHF/DSS.

- A rapid response to platelet and fresh frozen plasma (FFP) transfusion is reported in a study. The types of fluids used are -

- **Crystalloid**: Five per cent dextrose in lactated Ringer's solution, five per cent dextrose in acetated Ringer's solution five per cent dextrose in half strength normal saline solution and five per cent dextrose in normal saline solution.
- **Colloidal:** Dextran 40 and Plasma

- Anti-D has been used in children with DHF and severe refractory thrombocytopenia.
- In experimental study pre-feeding mice with trivalent chromium picolinate (CrP) in drinking water could abolish the adverse effects of DV infection on most of the hematological parameters.
- *Hippophae rhamnoides* (Sea buckthorn, SBT) leaf extract has been shown to have a significant anti-dengue activity.
- Mycophenolic acid (MPA) inhibits Dengue virus infection. The drug currently is used as an immunosuppressive agent to inhibit Dengue virus antigen expression, RNA replication & virus production. Early steps in viral infection, such as viral entry and nucleocapsid uncoating are not primary target. It interfere with viral protein synthesis in the amplification phase. Quantitive RT-PCR demonstrated that MPA prevents the accumulation of viral +ve & -ve strand RNA as the infection proceeds.

❖ RISK FACTORS OF DENGUE :

1. Tropical & subtropical areas.
2. Immune status of a person.
3. Previously infected individual will have greater chances of developing DHF if infected again.
4. All 4 dengue serotypes i.e., DEN-1, DEN-2, DEN-3 and DEN-4, produce clinically identical disease and all can produce DHF and DSS in decreasing order of frequency: serotypes 2, 3, 4, & 1.
5. People who develop DHF have a 5% chance of death but if they go on to develop DSS then; the mortality rate can rise as high as 40%.
6. Effective mosquito control efforts are underway in most countries where dengue is prevalent.
7. Public health systems to detect & control epidemics are deteriorating around the world.
8. Increasing population of cities in the tropical countries has led to overcrowding, urban decay and sub standard sanitation, thereby allowing more mosquitoes to live closer to people and increasing the chance of infection.
9. The increase in non-biodegradable plastic packaging and discarded tires acts as a new breeding sites for mosquitoes.
10. Increased jet air travel is helping people infected with dengue. The International Air Travel Association forecasts that international passenger numbers will grow from 1.11 billion in 2011 to 1.45 billion in 2016, with the dengue-endemic regions of Latin America, Africa and the Asia-Pacific region representing three of the top five fastest growing regions [28].
11. Viruses to move easily from city to city by immigration.

❖ VACCINE FOR DENV :

Dengue vaccines have been under development since the 1940s, but a tetravalent vaccine which simultaneously provides long-term protection against all DV serotypes is round the corner. A tetravalent antigen was designed by splicing the EDIIIs (one of the Envelop Domains) of DENV-1, DENV-2, DENV-3 and DENV-4 using flexible pentaglycyl linkers. A synthetic gene encoding this tetravalent antigen was expressed in Pichiapastoris and purified to near homogeneity. Efforts are underway to present the tetravalent antigen on a chimeric VLP (Virus-Like Particle) platform. Some promising dengue antigens have been developed using different systems.

The first dengue vaccine, Dengvaxia (CYD-TDV) by Sanofi Pasteur, was first registered in Mexico in December, 2015.CYD-TDV is a live attenuated tetravalent chimeric vaccine made using recombinant DNA technology by replacing the PrM (pre-membrane) and E (envelope) structural genes of the yellow fever attenuated 17D strain vaccine with those from each of the four dengue serotypes. Ongoing phase III trials in Latin America and Asia involve over 31,000 children between the ages of 2 and 14 years. In the first reports from the trials, vaccine efficacy was 56.5% in the Asian study and 64.7% in the Latin American study in patients who received at least one injection of the vaccine. Efficacy varied by serotype. There are approximately five additional vaccine candidates including DENVAX, TETRAVAX-DV, and TDEN-PIV under evaluation in clinical trials, including other live-attenuated vaccines, as well as subunit, DNA and purified inactivated vaccine candidates.

PREVENTION

Physical control :

- **GIS mapping of dengue foci** - Among the advanced techniques used for location of DENV, GIS mapping has been efficient in locating dengue concentrations. By locating dengue Seri-positive cases within the study area, dengue transmission can be prevented by locating dengue foci, and then treating them with diverse preventive strategies [29]. Kittayapong et al., showed that GIS mapping not only allowed better surveillance and community-based intervention programs for suppressing dengue; it also determined the rate of successful control in the mapped areas. In their study, surveillance of the mapped dengue foci determined the major breeding sites of A. *aegypti* mosquitoes to be water containers and bath basins [30].

- **Focused and effective surveillance** - Surveillance provides fundamental information on the assessment of risk, outbreak reaction, program evaluation and guidance, as well as delivers timely responses to prevent and control dengue [31]. Surveillance enables the understanding of spatiotemporal distribution of dengue cases, and provides entomological and epidemiological links for better planning [32]. On the other hand, these programs are not focused on the elimination of dengue vector [33]. The eruption of dengue in Singapore, after decades of surveillance, indicated unsustainable vector control measures and ineffective surveillance in 2005 [34]. An effective surveillance system aiming at vector identification [35] and eradication [33], providing the underlying information regarding vector concentration and its breeding, will prove beneficial in controlling vector species.

- **Determination of oviposition sites** - As determined by Morrison et al., *A. aegypti* females lay eggs above the water in containers or jars and so on for their survival improvement. To detect and reduce the population density of dengue vectors, it is necessary to determine the behavioral pattern of vectors. Wong et al., studied the oviposition pattern of A. *aegypti* and reported that strong intra-specie affinity may be an indication of targeting vector specie. Moreover, once the oviposition sites have been determined, introduction of strategies that eliminate mosquito population at a later developmental stage will increase the efficacy of control strategies [36]. Recently, introducing oviposition-based innovative techniques have shown promising results in intensifying control of vector species [37].

- **Community-based control programs** - Community-based control programs are developed with the aim to educate the community about the measures for the extermination of mosquito breeding sites. People in a community are divided into various groups depending upon their level of education and understanding [33]. The significance of community-based programs for elimination of dengue mosquitoes in Kerala district [38], Mexico [38], and Cuba [38] has been proven in the form of elevated awareness among the communities. Through community involvement, a variety of techniques can be integrated for maximum control of vector population [39], such as, the combination of community-based program and chemical control of *A. aegypti* have yielded significant results in Cuba [40].
- **Education of prevention strategies** - It has been noted that the success of community-based strategies depends upon the knowledge, education, and behavior of the people, and strategies involved [41]. Education serves as a basis for an ability of an individual to identify and deal with vector habitats, and use preventive measures. Madeira et al. emphasized that distribution of information brings awareness in order to control dengue, and provides necessary measures for the destruction of vector habitats [42]. A recent study in Thailand showed that education of prevention strategies through media also played a vital role in developing awareness [43].

Biological control :

- **Paratransgenesis and use of *Wolbachia*** - Nowadays, genetic control of *A. aegypti* has risen as a set of promising techniques, among which paratransgenesis is the popular method [44]. This approach utilizes genetically-modified symbiotic bacteria that are reintroduced in the vector to colonize the vector population, hence limiting the transmission of disease [44]. These genetically modified bacteria cause harmful effects in the host body, deregulate its sexual cycle, decrease the host competence and interfere with the developmental processes of the vector species, thereby suppressing the vector population [44]. As reported in the study by Jeffery et al., the most effective bacterial agents used is *Wolbachia* [45], which is a reproductive parasite interfering with the cellular and reproductive mechanisms of the vector species [44].

- **Vector species genetic modification** - The genetic methods for the control of *A. aegypti* aim at suppressing the population and its replacement or transformation. Therefore, the aim is designed to provide an alternate that could be accounted for providing an effector gene for reduction and inhibition of disease transmission [46]. The field release of genetically modified mosquito species in Brazil showed an 85% decline in *A. aegypti* population [47], indicating that genetically modified vector species are innovative and feasible methods used for blocking the transmission of mosquito-borne diseases [48].
- **Use of sterile insect technique (SIT)** - As the name indicates, SIT refers to the release of laboratory-sterilized male vectors in the target population. Once released, these male mosquitoes help in suppressing the fecundity rate in female mosquitoes and, consequently control the vector density in urban environments[49]and transmission of vector-borne diseases[49]. According to Oliva et al., SIT is a promising strategy that helps in prevention and control of mosquito-borne diseases. After examining the irradiation effect on sterile male, they stated that sterile males were potential competitors and can help suppress the number of offsprings.
- **Use of larvivorous fish and Crustacean** - Since the larvae of dengue vectors reside in open water bodies, use of larvivorous fish, such as *Poecilia reticulate*[50] and *Mesocyclops formosanus*[30] comes as a cost-effective, eco-friendly, and innovate strategy in controlling the population of *A. aegypti*[33] . A successful study in Cambodia was carried out to evaluate the efficacy of introducing larvivorous guppy fish (*Poecilia reticulate*) into heavily infested water containers. It showed that the guppy fish in test houses reduced vector larval population by 79% as compared to control houses, thus indicating successful implementation of this strategy [51].

Chemical control :

- **Use of insecticides and plant derivatives** – The insecticides have been utilized for mosquito control for many decades. These insecticides became the most commonly used integrated strategy; nevertheless, the continuous use developed resistance in the target vector population, and can induce negative impacts on the environment [44]. To counter the effects of these compounds, researchers developed alternative control method i.e., introduction of plant-based insecticides that can sustain and induce less toxicity in environment than synthetic insecticides [52]. These plant-based insecticides can be developed from different plant parts (leaves, stem, roots) and/or herbal extracts, such as, *Cipadessa baccifera* [53], *Callistemon rigidus* [53], *Erythrina indica* and *Asparagus racemosus* [53]. Furthermore, these plant derivatives are not only limited to produce insecticides; however, they have also proved their efficiency as potential repellents against *A. aegypti* [53].
- **Use of insect growth regulators (IGRs)** - Among other known chemical compounds, insect growth regulators (IGRs) are used for hindering the growth and development in insects. During early stages of development, IGRs induce changes that kill the insect before becoming an adult. There are number of IGRs such as, diflubenzuron, endotoxins, and methoprene that have been used to counter viral infections spread by *A. aegypti* [33]. According to Lau et al., field population of vector species develops resistance to certain IGRs; and in their study, they found that cyromazine showed effective results in attenuating larval population indices of *A. aegypti* [54].
- **Use of pheromones as "attract-and-kill" approach** -The practical application of pheromones as a part of integrated pest management (IPM) has been well-documented in various fields. In a recent integrated approach using pheromones, also termed attracticides, and IGRs, Nagpal et al., demonstrated the prevention of developmental stages from eggs to adults. In this study, larvae in test containers were found in a greater number than controls containers, which indicated that using attracticides hampers the progression of adulthood in *A. aegypti* and is effective in field conditions. Another study developed an uncomplicated "lethal lure control" based on attract-and-kill strategy and found that the pheromone (caproic acid)-insecticide (temephos) combination not only attracted mosquitoes, but also restricted hatching of eggs and killed the larvae, thus elaborating its significance[55].

➢ Development of immunotherapy and vaccines -

Although no specific vaccine for dengue has been licensed at commercial scale, several candidates have been undergoing a developmental phase. Some of these are discussed below:

CURRENT VACCINE CANDIDATES FOR DENGUE PREVENTION[56].

Vaccine type	Developer	Process	Progress
Live, attenuated chimeric (recombinant)	Acambis/Sanofi Pasteur	Insertion of genes coding for DENV structural proteins into a yellow fever virus (17D) backbone.	Phase III tetravalent—leading candidate
	Centre for Disease Control (CDC)/Inviragen	Insertion of serotype genes into serotype II (DENV2-PDK53) DNA backbone.	Phase II monovalent
	National Institutes of Health (NIH)/University of Maryland	Insertion of serotype II and III genes into safer, more immunogenic serotype I and IV DNA backbone. Live attenuated DENV Delta-30 mutation	Phase I tetravalent

Vaccine type	Developer	Process	Progress
Live, traditionally attenuated	Walter-Reed Army Institute of Research (WRAIR)/GlaxoSmithKline (GSK)	Attenuation achieved by growing the virus in cultured cells and selecting strains	Phase II tetravalent; technical issues
	Mahidol Institute/Sanofi Pasteur		Phase II tetravalent
Inactivated	GSK	Viruses cultured and killed	Phase I tetravalent
Subunit	Hawaii Biotech	Viral immunogenic envelope is combined with viral non-structural protein antigens to produce recombinant 80% E subunit vaccine	Phase I tetravalent
DNA	WRAIR	Dengue prM-E DNA vaccine incorporating membrane and envelope genes into a plasmid vector	Phase I monovalent

METHODOLOGY

OVERVIEW OF THE PROCESS

BLOOD WERE COLLECTED IN EDTA VIALS (ARCHIVE SAMPLES)

↓

SAMPLE SERA SUBJECTED TO NS1 ELISA USING PANBIO DENGUE NS1 MICROLISA ELISA

↓

ELISA POSITIVE SAMPLES SUBJECTED TO RNA ISOLATION BY MACHEREY NAGEL (MN) NUCLEOSPIN VIRAL RNA MINI KIT

↓

SEROTYPING WAS PERFORMED USING RT & SEMI-NESTED PCR, LANCIOTTI, 1992

↓

NESTED –PCR PRODUCT SUBJECTED TO AGAROSE GEL ELECTROPHORESIS ON BIO-RAD SYSTEM

↓

PCR PRODUCTS ANALYZED IN TRANSILLUMINATOR ON GEL DOCCUMENTATION SYSTEM

↓

POSITIVE RESULT CONCLUDES SAMPLE SERUM TO BE DENGUE RNA POSITIVE

➢ MATERIALS AND METHODS:

1. COLLECTION OF BLOOD SERUM SAMPLE-

51 NS1 poaitive human serum samples were randomly selected from the archive samples, collected from Salt Lake State Divisional Hospital of Kolkata and stored in -80°C cooler of ICMR-NICED Virus Laboratory, Kolkata for the detection of dengue serotype in recent dengue outbreak in Kolkata 2017.

2. DETECTION OF ANTIGEN USING THE PANBIO DENGUE NS1 ELISA –

- **PRINCIPLE:**

Detection of dengue serotype from 2017 dengue outbreak in Kolkata with the help of Dengue NS1 antigen capture ELISA and Serotyping. Serum from the collected blood samples were separated and were stored at -20°C.

ELISA (Enzyme-Linked Immunosorbent Assay) is a plate-based assay technique designed for detecting and quantifying substances such as peptides, proteins, antibodies and hormones. DENGUE NS1 Ag MICROLISA is designed for in vitro qualitative detection of Dengue NS1 antigen in human serum or plasma and is used as a screening test for testing of collected blood samples suspected for DENGUE. The kit detects all four subtypes; DENV1, DENV2, DENV3 & DENV4 of Dengue Virus.

DENGUE NS1 Ag MICROLISA is a solid phase enzyme linked immunosorbent assay(ELISA) based on the "Direct Sandwich" principle.ELISAs are typically performed in 96-well polystyrene plates, which will passively bind antibodies and proteins. The microwells are coated with Antidengue NS1 antibodies with high reactivity for Dengue NS1 Ag. The samples are added inthe wells followed by addition of enzyme conjugate (monoclonal anti-dengue NS1 antibodies linked to Horseradish Peroxidase (HRPO).

A sandwich complex is formed in the well wherein dengue NS1 (from serum sample) is "trapped" or "sandwiched" between the antibody and antibody HRPO conjugate. Unbound conjugate is then washed off with wash buffer. The amount of bound peroxidase is proportional to the concentration of dengue NS1 antigen present in the sample. Upon addition of the substrate buffer and chromogen, a blue colour develops.

The intensity of developed blue colour is proportional to the concentration of dengue NS1 antigen in sample. To limit the enzyme-substrate reaction, stop solution is added and a yellow colour develops which is finally read at 450nm spectrophotometrically.

- **MATERIALS REQUIRED:**

i. Microwells
ii. Diluent
iii. Enzyme Conjugate (Containing Monoclonal Anti-Dengue NS1 linked to horseradish peroxidase with protein stabilizers)
iv. Wash Buffer (Concentrated Phosphate buffer)
v. TMB Substrate (TMB, to be diluted with TMB Diluent H_2O_2 before use)
vi. Stop Solution (Ready to use, 1N H_2SO_4)
vii. Plate Sealers (Adhesive sheets to cover the microwells during incubation)
viii. microwells during incubation
ix. Micropipettes and microtips
x. Elisa reader
xi. Elisa washer
xii. Distilled or deionized water
xiii. Incubator 37°C
xiv. Graduated Cylinders, for reagent dilution
xv. Vials to store the diluted reagent
xvi. Sodium hypochlorite solution
xvii. Disposable gloves

- **PROCEDURE:**

The procedure according to the kit is as follows-

1. Serum was collected by separating clot from the blood sample with the help of centrifugation.

2. Serum was diluted in 1:100 ratio with serum dilution buffer.

3. Wells coated with anti dengue NS1 antibody were washed thrice by wash buffer.

4. 50µl of diluted samples were transferred to appropriate wells. Equal amount of positive and negative controls&Calibratorwere added to respective wells.

5. The plate was kept in a humified box(A bread box with a soaked tissue paper) and was incubated at 37°C for 1hour

6. After incubation, the plate was washed six times with wash buffer. The plate was tapped on a tissue paper after last wash

7. 100 µl of anti dengue NS1 antibody linked to HRP was added to each well. Then the plate was kept in a humified box for30mins. Step 6 was repeated

8. Add 150 µl of working substrate (TMB) solution in each well

9. Incubate at room temperature (20-30°C) for 30 min. in dark.

10. Add 100 µl of stop solution

11. The plate was incubated at room temperature for color development which normally takes 10minutes.

12. Optical density was measured at 450nm using an ELISA reader

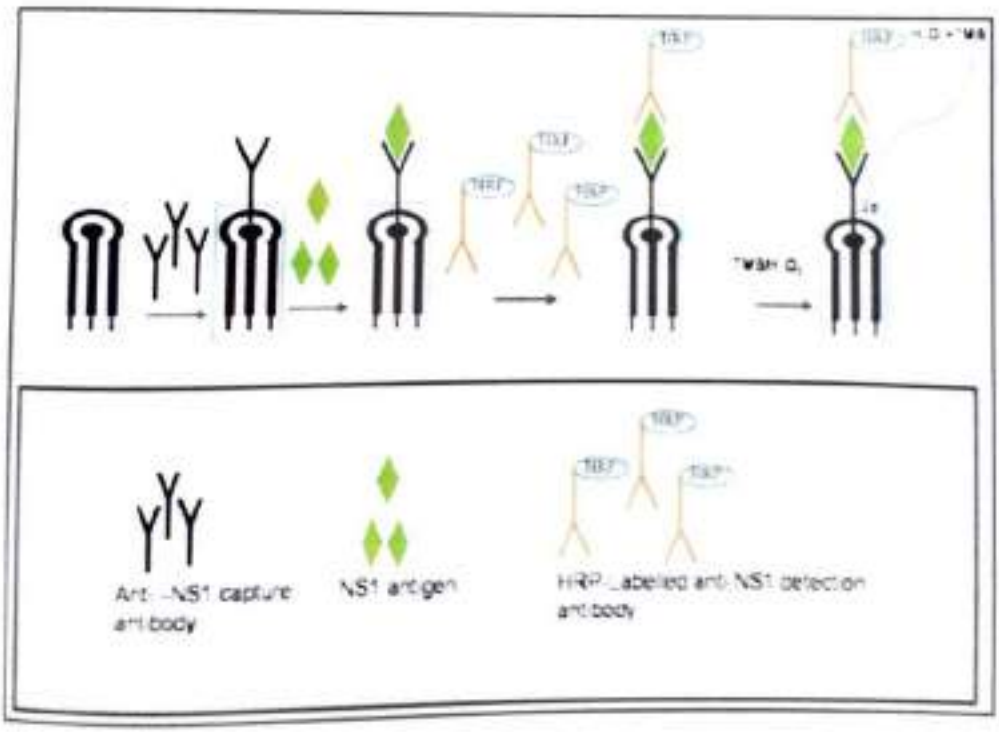


Fig 13: Mechanism of ELISA assay for detection of Dengue NS1 Antigen

ASSAY PLATE

	1	2	3	4	5	6	7	8
A	DEN-055	DEN-056	DEN-057	DEN-058	DEN-059	DEN-060	DEN-061	DEN-062
B	DEN-063	DEN-064	DEN-065	DEN-066	DEN-067	DEN-068	DEN-069	DEN-070
C	DEN-071	DEN-072	DEN-073	DEN-235	DEN-236	DEN-237	DEN-238	CAL-1
D	DEN-239	DEN-240	DEN-241	DEN-242	DEN-243	DEN-244	DEN-245	CAL-2
E	DEN-246	DEN-247	DEN-248	DEN-249	DEN-250	DEN-251	DEN-252	CAL-3
F	DEN-253	DEN-254	DEN-255	DEN-256	DEN-257	DEN-258	DEN-259	+VE
G	DEN-260	DEN-261	DEN-262	DEN-263	DEN-264	DEN-265	DEN-266	-VE

-Calibration Factor

-Positive control

-Negative control

3. VIRAL RNA ISOLATION-

- **PRINCIPLE:**

Viral RNA can be purified from plasma (treated with anticoagulants other than heparin), serum, and other cell-free body fluids. In this experiment MACHEREY-NAGEL Viral RNA Mini Kit (Spin Protocol) was used. The kit combines the selective binding properties of a silica gel- based membrane with the speed of micro-spin technology and is suited for simultaneous processing of multiple samples. The samples were first lysed under the highly denaturing conditions provided by RAV1 lysis buffer (containing guanidine thiocyanate) to inactivate RNases and to ensure isolation of intact viral RNA. Carrier RNA, added to Buffer RAV1, improves the binding of viral RNA buffering conditions are then adjusted to provide optimum binding of the RNA to the QIAamp membrane, and the sample is loaded onto the MN Mini spin column. Carrier RNA improves binding and recovery of low-concentrated viral RNA. The RNA binds to the membrane, and contaminants are efficiently washed away in two steps using two different wash buffers (RAW and RAV3). High-quality RNA is eluted in a special RNase-free buffer, ready for direct use or safe storage.
The use of two different wash buffers, RAW and RAV3, significantly improves the purity of the eluted RNA by removing the contaminations (potential PCR inhibitors) like salts, metabolites and soluble macromolecular cellular components. RAW contain guanidine hydrochloride, which can form highly reactive compounds when combined with bleach. The elution buffer is RNase-free water that contains 0.04% sodium azide to prevent microbial growth and subsequent contamination with RNases.

- **MATERIALS:**
  - Reagent:
    i. Buffer RAV1 (Lysis buffer)
    ii. Carrier RNA (It enhances binding of viral RNA to the membrane in case of low viral titer, as well as limits possible degradation of viral RNA due to any residual RNase activity)
    iii. Absolute Ethanol
    iv. Buffer RAW (Wash buffer 1)
    v. Buffer RAV3 (Wash buffer 2)
    vi. Elution buffer

- Apparatus:
  - i. Micropipettes
  - ii. Sterile microtips
  - iii. 1.5 ml eppendorfs
  - iv. Thermomixer
  - v. Microcentrifuge
  - vi. Nucleospin Mini Spin Columns

- **PROCEDURE:**

✓ **LYSIS »**

1. 150 µl of plasma was added to the 1.5 ml of eppendorf
2. 600 µl of prepared lysis buffer (1ml RAV1 containing carrier RNA) was pipetted into the eppendorff
3. It was vortex at 70°C temperature in 300rpm for 10 min
4. 600 µl of ethanol (96–100%) was added to the sample, and mixed it by pulse-vortexing for 15 seconds
5. After mixing, the tube was briefly centrifuged to remove drops from inside the lid

✓ **BINDING »**

1. After the lysis step, 680µl of the solution from the eppendorfs was added to the Mini Columns (in a 2 ml collection tube) without wetting the rim
2. Then the columns were centrifuged at 8000 rpm for 1 min
3. The Mini columns were then placed into a clean 2 ml collection tube, and the tubes containing the filtrate were discarded
4. The binding step was repeated

✓ **WASHING »**

1. 500 µl of washing buffer RAW was added
2. Then the Mini columns were centrifuged at 8000 rpm for 1 min and the QIAamp Mini columns were placed in a clean 2 ml collection tube (provided), and the tubes containing the filtrate were discarded
3. 700 µl of washing buffer RAV3 was added

4. Then the Mini columns were centrifuged at full speed 14,000 rpm for 3 min.
5. The Mini columns were placed into a clean 2 ml collection tube, and the tubes containing the filtrate were discarded.
6. The blank tubes were then centrifuged at 11,000 rpm for 1 min.
7. The Mini columns were placed in clean 1.5 ml of eppendorfs. The old collection tubes containing the filtrate were discarded.

✓ **ELUTION »**

1. After washing 50µl of Elution Buffer (kept at 72°C for few minutes) was added into the tubes, incubated for 5 minutes at room temperature and centrifuged at 8000 rpm for 1 min.
2. The purified RNA samples were used as the substrate for Reverse Transcription Polymerase Chain Reaction and stored at -80°C for RNA stability.

Fig 14: RNA ISOLATION (MACHEREY-NAGEL Viral RNA Mini Kit)

- **PRECAUTION :**

a. All the centrifugation steps must be carried out at room temperature (15-25°C).

b. The samples and the Equilibrate Buffer RAV1 should be equilibrated to room temperature (15-25°C) before starting the protocol.

c. Buffer RAW and Buffer RAV3 must be prepared according to the instructions.

d. Thermo mixer temperature must be set at 72°C.

e. The carrier RNA must be lyophilized by mixing with 1 ml RAV1, and then this solution is poured in the remaining RAV1 and is mixed well.

f. The procedure must be carried out aseptically to prevent RNase contamination, hand gloves & 75% alcohol for sterilization must also be used for this.

4. REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR) -

- **PRINCIPLE :**

The polymerase chain reaction (PCR) is a scientific technique in molecular biology to amplify a single or few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.RT-PCR utilizes a pair of primers, which are complementary to a defined sequence on each of the two strands of the cDNA. These primers are then extended by a DNA polymerase and a copy of the strand is made after each cycle, leading to logarithmic amplification.

RT-PCR utilizes a pair of primers, which are complementary to a defined sequence on each of the two strands of the cDNA. These primers are then extended by a DNA polymerase and a copy of the strand is made after each cycle, leading to logarithmic amplification. RT-PCR includes three major steps. The first step is reverse transcription (RT), in which RNA is reverse transcribed to cDNA using reverse

transcriptase. This step is very important in order to perform PCR since DNA polymerase can act only on DNA templates. The RT step can be performed either in the same tube with PCR (one-step PCR) or in a separate one (two-step) using a temperature between 40°C and 50°C, depending on the properties of the reverse transcriptase used. The next step involves the denaturation of the ds DNA at 95°C, so that the two strands separate and the primers can bind again at lower temperatures and begin a new chain reaction. Then, the temperature is decreased until it reaches the annealing temperature which can vary depending on the set of primers used, their concentration, the probe and its concentration (if used), and the cations concentration. The main consideration, of course, when choosing the optimal annealing temperature is the melting temperature (Tm) of the primers and probes (if used). The annealing temperature chosen for a PCR depends directly on length and composition of the primers. This is the result of the difference of hydrogen bonds between A-T (2 bonds) and G-C (3 bonds). An annealing temperature about 5 degrees below the lowest Tm of the pair of primers is usually used. The final step of PCR amplification is DNA extension from the primers. This is done with thermo stable Taq DNA polymerase, usually at 72°C, the temperature at which the enzyme works optimally. The length of the incubation at each temperature, the temperature alterations, and the number of cycles are controlled by a programmable thermal cycler. The analysis of the PCR products depends on the type of PCR applied. If a conventional PCR is used, the product is analysed by agarose gel electrophoresis.

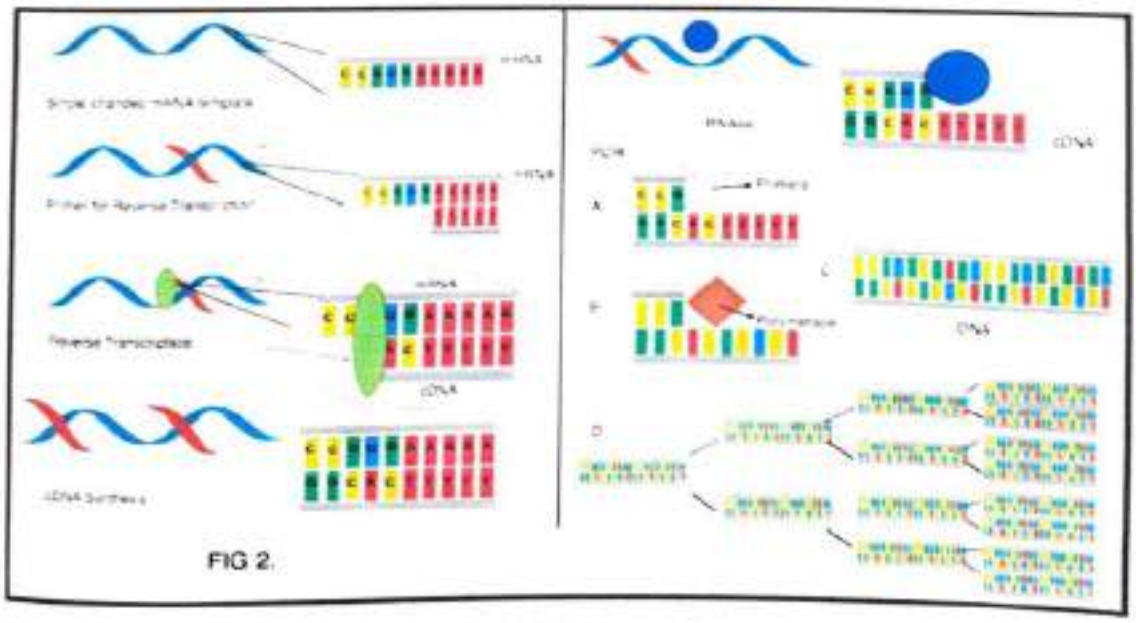


Fig 15: Mechanism of RT Polymerase Chain Reaction

- **MATERIALS REQUIRED :**

  i. Previously isolated viral RNA samples
  ii. PCR tubes.
  iii. Micropipettes and autoclaved tips, minicooler.
  iv. PCR machine (Thermo cycler).
  v. Biosafety Hood.
  vi. Mastermix
  vii. Nuclease free water
  viii. 10x RT-PCR buffer
  ix. Forward primer (Primer D1)
  x. Reverse primer (Primer D2)
  xi. dNTPs(10mM)
  xii. $MgCl_2$
  xiii. AMV Reverse Transcriptase (Promega, Madison, WI, USA)
  xiv. Taq DNA polymerase (Fermentas Inc., USA).

- *MASTER MIXTURE -*

Components	Volume for 1 reaction(μl)
Milipore water	12
10 x PCR buffer-II	2
dNTPs (10mM)	1.6
MgCl2 (25 mM)	1.2
Primer D1(10nM)	1
Primer D2(10nM)	1
AMV RT	0.04
Taq Polymerase	0.1
TOTAL	**18.94**

- **PROCEDURE :**

1. Target RNA was amplified in 20 µl volumes for each sample with 18µl master mix containing the following components: 2µl of 10x PCR buffer-II, 1.6µl of 10mM dNTPs (Life Technologies, Foster City, CA), 1µl each of 10mM of forward and reverse primers (D1 and D2), 1.2 µl of 25mM of MgCl2 (Life Technologies), 0.04U/µlof AMV RT (Promega, Madison, WI), 0.1U/µl of Taq Polymerase (Life Technologies) and 2 µl of extracted RNA.
2. Then the reaction mixture was mixed well by a short spin.
3. After spin reaction tubes were subjected to the PCR machine the following programe was set for reverse transcription as well as polymerase chain reaction specific for Dengue virus reaction.
4. The reaction conditions were 1 hr at 42°C, initial denaturation at 94°C for 5 min followed by 30 cycles, 94°C for 30 sec, 55°C for 1 min, 72°C for 1.15 min followed by final extension at 72°C for 10 min.

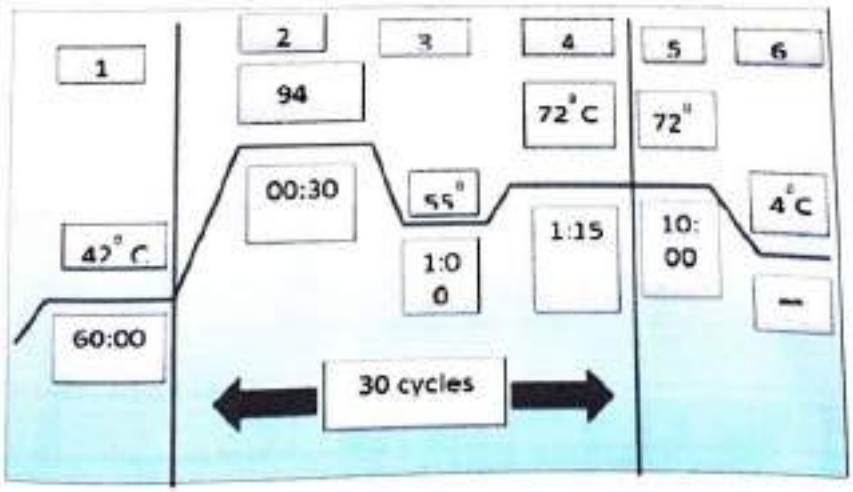


Fig 16: Reaction Condition of Reverse Transcriptase Polymerase Chain Reaction

5. SEMI-NESTED PCR -

- **PRINCIPLE :**

The sensitivity and specificity of PCR can be increased by using nested PCR (n PCR). In n PCR, two separate amplifications are used. The first uses a set of primers that yields a large product, which is then used as a template for the second amplification. The second set of primers anneal to sequences within the initial product producing a second smaller product. The primers for the second round of amplification are either both different from the first set or both located within the amplified DNA region. If only one of the second round primers is located within the amplified region and is used together with one of the first round primers, it is termed as semi-nested PCR. Nested PCR increases the specificity of the reaction because formation of the final product depends upon the binding of two separate sets of primers, which may preclude the need for verification of the PCR product by blotting, restriction digestion or sequencing. The second set of primers also serves to verify the specificity of the first product.

The Nested PCR used for Dengue stereotyping is basically semi nested PCR. Here the forward primer is also used in the RT-PCR reaction. But the reverse primers are serotype specific, i.e. the TS1 , TS2 , TS3 ,TS4 primers are specific for DENV-1 , DENV-2 , DENV-3 , DENV-4 respectively. All the four primers are used in a single PCR reaction as reverse primer. The 4 primers give the amplification of different sizes. **TS1 – 482 base pair (bp) for DENV-1 , TS2 – 119 bp for DENV-2 , TS3 – 290 bp for DENV-3 , TS4 – 392 bp for DENV-4 PCR** product.

- **MATERIALS REQUIRED :**

i. The product of RT –PCR.
ii. Autoclaved dilution tubes.
iii. The other requirements are as same as the requirements of RT-PCR reaction.
iv. Master mixture for DENV Nested PCR

- *MASTER MIXTURE* -

Components	Volume for 1 reaction(µl)
Milipore water	11
10 x PCR buffer-II	2.5
dNTPs (10mM)	2.0
MgCl2 (25 mM)	2.5
Primer D1(10nM)	1
Primer TS1 (10nM)	1
Primer TS2 (10nM)	1
Primer TS3 (10nM)	1
Primer TS4 (10nM)	1
Taq Polymerase	0.125
TOTAL	**23.125**

- **PROCEDURE :**

1. Serotyping of dengue virus was conducted by nested PCR using 1:100 times dilution of the first round RT-PCR product as the template.
2. The nested PCR was performed in a total volume of 25µl using 2.5µl of 10x PCR buffer-II, 2.0 µl of 10mM dNTPs (Life Technologies), 2.5 µl of 25mM MgCl2 (Life Technologies), 1 µl each of 10mM of forward and reverse primers (D1, TS1, TS2, TS3, and TS4), 0.125U/µl of Taq Polymerase (Life Technologies) and 2µlof first round diluted product.
3. Then the reaction mixture was mixed well by a short spin.
4. After spin reaction tubes were subjected to the PCR machine, the following program was set for Nested PCR specific for Dengue virus reaction.
5. The PCR was performed at initial denaturation of 94°C for 10 min followed 94°C for 30 sec, 55°C for 1 min and 72°C for 2 min for 30 cycles with a final extension at 72°C for 1 min.

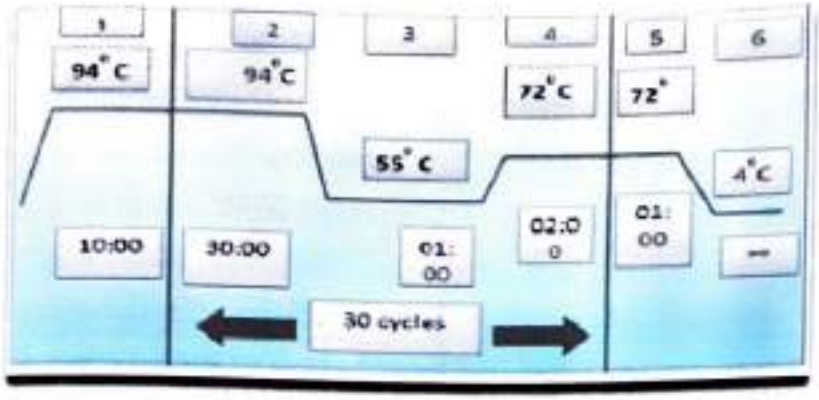


Fig 17: Reaction Condition of NESTED Polymerase Chain Reaction

6. AGAROSE GEL ELECTROPHORESIS –

- **PRINCIPLE :**

Agarose gel electrophoresis is performed for the confirmation of the presence of viral RNA in the sample. Agarose gel electrophoresis is a routinely used method for separating proteins, DNA or RNA. Nucleic acid molecules are size separated by the aid of an electric field where negatively charged molecules migrate toward anode (positive) pole. The migration flow is determined solely by the molecular weight, where small weight molecules migrate faster than larger ones [57]. In addition to size separation, nucleic acid fractionation using agarose gel electrophoresis can be an initial step for further purification of a band of interest. Extension of the technique includes excising the desired "band" from a stained gel viewed with a UV transilluminator. In order to visualize nucleic acid molecules in agarose gel ethidium bromide or SYBR Green are commonly used dyes.

- **MATERIALS REQUIRED :**

i. Gel casting apparatus
ii. Comb
iii. Gel casting tray
iv. Weighing machine.
v. Agarose
vi. Spatula.& Micropipette

vii. TBE buffer (1X)
viii. Beaker & Conical flask
ix. Microwave oven.
x. Ethidium bromide.
xi. Electrophoresis apparatus
xii. Tris Borate EDTA Buffer (6X)
xiii. Gel loading dye (6X)
xiv. Product of Nested PCR.
xv. DNA ladder (100bp)
xvi. Negative control & DENV- 1-4 RNA Positive Control
xvii. BIO RAD GEL DOC for photography of gel after electrophoresis.

- **PROCEDURE :**

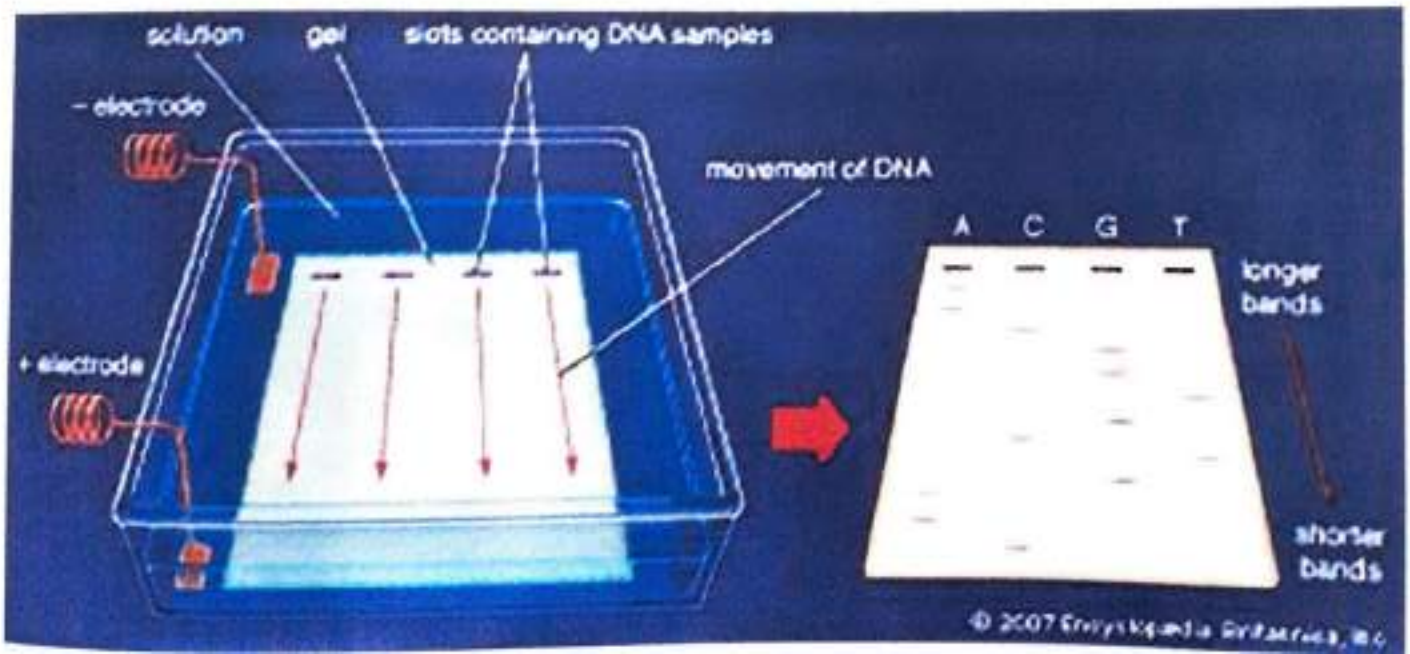
1. All the gel casting apparatus were cleaned and fixed in place.
2. Required amount of 1X Tris Borate EDTA (TBE) buffer was prepared from 10X TBE buffer. 1.5% agarose was measured and dissolved in 100ml amount of the prepared 1X buffer.
3. The agarose was dissolved completely in the buffer by heating within microwave oven, and then it was allowed to cool to a certain extent.
4. 1.5µL Ethidium bromide was added to it and mixed gently and poured in the gel casting apparatus (settled previously with a comb) and care was taken from forming any air bubbles.
5. After solidification of gel the comb was removed without damaging the shape of the wells.
6. The gel was then transferred to electrophoresis apparatus and it was sunk with 1X TBE buffer.
7. 10-20µl sample are mixed with loading dye and was loaded to respective wells. 100bp DNA ladder, positive and negative control were loaded onto the first three wells respectively.
8. The power supply was switched on and maintains the current at 150mA. The sample was allowed to travel in the gel for certain distances. After proper migration the current was switched off.
9. Gel was observed in Gel Doc under UV-transelluminator mode.

PRECAUTIONS :

a) Loading of samples to the wells in agarose gel should be done very carefully so that the wells' shape does not get distorted.

b) Contact with Ethidium Bromide should be prevented as it is a potent carcinogen.

c) There are limits to electrophoretic techniques. Since passing current through a gel causes heating, gels may melt during electrophoresis. Electrophoresis is performed in buffer solutions to reduce pH changes due to the electric field, which is important because the charge of DNA and RNA depends on pH, but running for too long can exhaust the buffering capacity of the solution.



RESULT

❖ NS1 ELISA-

Optical Density (O.D) values of the test samples at 450 nm.

	1	2	3	4	5	6	7	8
A	0.343	0.258	0.500	0.325	0.635	0.566	0.540	0.339
B	0.334	0.376	0.328	0.346	0.382	0.348	0.263	0.312
C	0.307	0.380	0.26	0.256	0.333	0.658	0.531	0.514
D	0.373	0.357	0.465	0.321	0.362	0.334	0.341	0.463
E	0.301	0.317	0.425	0.345	0.356	0.389	0.325	0.457
F	0.356	0.317	0.405	0.259	0.277	0.284	0.296	0.451
G	0.378	0.246	0.348	0.377	1.563	0.371	0.299	0.03

-Calibration Factor

-Positive control

-Negative control

CALCULATIONS:

- Cut-off value = Mean absorbance of Calibrator × Calibration Factor.
- Mean of absorbance of Calibrator =(0.514+0.463+0.457)/3=0.478
- Calibration Factor = 0.53
- Cut –off value = 0.478 × 0.53 =0.253

Values greater than cut-off (>0.253) value considered as NS1 Antigen Positive.

Hence, 51 Samples are NS1 antigen positive out of 51 samples. 51 samples had been taken randomly among 285 samples which were already tested for NS1 Ag serology.

❖ Nested RT-PCR and DENV Serotype -

The Results and the representative photograph of 2 gels are on the following page-

Fig 19: Representative picture of GEL-1

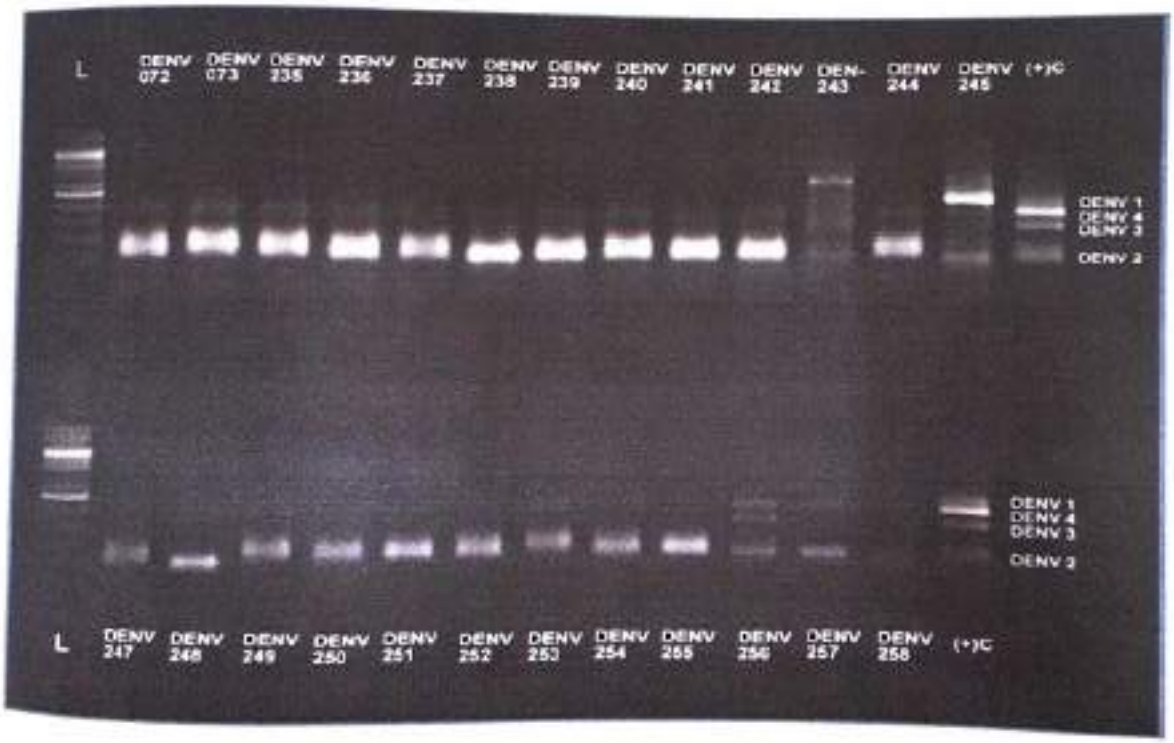


Fig 20: Representative picture of GEL-2

NOTE: - In the above mentioned representative pictures 33 samples are shown among the 50 samples. Here, 'L' stands for Ladder (1000bp), (+) C stands for positive control. 1st band represents DENV 1- 482bp, 2nd band represents DENV 4- 392bp, 3rd band represents DENV 3 – 290bp and 4th band represents DENV 2 – 119bp.

INTERPRETATION

All samples out of 51 NS1 positive samples were rechecked and all samples gave the positive result in Dengue NS1 antigen testing Experiment. Out of 51 Dengue NS1 positive samples, 84% samples gave positive for DENV 2 Serotype, 14% samples gave positive for DENV 4 serotype, 6% samples are DENV 1 and 4% are DENV 3.

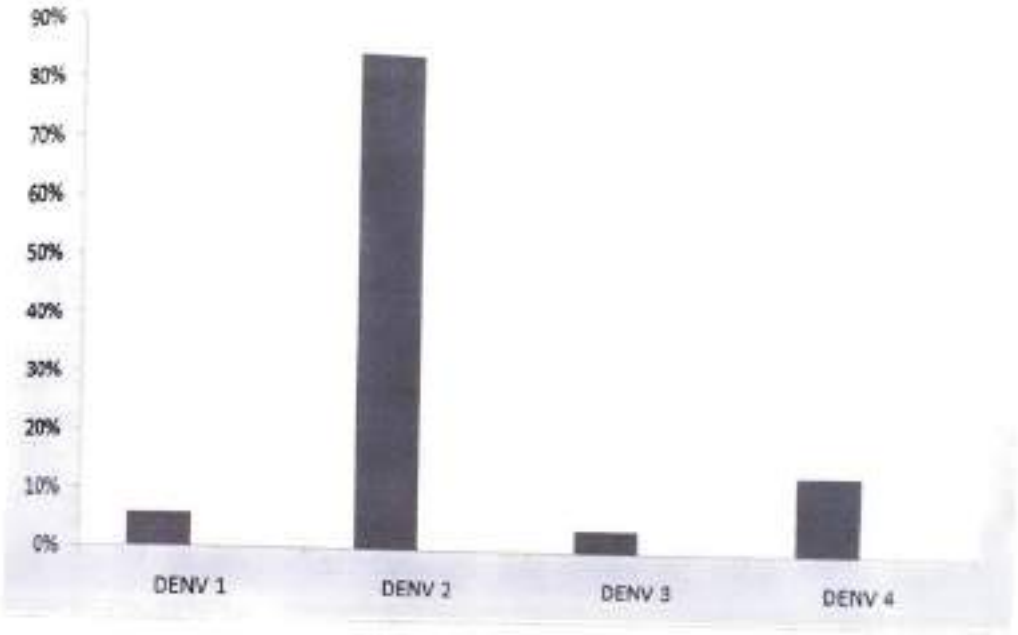


Fig 21: Graphical representation of Dengue serotypes.

From the above graph, we can state that DENV 2 is the most occurring Dengue serotype in Kolkata district, followed by DENV 4. From few samples we found that the DENV 1 and DENV 3 serotypes are also less occurring.

DISCUSSION

Outbreaks of Dengue have been recorded in India on several occasions, including Kolkata. In this study the samples were of the year 2017 collected from Salt Lake State Divisional Hospital of Kolkata, showing similar symptoms of Dengue fever.

The result of serotyping suggests that the major circulating Dengue serotype in Kolkata in the year of 2017 was DENV-2 followed by DENV-1 and Denv-4. The DENV-3 serotype may not be prevalent during that time in Kolkata in regard with the sample collected.

Hence, the study proves that the Dengue is emerging as a major health problem round the year in Kolkata and is now proving to be an endemic health problem that draws major concern for its fast diagnosis and treatment .Serotyping also solves the problem of cross reactivity and the cases of multiple infection of different Dengue serotypes that causes serious health problem. For that reason, monitoring of dengue virus is important.

Hence, it needs a continuous surveillance and further epidemiological data on any other unknown fever outbreak.

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ISOLATION AND CHARACTERIZATION OF PROTEASE PRODUCING BACTERIA FROM SOIL

SUBMITTED FOR THE PARTIAL FULFILLMENT OF THE AWARD OF MSc.IN MICROBIOLOGY

BY

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3RD SEMESTER, ROLL-BNC/MCB-/-III

NO-004, REGISTRATION NO 1031521401163

OF 2018-2020

UNDER SUPERVISION OF

Mrs.PARAMA DAS GUPTA

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Nirlipta Saha.

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Date : 16/12/19.

To Whom It May Concern

This is to certify that Ms. Nirlipta Saha of P.G. Department of Microbiology, Bidhannagar College, has successfully completed her project entitled "Isolation and characterization of protease producing bacteria from soil" under my supervision from 6th July 2019 to 4th October 2019.

I wish her every success in his future.

Parama Das Gupta.

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INTRODUCTION

Proteases are known as peptidyl–peptide hydrolases which catalyze the hydrolysis of peptide bond from protein molecule [1]. Protease is one of the most important enzymes known and is of great significance having approximately 60 % of the total industrial enzyme market. This proteolytic enzyme constitute one of the most important group of industrial enzymes and in recent years, the use of alkaline proteases in a variety of industrial processes involving detergents, food, leather and silk has increased remarkably [2][3].Bacterial proteases are preferred as they grow rapidly, needless space, can be easily maintained and are accessible for genetic manipulations [4].Currently, a large proportion of commercially available proteases are derived from *Bacillus* strain [5].

This vast diversity of proteases, in contrast to the specificity of their action has attracted worldwide attention in attempts to exploit their physiological and biotechnological applications [6].To meet the upward trend in demand, studies on cost effective production of industrially important enzymes have become the need of today.Proteases of commercial importance are produced from microbial, animal and plant sources [7], but proteases from microbial sources are preferred to the enzymes from plant and animal sources since they posses almost all characteristics desired for their biotechnological applications [8] and because of their fast growth rate, easy to manipulate for getting highly stable enzymes through genetic engineering and requires shorter time for production and purification steps [9]. Microbial proteases account for approximately 40% of the total worldwide enzyme sales.

Proteases execute a large variety of functions and have important biotechnological applications. Proteases represent one of the three largest groups of industrial enzymes and find application in detergents, leather industry, food industry, pharmaceutical industry and bioremediation processes [10].

Several *Bacillus* species involved in protease production are e.g. *B. cereus, B. sterothermophilus, B. mojavensis,B. megaterium and B. subtilis* [11] Probably the largest application of proteases is in laundry detergents, where they help removing protein based stains from clothing [12]. In textile industry, proteases may also be

MATERIALS AND METHODS

1.REQUIREMENTS-Culture,Distilled water, Glass wares, Micro centrifuge tubes,Micropipettes,Centrifuge,Spectrophotometer,Refrigerator,Autoclave,Laminar air flow, Incubator,Thermometer,Heater etc.

2. FOR ENZYME ESTIMATION-Casein, 4N NaOH, 1N HCL,10%TCA,Folin reagent,0.5M Sodium bicarbonate

3. FOR SKIMMED MILK AGAR PLATE-Skimmed milk powder-28 gm, Tryptone-5 gm, Yeast extract-2.5 gm, Dextrose-1 gm, Agar powder-15 gm, distilled water-1000 ml

4. FOR PROTEASE PRODUCTION MEDIA-$Cacl_2$ -0.1 gm, K_2HPO_4-0.5 gm, Yeast extract-0.2 gm, Peptone-10 gm, $MgSO_4.7H_2O$-0.1 gm, Dextrose-1 gm, distilled water-1000ml

5. FOR GRAM STAINING- Crystal violet, Gram's iodine, 95% Alcohol, Safranine

6. FOR SIMPLE STAINING-Crystal violet

7. FOR NEGATIVE STAINING- Nigrosine

8. FOR ENDOSPORE STAINING-Malachite green, Safranine

9. FOR INDOLE TEST-

Composition of Tryptone broth: Tryptone-10gm, NaCl-5gm, Distilled water-1000ml,

Composition of kovac's reagent:

Paradimethyl aminobenzaldehyde-5 gm, Amyl alcohol-75 ml, Concentrated HCl-25 ml

10. FOR MR-VP TEST-

Composition of MR-VP broth: Peptone-7 gm, KH_2PO_4-5 gm,, Distilled water-1000 ml

Compositions of MR reagent: Methyl red-0.1%, Ethyl alcohol-300 ml, Distilled water-200ml,

VP reagent1: KOH-40%

VP reagent 2: Alpha napthol-5 gm, Ethyl alcohol-95 ml

11. FOR SIMMON CITRATE TEST-Simmons's citrate agar

12. FOR CATALASE TEST-H_2O_2-3%

13. FOR SUGAR FERMANTATION TEST:

Dextrose, Sucrose, Lactose (anyone of this for particular sugar test)-5 gm, Peptone-5 gm, Beef extract-3 gm, Distilled water-1000 ml

14.**FOR STARCH AGAR PLATES**-Starch-20 gm,

Peptone-5 gm, Beef extract-3gm, Agar powder-20 gm, Distilled water-1000 ml

15. FOR UREA AGAR SLANTS-Urea base powder, Urea powder

20. FOR SYNTHETIC MEDIA:

K_2HPO_4 -2.5 gm, KH_2PO_4-2.5 gm, Ammonium dihydrogen phosphate-1 gm, $MgSO_4.7H_2O$ -2 gm, $FeSO_4.5\ H_2O$ -0.01 gm, $MnSO_4.\ H_2O$ -0.007 gm, Dextrose-10 gm, Distilled water-1000 ml

FOR DIFFERENT CARBON SOURCE I USED Sodium carbonate, Sucrose, Starch, Maltose, Manitol

FOR DIFFERENT NITROGEN SOURCE I USED Sodium nitrate, Ammonium chloride, Beef extract, Tryptone, Casein, and Ammonium sulfate

FOR SALT CONCENTRATION-different concentration of NaCl used like 0.05%,0.1%,0.5%,1%,2%,5%,10%

FOR DIFFERENT AMINO ACID SOURCE I USED Glycine, Arginine, Phenylalanine, Cystine

FOR DIFFERENT PH RANGE I USED ph3, ph5, ph6, ph7, ph8, ph9

21. FOR ANTIBIOTIC RESISTANCE TEST:

Mueller Hinton, Chloramphenicol, Tetracycline, Ampicillin, Streptomycin

22. FOR DNA ISOLATION-3(M) sodium acetate, 2.5 mM tris HCL, 500 mM EDTA, 1M tris-HCL, 10% SDS,1N NaOH solution, concentrated HCL, Chloroform, Ethanol, Tris saturated phenol.

23. FOR PCR: $MgCl_2$, 5x Reaction buffer, DNTP, 27f primer, 1492r primer, Taq DNA polymerase

ISOLATION OF A PROTEASE PRODUCING ORGANISM

SOURCE OF SAMPLE COLLECTION: Soil samples were collected from Bidhannagar Government College campus soil sample were collected below 5-6 cm depth aseptically.

ISOLATION OF PROTEASE PRODUCING BACTERIA: The techniques used for isolation of bacteria is serial dilution and spread plate method.1 gm of soil sample was weighed and serial dilution(10^{-1} to 10^{-5}) of each soil sample were carried out in 0.9% saline solution of each aliquot was spread on skimmed milk agar(1%) plate at temperature 37^0c for 48 hours. The zone of hydrolysis was noted. The colony showing highest zone of proteolysis was selected for further study. The colony was grown on nutrient agar plate repeatedly and preserved on nutrient agar slant at 4^0c.Based on the morphological and biochemical tests the bacterial isolates was identified [17]

IDENTIFICATION OF BACTERIA: The identification of bacteria was carried out by morphological studies i.e. staining, motility test, culture characteristics on agar plates like colony morphology i.e.shape, size, colour etc. and also Grown in broth. Biochemical tests i.e. Indole, Methyl red, VP, Sugar fermentation, Nitrate reduction, Gelatin hydrolysis test etc. and physiological tests i.e. carbon sources, amino acid sources etc. are also carried out and Chromosomal DNA is isolated and further molecular study is going on.

QUANTITATIVE ESTIMATION OF PROTEASE ENZYME:

PREPARATION OF CASEIN SOLUTION: Casein was used as substrate .It was prepared from alkali soluble casein which was dissolved in 80ml distilled water. The insoluble portion was dissolved by addition of 4N NaOH.The PH was adjusted to 9 approximately. Then 1N HCL is added to adjust the ph at 7.6 and the total volume was adjusted with distilled water at volumetric flask. [18]

ENZYME PREPARATION: The protease producing bacterial colony was inoculated in production media. It was incubated at 37^0c for 48 hours.2 ml of culture subjected to centrifugation at 10000 RPM for 10 minutes. The supernatant was used as enzyme preparation for further study.

PROTOCOL FOR TYROSINE STANDARD CURVE:

Stock concentration = 200 µg/ml

Na_2CO_3 solution = 0.5 M (in 100 ml of distilled water 5.299 gm of Na_2CO_3 was added)

Folin-Ciocalteau reagent = 1:1

TEST TUBE NO.	L-TYROSINE (ml)	DISTILLED WATER (ml)	L-TYROSINE CONCENTRATION (µg/ml)	SODIUM BICARBONATE	INCUBATION TIME(Min)	FOLIN MIXTURE(ml)	INCUBATION TIME(min)
Blank	0	1	0	2.5	20	0.5	15
T1	0.05	0.95	10	2.5	20	0.5	15
T2	0.1	0.9	20	2.5	20	0.5	15
T3	0.2	0.8	40	2.5	20	0.5	15
T4	0.3	0.7	60	2.5	20	0.5	15
T5	0.4	0.6	80	2.5	20	0.5	15

ESTIMATION OF PRODUCT FORMED

1.1.0.1 ml of bacterial culture is inoculated from the fresh broth to the production media and allowed to grow overnight.

1.2. 2 ml of overnight grown culture is taken in a micro centrifuge tube and centrifuged at 10000 rpm for 10 minutes .

1.3. Then the supernatant is collected.

1.4.1 ml of 1% casein and 1 ml of supernatant is mixed and incubated for 10 mins.

1.5.1 ml of 1% casein and 1 ml of distilled water is mixed and it is considered as blank.

1.6. after 10 mins. Of incubation 3 ml of 10 TCA solutions is added and the some amount of sample collected in fresh MC tube.

1.7. It is centrifuged at 5000 RPM for 15 mins.

1.8. Then 0.5 ml of supernatant is collected in a test tube and 2.5 ml of 0.5M Na_2CO_3 is added and incubated for 20 mins.

1.9. Then 0.5 ml of 1:1 Folin reagent is added and incubated for 20 mins in dark.

2.0. Then the O.D. is measured under 660 nm and graph is plotted.

METHOD OF SIMPLE STAINING

1. Using a sterilized inoculating loop, loopful of liquid suspension containing bacteria is transferred to a slide (clean grease free microscopic slide) and a smear is formed using the inoculation loop and let it be dried.
2. The smear cautiously heat fixed by passing the underside of the slide through the burner flame two or three times. It fixes the cell in the slide. Do not overheat the slide as it will distort the bacterial cells.
3. the smear is flooded with CRYSTAL VIOLET and allowed the dye to remain in the smear for approximately one minute (Staining time is not critical here; somewhere between 30 seconds to 2 minutes should give you an acceptable stain, the longer you leave the dye in it, the darker will be the stain).
4. Using distilled water, excess crystal violet is gently washed off from the slide and the back of the slide is blotted the stained surface with blotting paper.
5. The stained smear is placed on the microscope stage smear side up and focused the smear using the 10X objective and immersion oil is applied directly to the smear, and focus the smear under oil with the 100X objective.

METHOD OF NEGATIVE STAINING

1. A drop of nigrosine is added and placed it in the middle of the Clean & Grease free Glass slide.

2. Now, with the help of Sterilized inoculating a small portion of the specimen transferred to the slide containing a drop of dye and mixed well the specimen with the dye using the sterilized straight wire and spread evenly over an area of about 1 – 2 cm.

3. The smear is allowed to air dried and then observed under the microscope under oil with 100X objectives.

METHOD OF GRAM STAINING

1. Air-dried, heat-fixed smear of cells are flooded for 1 minute with crystal violet staining reagent for 1 minute.
2. Slide is washed in a gentle and indirect stream of tap water for 2 seconds.
3. Slide is flooded with the mordant: Gram's iodine. Waited for 1 minute.
4. Slide is washed in a gentle and indirect stream of tap water for 2 seconds.
5. Slide is flooded with decolorizing agent (Acetone-alcohol decolorizer) and waited for 10-15 seconds. It is added drop by drop to slide until decolorizing agent running from the slide runs clear.
6. Slide is flooded with a counter stain, safranin an waited 30 seconds to 1 minutes and is washed in a gentle and indirect stream of tap water until no color appears in the effluent and then blot dry with absorbent paper.
7. The result was observed under oil immersion objective (100x) using a bright field microscope.

METHOD OF ENDOSPORE STAINING

1. Smears of organism is prepared to be tested for presence of endospores on a clean microscope slide and air dried and heat fixed and added few drops of malachite green dye.
2. The slide (smear side up) is placed on a wire gauze on a ring stand and heated gently till it starts to evaporate (either by putting the slide on a staining rack that has been placed over a boiling water bath or via Bunsen burner) for about 3-5 minutes A drop or two of malachite green is added to keep it moist, but don't add so much at one time that the temperature is appreciably reduced.
3. After 5 minutes the slide is removed carefully from the rack and allowed to cool to room temperature for 2 minute and rinsed the slide thoroughly with tap water (to wash the malachite green from both sides of the microscope slide).
4. It is stained with safranin for 2 minutes and is rinsed both side of the slide to remove the secondary stain and air dried and observed the bacteria under oil immersion objective.

METHOD OF MOTILITY TEST

1. A semi-solid nutrient agar medium with 0.8% agar is prepared. The media is melted and dispensed into 5 test tubes
2. Then the test tubes are autoclaved under pressure 15psi for 15 minutes.
3. The test tubes are allowed to solidify in vertical position.
4. After that a loopful of culture is inoculated straight through the stab and incubated for 24 hours.

PROCEDURE OF INDOLE TEST:

1. 50 ml of tryptone broth is prepared and dispensed into 5 tubes.

2. They are autoclaved under 15 psi for 15 minutes and allowed to cool down

3. Then 0.1 ml of culture are inoculated to the 4 of the tubes and the remaining tube is used for negative control. And the tubes are allowed to incubate for 24 hours.

4. After 24 hours incubation few drops of Kovac's reagent is added to the control and test samples and the result is observed.

PROCEDURE OF METHYL RED TEST:

1.50 ml of MR-VP broth is prepared and dispensed into 5 tubes.

2. They are autoclaved under 15 psi for 15 minutes and allowed to cool down

3. Then 0.1 ml of culture are inoculated to the 4 of the tubes and the remaining tube is used for negative control.

4. Tubes are allowed to incubate for 24 hours.

5. After 24 hours incubation few drops of methyl-red reagent is added to the control and test samples and the result is observed.

5.3. PROCEDURE OF VOGES-PROSKAUER TEST:

1.50 ml of MR-VP broth is prepared and dispensed into 5 tubes.

2. They are autoclaved under 15 psi for 15 minutes and allowed to cool down.

3. Then 0.1 ml of culture are inoculated to the 4 of the tubes and the remaining tube is used for negative control.

4. Tubes are allowed to incubate for 24 hours.

5. After 24 hours incubation few drops of VP_1 and VP_2 reagent is added one after another and allowed to react for 30 minutes and the result is observed.

PROCEDURE OF CITRATE TEST:

1.50 ml of Simmons's citrate agar medium is prepared and malted and dispensed into 5 tubes.

2. They are autoclaved under 15 psi for 15 minutes and allowed to cool down in the slanted position.

3. Then a loopful of culture is streaked into four of the slants and the remaining is considered as negative control.

4. Tubes are allowed to incubate for 24 hours.

5. After 24 hours incubation the result is observed.

PROCEDURE OF CATALASE TEST:

1. It is prepared 50 ml of nutrient agar medium with 2.5% agar powder and malted and dispensed into 5 tubes.

2. They are autoclaved under 15 psi for 15 minutes and allowed to cool down in the slanted position.

3. Then a loopful of culture is streaked into four of the slants and the remaining is considered as negative control. And the tubes are allowed to incubate for 24 hours.

5. After 24 hours incubation 0.5 ml of 3% H_2O_2 solution is added drop wise and the result is observed.

PROCEDURE OF OXIDASE TEST:

1. It is prepared 50 ml of nutrient agar medium with 2.5% agar powder and malted and dispensed into 5 tubes.

2. They are autoclaved under 15 psi for 15 minutes and allowed to cool down in the slanted position.

3. Then a loopful of culture is streaked into four of the slants and the remaining is considered as negative control.

4. Tubes are allowed to incubate for 24 hours.

5. After 24 hours of incubation the loopful of colony is taken and its rubbed on the oxidase disc and the result is observed.

SUGAR FERMENTATION TEST

PROCEDURE OF LACTOSE FERMENTION TEST:

1.50 ml of lactose broth is prepared and dispensed into 5 tubes. From each tubes some of the broth media is filled with the micropipettes into the Durham's tubes and they are inserted into the each test tubes.

2. They are autoclaved under 15 psi for 15 minutes and allowed to cool down

3. Then 0.1 ml of culture are inoculated to the 4 of the tubes and the remaining tube is used for negative control.

4. Tubes are allowed to incubate for 24 hours.

5. After 24 hours incubation the result is observed.

PROCEDURE OF DEXTROSE FERMENTATION TEST:

1.50 ml of dextrose broth is prepared and dispensed into 5 tubes. From each tubes some of the broth media is filled with the micropipettes into the Durham's tubes and they are inserted into the each test tubes.

2. They are autoclaved under 15 psi for 15 minutes and allowed to cool down

3. Then 0.1 ml of culture is inoculated to the 4 of the tubes and the remaining tube is used for negative control.

4. Tubes are allowed to incubate for 24 hours.

5. After 24 hours incubation the result is observed.

PROCEDURE OF SUCROSE FERMENTATION TEST:

1.50 ml of sucrose broth is prepared and dispensed into 5 tubes. From each tubes some of the broth media is filled with the micropipettes into the Durham's tubes and they are inserted into the each test tubes.

2. They are autoclaved under 15 psi for 15 minutes and allowed to cool down

3. Then 0.1 ml of culture are inoculated to the 4 of the tubes and the remaining tube is used for negative control.

4. Tubes are allowed to incubate for 24 hours. After 24 hours incubation the result is observed.

PROCEDURE OF TEST FOR DETECTION OF H_2S GAS PRODUCTION:

1. It is prepared 50 ml of triple sugar iron agar medium with 1.5% agar powder and melted and dispensed into 5 tubes.

2. They are autoclaved under 15 psi for 15 minutes and allowed to cool down in the slanted position.

3. Then a loopful of culture is streaked into four of the slants and the remaining is considered as negative control. Tubes are allowed to incubate for 24 hours. After 24 hours incubation the result is observed.

PROCEDURE OF TEST FOR DETECTION OF PHOSPHATE SOLLUBILIZATION:

1.100 ml of Pikovskaya agar medium is prepared and melted.

2. They are autoclaved under 15 psi for 15 minutes and 20 ml of medium is poured into four sterile petriplates and allowed to solidify.

3. Then a loopful of culture is streaked in single line to three of the plates and the remaining is considered as negative control. Plates are allowed to incubate for 72 hours and the result was observed.

PROCEDURE OF TEST FOR DETECTION OF NITRATE REDUCTION:

1.50 ml of nitrate broth is prepared and dispensed into 5 tubes.

2. They are autoclaved under 15 psi for 15 minutes and allowed to cool down.

3. Then 0.1 ml of culture are inoculated to the 4 of the tubes and the remaining tube is used for negative control.

4. Tubes are allowed to incubate for 24 hours.

5. After 24 hours incubation few drops of sulfanilic acid solution (reagent 1) and alpha-naphthylamine solution (reagent 2) are added one after another and the result is observed.

PHYSIOLOGICAL TEST:

PROCEDURE OF TEST FOR DETECTION OF BACTERIAL GROWTH WITH VARIOUS CARBON SOURCE:

1.30 ml of synthetic media with each carbon source prepared and dispensed into 3 tubes.

2. They are autoclaved under 15 psi for 15 minutes and allowed to cool down.

3. Among the each set of carbon source 0.1 ml of culture are inoculated to the 2 of the tubes and the remaining tube is used for negative control. Tubes are allowed to incubate for 24 hours.

5. Then their O.D is measured under 580 nm. And a bar graph is plotted.

PROCEDURE OF TEST FOR DETECTION OF BACTERIAL GROWTH WITH VARIOUS NITROGEN SOURCE:

1.30 ml of synthetic media with each nitrogen source prepared and dispensed into 3 tubes.

2. They are autoclaved under 15 psi for 15 minutes and allowed to cool down.

3. Among the each set of nitrogen source 0.1 ml of culture are inoculated to the 2 of the tubes and the remaining tube is used for negative control. Tubes are allowed to incubate for 24 hours.

5. Then their O.D is measured under 560 nm. And a bar graph is plotted.

PROCEDURE OF TEST FOR DETECTION OF BACTERIAL GROWTH WITH VARIOUS SALT CONCENTRATIONS:

1.30 ml of synthetic media with each salt concentration prepared and dispensed into 3 tubes.

2. They are autoclaved under 15 psi for 15 minutes and allowed to cool down.

3. Among the each set of particular salt concentration 0.1 ml of culture are inoculated to the 2 of the tubes and the remaining tube is used for negative control.

4. Tubes are allowed to incubate for 24 hours.

5. Then their O.D is measured under 560 nm. and a bar graph is plotted.

PROCEDURE OF TEST FOR DETECTION OF BACTERIAL GROWTH WITH VARIOUS AMINO ACIDS:

1. Synthetic media without nitrogen source was autoclaved and dispensed into 12 test tubes.

2. Each of the amino acids were prepared of 1% stock concentration.

3. Amino acid solution was filtered and 1 ml of each solution was added to each set of the tube.

4. Among the each set of particular amino acid, 0.1 ml of culture are inoculated into 2 of the tubes and remaining tube is negative control.

5. Tubes are allowed to incubate for 24 hours and their O.D is measured under 560 nm and a bar graph is plotted.

PROCEDURE OF TEST FOR DETECTION OF BACTERIAL GROWTH WITH VARIOUS pH RANGE:

1.30 ml of synthetic media for each pH is prepared and dispensed into 3 tubes.

2. They are autoclaved under 15 psi for 15 minutes and allowed to cool down.

3. Then the each media is adjusted according to desired ph with the addition of HCL and 1N NaOH solution. Among the each set of particular pH 0.1 ml of culture are inoculated to the 2 of the tubes and the remaining tube is used for negative control. Tubes are allowed to incubate for 24 hours.

5. Then their O.D is measured under 560 nm and a bar graph is plotted

PROCEDURE FOR TEST OF ANTIBIOTIC RESISTANCE WITH DIFFERENT ANTIBIOTICS AND THEIR CONCENTRATION:

1. Mueller Hinton agar media is prepared and autoclaved and poured into 8 petriplates and solidified.

2. Each of the antibiotic stock is prepared with the concentration of 2 mg/ml.

3. 0.1 ml of culture is spread on the plates.

4. 3 wells are cut by the cork borer into each plates.

5. 0.1 ml of different concentrations of antibiotic from a particular stock concentration is dispensed into wells of plates in a particular pattern like one of the plate having concentration of 100 microgram/ml, 300 microgram/ml and 500 microgram/ml and the another plate of that set having antibiotic concentration of 200 microgram/ml, 400 microgram/ml and a control.

6. Then the plates are allowed to incubate for 24 hours and the zone of inhibition is observed.

PROCEDURE OF DNA ISOLATION:

i. 1.5ml of culture was centrifuged at 10,000 rpm for 10 minutes.

ii. The pellet was suspended in 500µL TE buffer of pH8.0 (containing 10mMTris-HCl of pH8.0 and 1mM EDTA of pH8.0).

iii. To the mixture lysozyme was added at 1mg.mL-1 concentration and incubated at 37°C for 1hr. To remove the RNA contamination RNaseA was added to the mixture at a concentration of 20 µgmL-1.

iv. 1% SDS was added to the mixture and incubated at 55°C for 15 minutes.

v. Equal volume of Tris saturated phenol was added to the mixture and mixed well by inverting the tube.

vi. Phase separation was performed by centrifuging the mixture at 10,000 rpm for 10 minutes.

vii. The aqueous phase was collected in a fresh centrifuge tube and a 1:1 mixture of Tris saturated phenol and chloroform was added at equal volume.

viii. The aqueous phase was separated and collected as above and an equal volume of chloroform was added to remove trace amount of phenol present if any.

ix. The aqueous phase was again separated and collected in fresh tube.

x. 0.3M sodium acetate and 2.5volume chilled absolute ethanol was added to facilitate the precipitation of the nucleic acids.

xi. After 1hour incubation at -20°C centrifugation was performed at 15,000 rpm for 20 minutes.

xii. The pellet was washed with 70% ethanol twice and air dried.

xiii. Finally the genomic DNA was dissolved in 30µl of 2.5mM Tris HCl (pH8.0)

AGAROSE GEL ELECTROPHORESIS FOR VISUALIZATION OF GENOMIC DNA

1% agarose gel was prepared and the isolated genomic DNA was visualized using the following protocol:

i. A stock solution of 50X TAE (Tris –Acetate-EDTA) buffer(pH8.0) was prepared. It contains 242g Tris base, 57.1mL glacial acetic acid and 100mL of 500mM EDTA (pH 8.0) per liter.

ii. It is diluted to 1X by mixing with appropriate volume of distilled water.

iii. Agarose low EEO (SRL) was weighed according to 1% concentration.

iv. Agarose was dissolved in 1X TAE by heating.

v. After cooling down to room temperature 3µl ethidium bromide was added per 100ml of the gel.

vi. The gel was then poured over the clean gel casting tray and a comb was placed accordingly.

vii. The gel was allowed to solidify at room temperature.

viii. After solidification the comb lifted up and the gel was placed on gel running tank filled with 1X TAE as running buffer.

ix. The side of the well was placed towards the negative electrode.

x. 10µl DNA sample was mixed with 1X gel loading dye that contains

Bromophenol blue and xylene cyanol as indicator.

xi. The mixture was carefully loaded in a well using micropipette.

xii. After completion the DNA was allowed to run through the gel powered by 100V current generated from a power pack.

xiii. As the forward dye bromophenol blue had crossed a definite distance, the current supply turned off and the band of the DNA was visualized by UV transilluminator.

RESULT

1. Isolation:

Figure 1: Zone of hydrolysis after 48 hours.

2. Cultural characterization:

COLONY SIZE	COLONY COLOUR	COLONY FORM	COLONY MARGINE	ELEVATION	CHARECTER
Small	**White**	**Peripheral edge**	**Entire**	**Flat**	**Opaque**

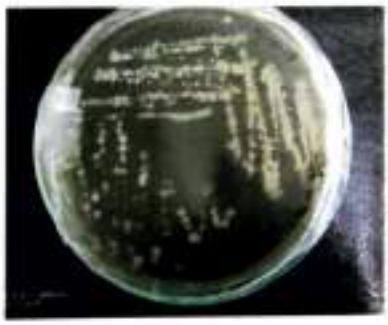


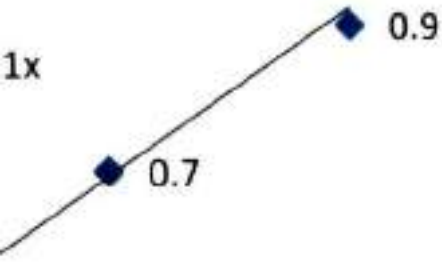
Figure 2: Colonies

Figure 3: Uniform broth culture

	0.244
	0.497
	0.7
	0.9

ATION VS. O.D

OF L-TYROSINE



60 80 1

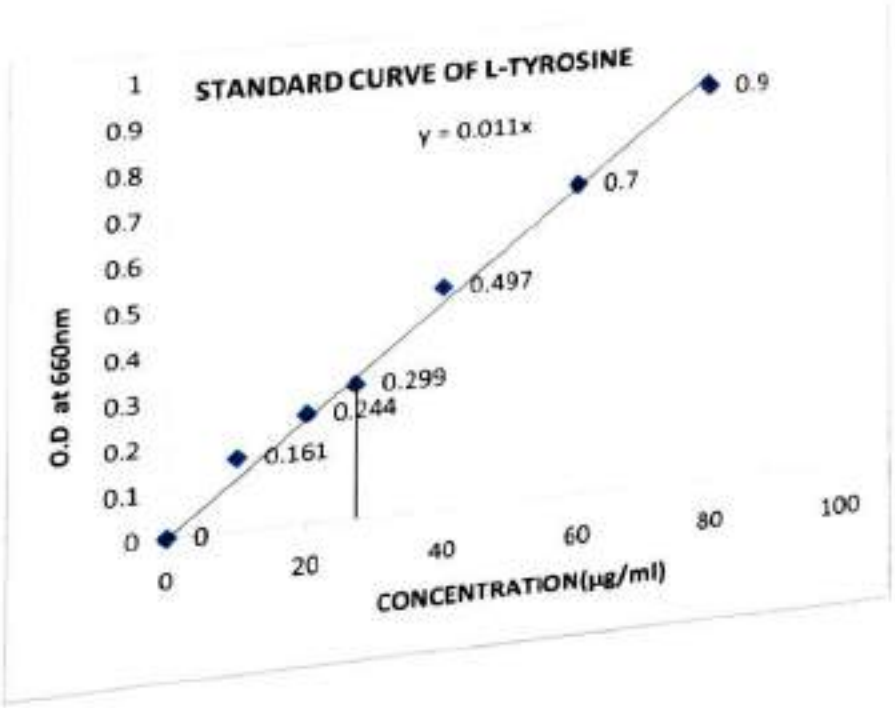
TION(µg/ml)

ESTIMATION OF PRODUCT FORMED

TEST TUBE	O.D AT 660 Nm	MEAN O.D.
T1	**0.301**	**0.299**
T2	**0.295**	
T3	**0.302**	

Graph is plotted against amount vs. O.D

Figure 4 : enzyme estimation



Conclusion: From the graph we get the data that the concentration of the product formed, 0.299/0.011=27.18 µg/ml

Enzyme activity, 27.18 µg/ml / 15 minuets = 1.812 µg/ml/Min

4. Morphological characteristics (Different Staining processes):

Figure 5: Simple

Figure 6: Negative

Figure 7: Gram

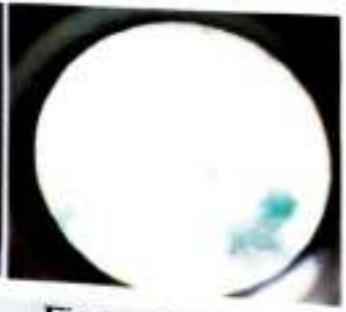


Figure 8: Endospore

SHAPE	ROD SHAPED
ARRANGEMENTS	MAJORITY ARE IN PAIR
GRAM CHARACTER	GRAM POSITIVE
ENDOSPORE	SPORES ARE TERMINALLY LOCATED
NEGATIVE STAINING	NO CAPSULE OBSERVED

MOTILITY TEST: Organism is Non motile.

5. Biochemical tests:

Figure 10: Indole test

Experimental tube

Figure 11: MR test

-ve control

-ve control Expt.tube

Figure 12: VP test

-ve control Expt.tube

Figure 13 : Citrate test

Expt.tube

-ve control

Figure 14: Catalase test

-ve control Expt.tube

Figure 15:Oxidase test

Experimental

Figure 16 : TSI test

-ve control

Expt.tubes

Figure 17:Urease test

-ve ctrl Expt.

Figure 18:Gelatine test

–ve control Expt. tube

Figure 19: Dextrose Fermentation

-ve control Expt.tubes

Figure 20: Sucrose Fermentation

-ve control Expt.tube

Figure 21: Lactose Fermentation

-ve control Expt.tube

Figure 22: Nitrate reduction test

-ve ctrl Expt.tube

Figure 23: Starch hydrolysis test

Figure 24: Phosphate solubilization test

Biochemical characterization

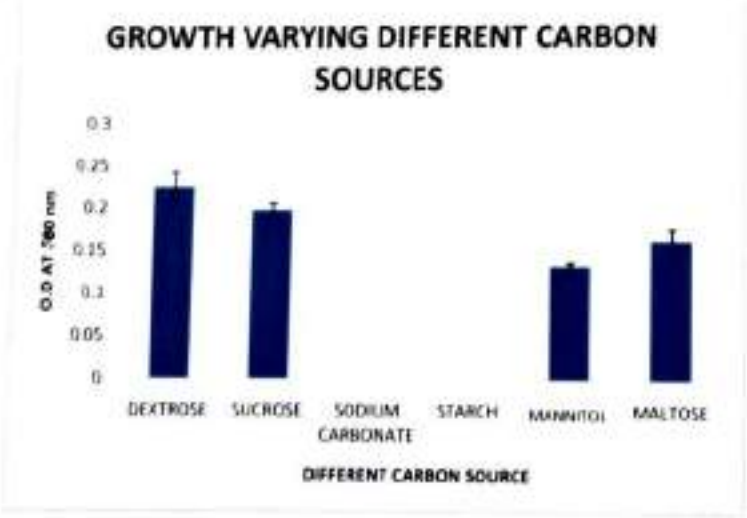
NAME OF THE TEST	RESULT
INDOLE	POSITIVE
MR	POSITIVE
VP	NEGATIVE
CATALASE	POSITIVE
CITRATE	POSITIVE
OXIDASE	POSITIVE
GLUCOSE FERMENTATION	POSITIVE (BOTH ACID AND GAS PRODUCED)
SUCROSE FERMENTATION	POSITIVE(NO GAS FORMATION)
LACTOSE FERMENTATION	NEGATIVE
UREASE	POSITIVE
TSI TEST	POSITIVE (FERMENTS GLUCOSE ,DOES NOT FORM H_2S)
GELATIN HYDROLYSIS	POSITIVE
AMYLASE PRODUCTION	NEGATIVE
PHOSPHATE SOLUBILIZATION	NEGATIVE
NITRATE REDUCTION	POSITIVE

6. Physiological Test

6.1. DIFFERENT CARBON SOURCE: O.D MEASURED AT 660 nm

CARBON SOURCES	O.D OF BLANK	O.D OF EXPERIMENTAL 1	O.D OF EXPERIMENTAL 2	MEAN O.D at 580 nm
DEXTROSE	0.000	0.207	0.242	0.224
SUCROSE	0.000	0.188	0.205	0.196
SODIUM CARBONATE	0.000	0.000	0.000	0.000
STARCH	0.000	0.000	0.000	0.000
MANNITOL	0.000	0.127	0.135	0.131
MALTOSE	0.000	0.148	0.174	0.161

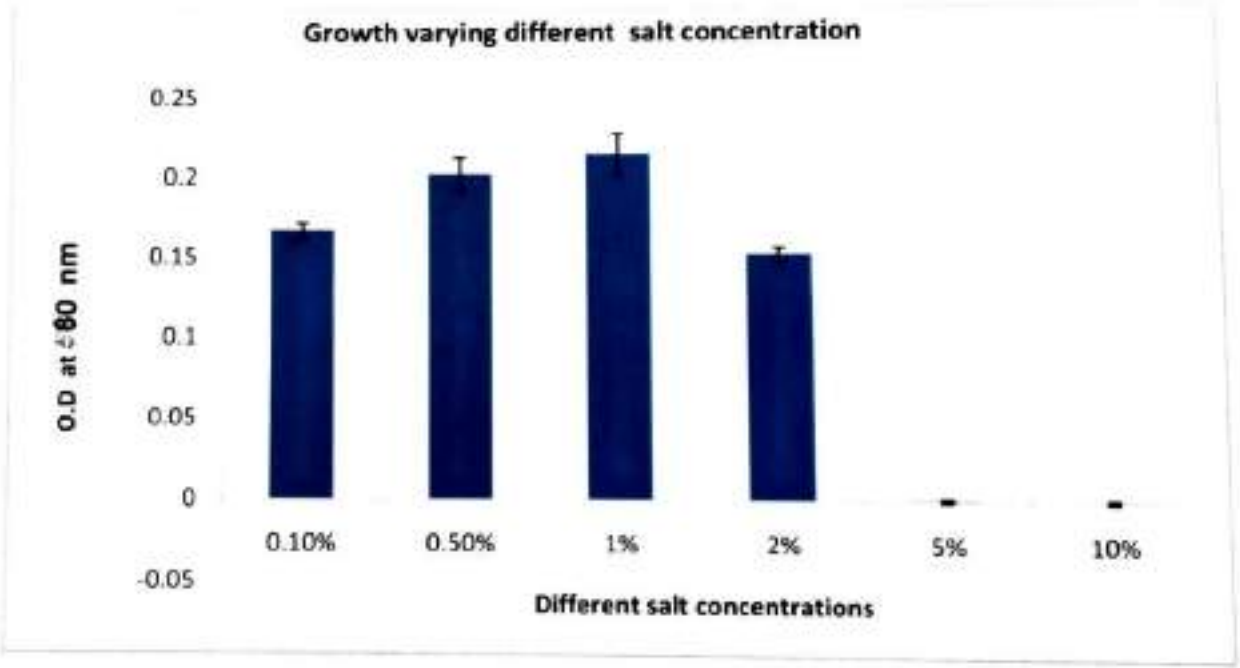
Figure 25: A COUMN GRAPH IS PLOTTED HERE.



6.2. DIFFERENT SALT PERCENTAGE: O.D MEASURED AT 660 nm

SALT PERCENTAGES	O.D OF BLANK	O.D OF EXPERIMENTAL 1	O.D OF EXPERIMENTAL 2	MEAN O.D at 580 nm
0.1%	0.000	0.162	0.172	0.167
0.5%	0.000	0.191	0.214	0.202
1%	0.000	0.228	0.202	0.215
2%	0.000	0.148	0.157	0.152
5%	0.000	0.007	0.005	0.0006
10%	0.000	0.003	0.001	0.0002

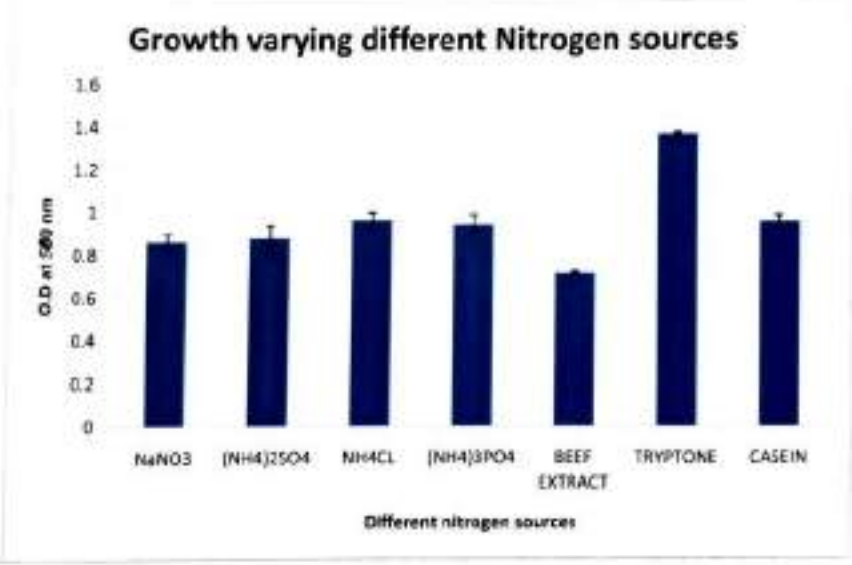
Figure 26: A COLUMN GRAPH IS PLOTTED HERE:



6.3. DIFFERENT NITROGEN SOURCE: O.D MEASURED AT 660 nm

NITROGEN SOURCE	O.D OF BLANK	O.D OF POSITIVE EXPERIMENTAL 1	O.D OF EXPERIMENTAL 2	MEAN O.D at 580 nm
$NaNO_3$	0.000	0.830	0.896	0.863
$(NH_4)_2SO_4$	0.000	0.822	0.938	0.88
NH_4Cl	0.000	0.995	0.933	0.964
$(NH_4)_3PO_4$	0.000	0.902	0.984	0.943
BEEF EXTRACT	0.000	0.717	0.707	0.712
TRYPTONE	0.000	1.363	1.376	1.36
CASEIN	0.000	0.982	0.933	0.95

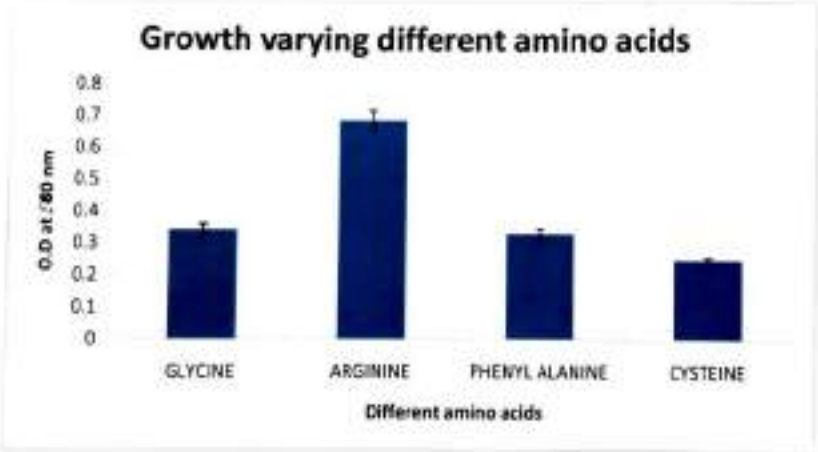
Figure 27: A COLUMN GRAPH IS PLOTTED HERE:



6.4. DIFFERENT AMINO ACID SOURCE: O.D MEASURED AT 660 nm

AMINO ACID SOURCE	O.D OF BLANK	O.D OF EXPERIMENTAL 1	O.D OF EXPERIMENTAL 2	MEAN O.D at 660 nm
GLYCINE	0.000	0.324	0.365	0.344
ARGININE	0.000	0.660	0.721	0.69
PHENYL ALANINE	0.000	0.353	0.308	0.33
CYSTEINE	0.000	0.259	0.240	0.249

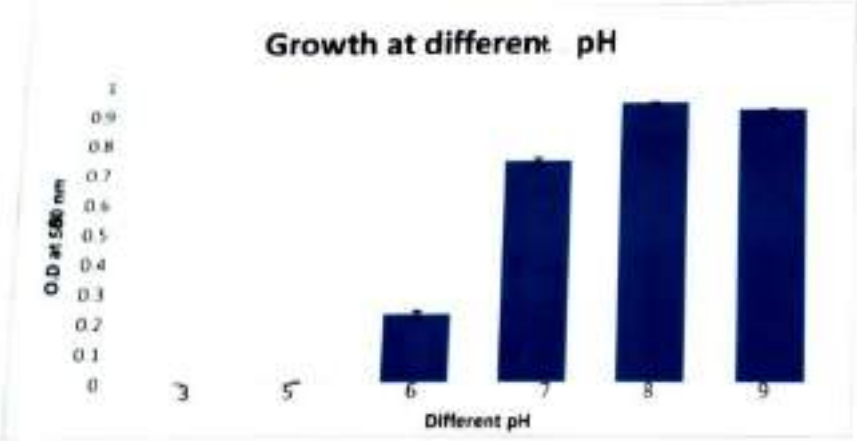
Figure 28: A COLUMN GRAPH IS PLOTTED HERE



6.5. DIFFERENT pH: O.D MEASURED AT 660 nm

ph	O.D OF BLANK	O.D OF EXPERIMENTAL 1	O.D OF EXPERIMENTAL 2	MEAN O.D at 580 nm
3	0.000	0.001	0.000	0.0005
5	0.000	0.005	0.000	0.0025
6	0.000	0.215	0.237	0.226
7	0.000	0.756	0.731	0.743
8	0.000	0.942	0.940	0.941
9	0.000	0.912	0.917	0.914

Figure 29: A COLUMN GRAPH IS PLOTTED HERE



7. DIFFERENT CONCENTRATIONS OF DIFFERENT ANTIBIOTICS:

7.1. FOR STREPTOMYCIN PLATES:

CONCENTRATION(µg/ml)	Zone diameter 1	Zone diameter 2	Zone diameter 3	MEAN DIAMETER(cm)
100	2.7	2.9	2.7	2.76
200	2.7	3	2.9	2.86
300	3	3.1	3	3.03
400	3.1	3	3	3.03
500	3.4	3.6	3.3	3.43

Figure 30: A COLUMN GRAPH IS PLOTTED HERE:

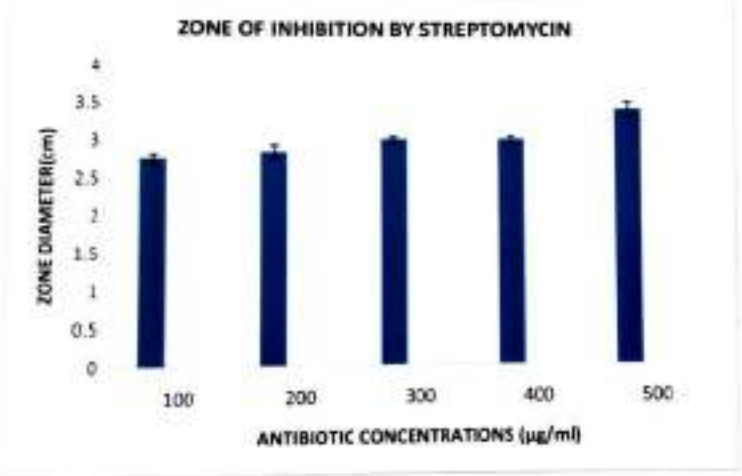
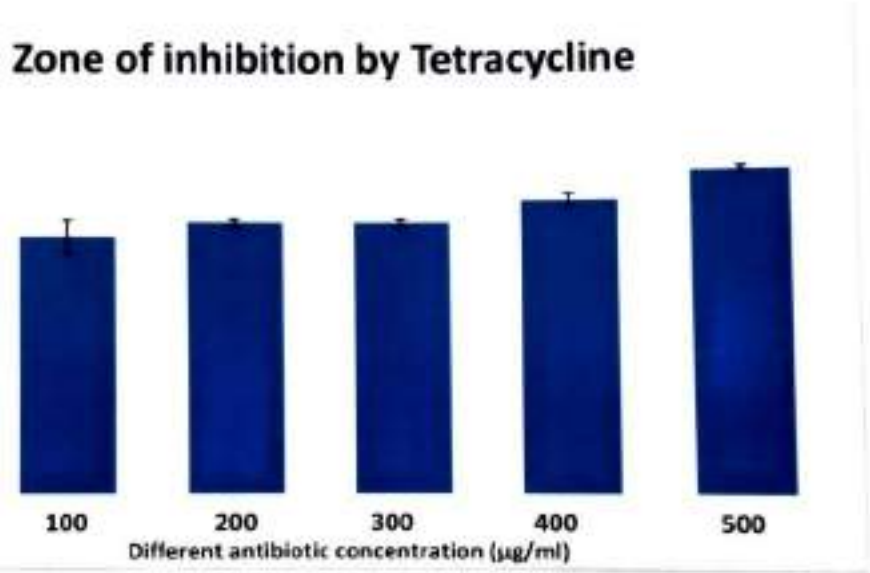


Figure 31: Zone of inhibition

2.4	3	2.6	2.67
2.8	2.9	2.8	2.83
2.9	2.8	2.8	2.83
3	3.2	3	3.06
3.3	3.3	3.2	3.36

COLUMN GRAPH IS PLOTTED HERE:



33: Zone of inhibition

IPICILLINE PLATES:

Figure 34: No zone of inhibition

6.4 FOR CHLORAMPHENICOL PLATES

CONCENTRATION(ug/ml)	Zone diameter 1	Zone diameter 2	Zone diameter 3	MEAN DIAMETER(cm)
100	2.5	2.5	2.8	2.6
200	2.6	2.6	2.9	2.7
300	**3**	**2.8**	**2.7**	**2.83**
400	3	2.8	2.9	2.9
500	**3.1**	**3.4**	**3.1**	**3.2**

Figure 35: A COLUMN GRAPH IS PLOTTED HERE

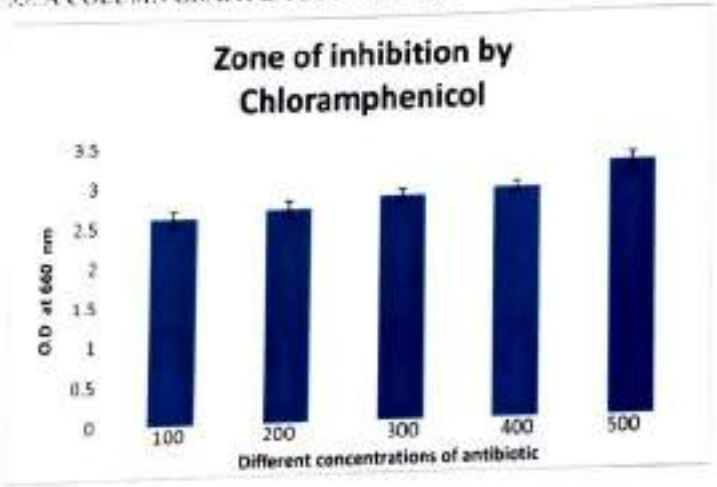


Figure 36: Zone of inhibition

8. ISOLATION OF BACTERIAL DNA

Figure 37: DNA band visible after agarose gel electrophoresis

9.PCR IMAGE

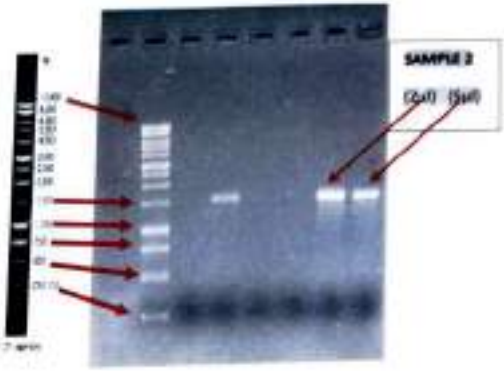


Figure 38: Image after PCR

DISCUSSION

Proteases are considered among the most important enzymes to be produced commercially and are of great significance. The present study is a preliminary screening report of soil sample obtain from garden area of West Bengal..Out of the all isolates the isolate with highest zone of hydrolysis was selected for protease production. Earlier reports show that the efficient protease from *Bacillus sp.* isolated from different sources.

The organism is gram positive which indicates the cell wall have thick peptidoglycan layer. The organism forms spores which we have seen under the microscope. It is non capsulated and non motile organism.

Bacterial growth was checked in different carbon sources, nitrogen sources, amino acids sources and different salt concentrations. Result observed in case of different carbon sources suggests the source of carbon affected growth of bacteria .Sucrose and maltose show high growth. They responded to organic nitrogen sources and found to better nitrogen sources for both growth and shows highest growth in presence of Tryptone. The organism show good growth and adaptability at 1% NaCl concentration. It is resistance to ampicillin and it gives optimum growth at alkaline ranges of pHs

My present study show positive result in MR test which mean that the organism utilizes glucose which is further metabolized through the mixed acid pathway to produce stable acids like lactic acid, formic acid, acetic acid. Indole positive result indicates bacteria have tryptophanase enzyme and Citrate positive result indicates bacteria can use citrate as sole carbon source and contain citrase enzyme.

In sugar fermentation test we can see the colour change from red to yellow which indicates that the organism produce gas from carbohydrate. Catalase positive result ensures the organism is aerobic and contain Catalase enzyme that mediates the breakdown of hydrogen peroxide into oxygen and water. Oxidase positive test ensure that the organism produce intracellular oxidase enzyme. Urease positive result indicates organism have a Urease enzyme which is able to split urea in presence of water to release ammonia and carbon dioxide.

Nitrate reduction test results positive which means organism is capable of producing the nitrate reductase enzyme which reduce nitrate to nitrite and further reduced to nitric oxide, nitrous oxide or nitrogen.TSI test results positive and indicates the organism ferment glucose

but do not ferment lactose or sucrose. It also shows the organism does not produce H_2S.The organism does not produce amylase enzyme and it is not capable to solubilize phosphate.

Protease produced by the isolate can be efficiently used for digestion or removal of protein. The isolate hydrolyzes Gelatin which is a protein obtained by hydrolysis of a collagen compound of the connective tissues of animals. So it is convenient that the isolate can be utilized effectively as industrial as well as pharmaceutical purposes. Protease hydrolyzes peptide links of proteins and peptide to form smaller unit of amino acids and it is produced both extracellular and intracellular. Protease plays an important role in baking, brewing, detergent industry, leather processing industry, meat tendering, peptide synthesis and medical diagnosis.

Bacterial DNA band is visible after the agarose gel electrophoresis and the 16S r RNA gene is amplified to be sequenced and the sample has been sent to sequencing which is under processing. After getting the result we will further investigate about the another capabilities of the organism and compare them with other organisms to get some potent source of various purposes and we will try to use them industrially in versatile manner.

CONCLUSION

Proteases are considered among the most important enzymes to be produced commercially and are of great significance. They have their applications in food, detergent, pharmaceuticals etc.The present study reports that the best growth observed after incubation of 48 hours. The organism secret protease which creates a zone of inhibition in skimmed milk agar medium and amount of the enzyme estimated is measured under spectrophotometer. It reports the colony character as whitish, opaque, small sized colonies. The microscopical view of Gram staining shows rod shaped gram positive bacteria with paired arrangements mostly. The result of endospore staining concludes spores are terminally located and negative staining reports absence of capsule. Motility test concludes bacteria is non motile. The report concludes it is motile and can hydrolyze Gelatin. It also conclude that the organism grow best in 1% NaCl concentration, Dextrose as carbon source, Tryptone as nitrogen source, Arginine as amino acid source. It gives highest growth at higher pHs, so they can be concluded as alkaliphilic organisms. It gives positive result in MR test, Catalase test, citrate test, glucose and fructose fermentation, Urease test and nitrate reductase test which confirms the chemical characterization of a protease producing organisms. As it gives positive result in oxidase test it can be concluded as aerobic. The organism also reports antibiotic resistance in ampicillin and sensitive in streptomycin, tetracycline and chloramphenicol.Further the report concludes that the bacterial DNA band is visible after the agarose gel electrophoresis and the 16S r RNA gene is amplified to be sequenced and the sample has been sent to sequencing which is under processing. The overall study give an idea of morphological, biochemical and physiological characteristics of a protease producing organism.

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[18 BACON F. CHOW; MARY-ANN PETICOLAS, 1948, 19-21]

Project Title::Prevalence and antimicrobial susceptibility pattern of Non-fermenting Gram-negative bacilli in all clinical samples from a tertiary care hospital of Eastern India

SUBMITTED FOR THE PARTIAL FULFILLMENT OF THE AWARD OF M.Sc. IN MICROBIOLOGY

BY

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Memo No: MICRO/231/21 Date: 27.7.2021

Certificate

This is to certify that ***Sayantan Banerjee*** *have successfully completed Non Stipendiary training in the Department of Microbiology from 08.04.2021 to 07.06.2021 and submitted a project on* ***"Prevalence and antimicrobial susceptibility pattern of Non-fermenting Gram-negative bacilli in all clinical samples from a tertiary care hospital of Eastern India"*** *under the guidance of Dr Kabita Choudhury.*

I wish him every success in life.

Department of Microbiology N.R.S.M.C.&H. Kol-14

Swagata Ganguly

Prof. (Dr.) Swagata Ganguly Bhattacharjee
Professor and Head,
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Content

Prevalence and antimicrobial susceptibility pattern of Non-fermenting Gram-negative bacilli in all clinical samples from a tertiary care hospital of Eastern India.

Abstract:

Introduction: Non-fermenting Gram-negative bacilli (NFGNB) was initially considered as non-pathogenic and contaminants but now have emerged as major pathogens and threat to the society because of increased isolation and emerging resistance to common antimicrobial agents.

Aims and objectives: The study aimed to determine the prevalence of NFGNB in all clinical samples submitted to the Department of Microbiology, NRS Medical College, Kolkata for a period of one year.

Materials and methods: The samples received were processed according to standard procedures and antimicrobial susceptibility testing done using Kirby Bauer disc diffusion method.

Results: 157 NFGNB was isolated from 725 bacterial isolates (21.65%). *Pseudomonas* species (n=84) was isolated most frequently followed by *Acinetobacter* spp (n=73). Male population was more affected compared to female population. *Pseudomonas* showed maximum sensitivity to Meropenem followed by Imipenem. Cephalosporins showed high degree of resistance.

Conclusion: It is necessary to identify NFGNB in tertiary care hospitals to take strict infection control measures to limit the spread of the underlying resistance mechanisms caused by these pathogens and to monitor their susceptibility pattern to guide the clinician for better care and management of patients. Since NFGNB are emerging as major community and hospital acquired infection, this study will help initiate proper empirical antimicrobial therapy.

Keywords: NFGNB, *Pseudomonas, Acinetobacter*, antimicrobial susceptibility.

Introduction:

Organisms which are aerobic, non-spore forming, Gram Negative rod and either do not take carbohydrates as their energy source or utilize them by various metabolic pathways except fermentation are known as Non-fermenting Gram-negative bacilli. Organisms show growth on slant of TSI (triple sugar iron)

medium but not in the butt part. These organisms never acidify the butt of the test media[1]. They are saprophytic in nature but can cause a significant number of infections particularly in hospitalized patients, immunocompromised hosts and patients with haematological malignancies[2]. NFGNB are known to account for about 15% of all bacterial isolates from a clinical Microbiology laboratory[3]. Inherent resistance of these bacterial agents to commonly used disinfectants and their tendency to colonize various surfaces have been pivotal in their emergence as important nosocomial pathogens[4].Currently *Pseudomonas aeruginosa* and *Acinetobacter baumannii* are the most commonly isolated non-fermenters pathogenic in humans. Infections caused by other species are relatively infrequent. Normally most of the infections caused by these organisms are secondary infections because their infections are mainly seen in patients already suffering from any other primary conditions like burns, prolonged antimicrobial therapy, patient on any immunosuppressive agents, old age etc[6]. Recent studies have shown that *Pseudomonas aeruginosa* is the second most common cause of nosocomial pneumonia and ventilator associated pneumonia[7,8]. Members of non-fermenting Gram-negative bacteria show resistance to a wide range of commonly used antibiotics by several mechanisms like antimicrobial inactivating enzymes, reduced access to bacterial targets and point mutations that change targets or cellular functions[9]. Due to increased frequency of isolation of NFGNB and the presence of antimicrobial resistance, the current study was undertaken to know the prevalence of NFGNB and their antimicrobial susceptibility pattern in our hospital setting.

Aims and objectives:

1. To know the prevalence of NFGNB among all clinical isolates.
2. To know the antimicrobial susceptibility of NFGNB in these clinical isolates.

Materials and Methods:

The study was conducted for a period of one year between January and December 2020 in the Department of Microbiology, NRS Medical College,

Kolkata. The samples submitted to the Department from various wards, ICU and outdoor were collected and processed routinely. The samples were blood, body fluids, urine, sputum, endotracheal tube secretions, central and peripheral line tips were inoculated into Blood agar and MacConkey agar and incubated at 37°C overnight at ambient air. The growth of bacterial colonies was identified the following day in respect to colony morphology, Gram staining and motility (Hanging drop method). It was then subjected to Catalase test, Oxidase test, Citrate test, Urease test, pigment production, Indole production, Methyl Red test, Voges Proskauer test, Triple sugar Iron test, Oxidation/ Fermentation test for Glucose, Lactose, Xylose, Mannitol and Maltose (Hugh and Leifson's media), Lysine and Ornithine decarboxylase and Arginine dihydrolase activity test etc. were done for isolation of the Non-fermentative Gram negative bacilli[10]. Antibiotic susceptibility was done by Kirby Bauer disc diffusion method following CLSI guidelines 2020[11]. The results were analysed and interpretation represented graphically. *Escherichia coli* ATCC 25922 & *Pseudomonas aeruginosa* ATCC 27853 were used as control organisms during the study[12].

Results:

Out of total 725 bacterial isolates, 157 NFGNB was isolated with an isolation rate of 21.51%. Of this 157, NFGNB, 84 isolates (53.84%) were *Pseudomonas* spp. and 73 isolates were (46.16%) were *Acinetobacter* spp

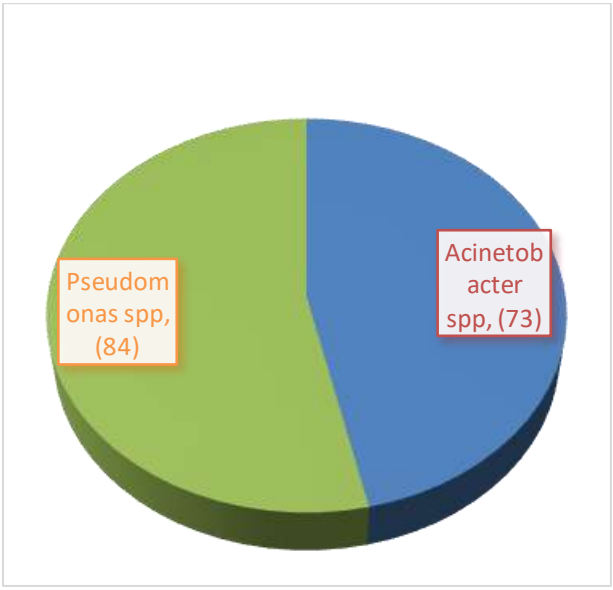

Figure 1: Distribution of NFGNB into type of isolated organism

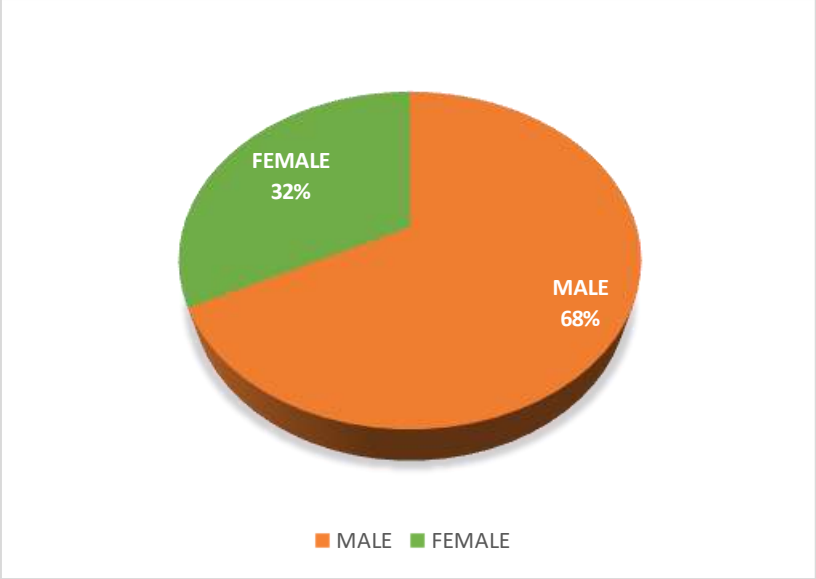

Figure 2: Gender distribution of isolated GNB

Males were more commonly affected with n=107 (68%) and females n=50(32%). Most of the NFGNB were isolated from pus (42%), followed by urine(21%), followed by body fluids(8%) and central & peripheral line tip(6%).

Table-1-Sources of samples with respective Number and corresponding Percentage

Sl no.	Source of sample	Number	Percentage (%)
1	Body Fluid	12	7.64%
2	Blood	7	4.45%
3	Oral Swab	1	0.63%
4	Urine	33	21.01%
5	Pathological	2	1.27%
6	Sputum	13	8.28%
7	Pus	66	42.03%
8	Et Tube Aspirate	8	5.09%
9	CSF	4	2.54%
10	Tip	11	7.00%

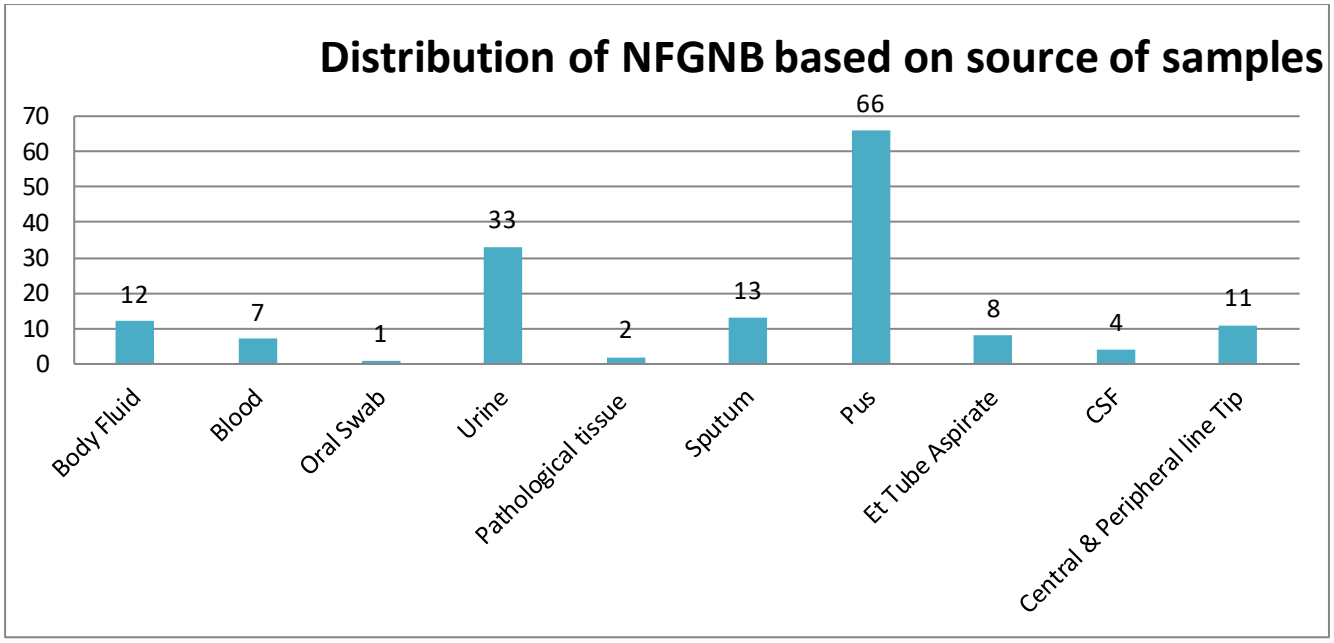

Figure 3: Distribution of NFGNB on basis of source of samples

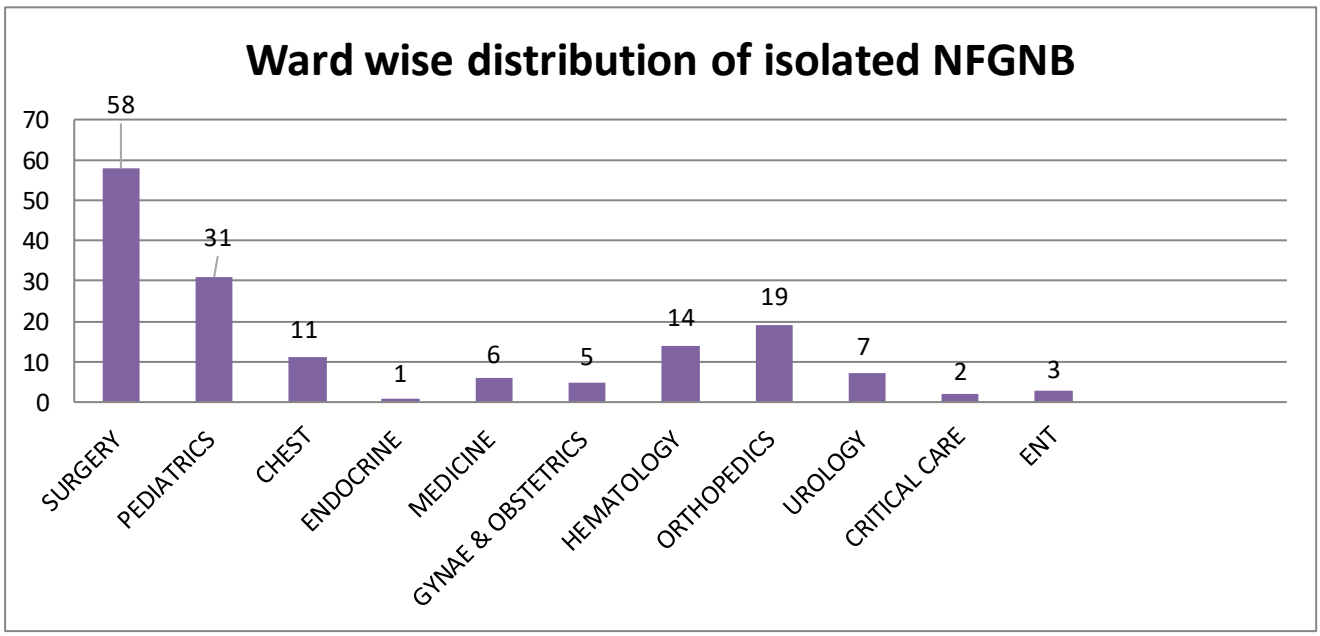

Figure 4: Ward wise distribution of isolated NFGNB

Most isolates of NFGNB were from the Department of Surgery (37%) followed by Paediatrics (20%) followed by Orthopaedics (12%).

Table 2: Sensitivity and resistance pattern of isolated NFGNB.

Antibiotics Used	*Pseudomonas* spp				*Acinetobacter* spp				Total (157 Samples)			
	Sensitive	Percentage	Resistant	Percentage	Sensitive	Percentage	Resistant	Percentage	Sensitive	Percentage	Resistant	Percentage
Cefepime	33	39.28%	51	60.72%	25	34.24%	48	65.76%	58	36.76%	99	63.24%
Imipenem	56	66.67%	28	33.33%	45	61.64%	28	38.36%	101	64.15%	56	35.85%
Meropenem	70	83.33%	14	16.67%	59	80.82%	14	19.18%	129	82.07%	28	17.93%
Amikacin	44	52.38%	40	47.62%	37	50.68%	36	49.32%	81	51.53%	76	48.47%
Gentamicin	41	48.80%	43	51.20%	34	46.57%	39	53.43%	75	47.68%	82	52.32%
Doripenem	68	80.95%	16	19.05%	61	83.56%	12	16.44%	129	82.25%	28	17.75%
Levofloxacin	28	33.33%	56	66.67%	27	36.98%	46	63.02%	55	35.15%	102	64.85%
Piperacillin Tazobactum	73	87.00%	11	13.00%	63	86.30%	10	13.70%	136	86.65%	21	13.35%
Ceftazidime	47	55.95%	37	44.05%	45	61.64%	28	38.36%	92	58.79%	65	41.21%
Ticarcillin+Clavulinic acid	30	35.71%	54	64.29%	29	39.72%	44	60.28%	59	37.71%	98	62.29%
Aztreonam	38	45.23%	46	54.77%	30	41.09%	43	58.91%	68	43.16%	89	56.84%
Cefoperazone+ Sulbactum	54	64.38%	30	35.62%	49	67.12%	24	32.88%	103	65.75%	54	34.25%

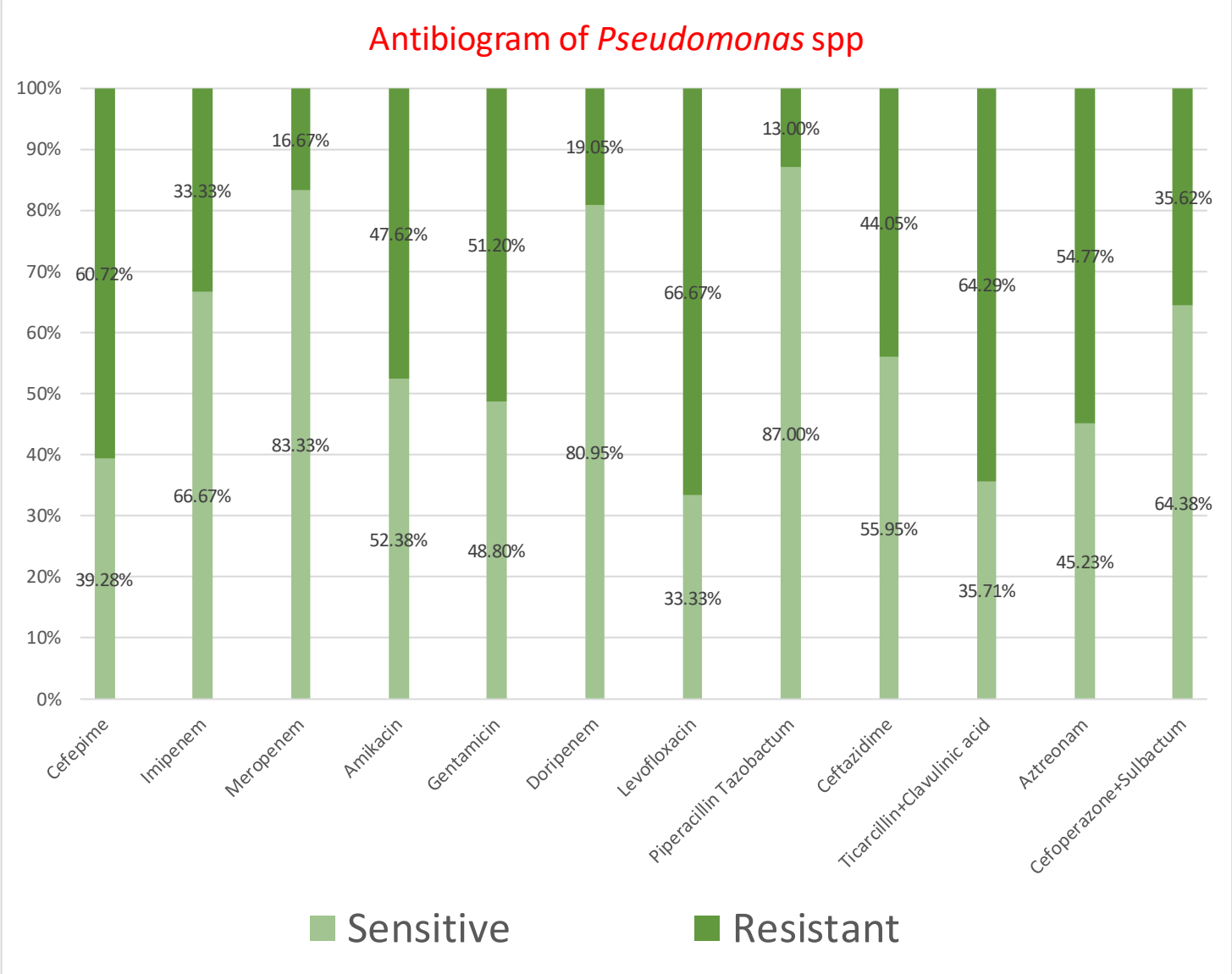

Figure 5: Antibiogram of *Pseudomonas* spp.

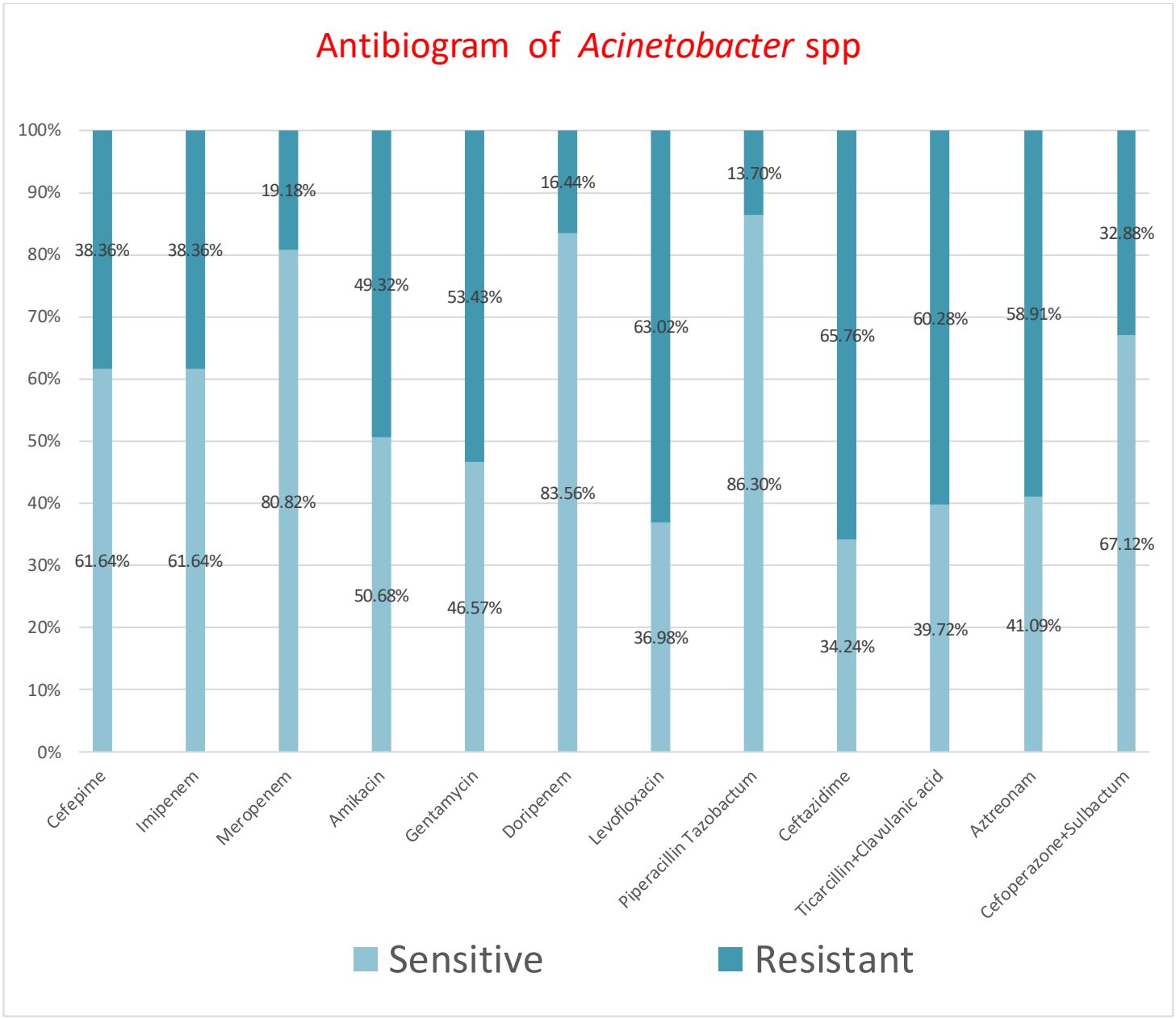

Figure 6: Antibiogram of *Acinetobacter* spp.

Discussion:

In our study, of the total 725 bacterial isolates ,157 isolates were NFGNB with a prevalence of 21.65%. This is similar to study conducted by Vijaya et al. (21.8%) and higher than study conducted by Anshu Shastry et al. (18.1%) and Amandeep Kaur et al (16.5%). Non-fermenter Gram Negative Bacilli are universal in nature and they are non-pathogenic or pathogenic in nature. They are basically attacked on immunocompromised persons. In the present study, isolation rate of NFGNB out of total samples 68% males and 32% females which is similar to Jayapriya et al study who has reported NFGNB isolates from males 71% and females 29%[13]. *Pseudomonas* spp and *Acinetobacter* spp were the common isolates from pus, urine, body fluids in samples. In our study the isolation rate of NFGNB from Pus sample is 42.03% which is lower than Malini et al(62.17%) study[14]. Isolation of NFGNB from urine sample is 21.01% in our study which is higher than Benanchinmardi et al 11% and Malini et al [15,14]. In case of our study Piperacillin Tazobactum is the most sensitive drugs and whose sensitivity around 86.30% for *Acinetobacter* spp and 87% for *Pseudomonas* spp but in study of Anshu et al its find 75% for *Pseudomonas* spp and 56.5% for *Acinetobacter* spp which is discordant of our study. In case of *Acinetobacter* spp sensitivity shows for Ceftazidime 61.64% and amikacin 50.68% which is similar to the Kaur et al study[16]and Gomathi et al study[17]. The antimicrobial sensitivity pattern of *Pseudomonas* spp showed 83.33% for Meropenem and 80.82% for *Acinetobacter* spp which is concordant with the Gomathi et al study showed 79.6% for *Pseudomonas* spp and 75% for *Acinetobacter* spp. In our study amikacin sensitivity shows 52.38% for *Pseudomonas* spp which is similar with Gomathi et al but in case of Malini et al showed 69.2% sensitivity which is discordant with our study. Gentamicin sensitivity has founded in our study for

48.8% and 46.57% for *Pseudomonas* spp and *Acinetobacter* spp. These result correlates with the findings of Gomathi et al[17].Doripenem sensitive among *Pseudomonas* spp and *Acinetobacter* spp was 80.95% and 83.56%.

Conclusion:

Non fermenter Gram Negative Bacilli (NFGNB) are available in nature. These organism mainly attacking the immunocompromised patients. Antimicrobial resistance is very common and now a days this is increasing rapidly. To conclude, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* are the foremost common NFGNB isolated in our study. these bacteria also have a great potential to survive in hospital environment therefore, improved antibiotic stewardship, good housekeeping, equipment decontamination, strict protocols for hand washing, isolation procedures need to be implemented to prevent emergence and spread of multidrug resistant NFGNB in health care settings. Our study highlights the actual fact that it's essential to ascertain the clinical relevance of the isolated NFGNB. Limitation Of our study is small samples size and durational study is very small. Hence the appropriate scenario cannot be determined. Similar studies with molecular characteristics should be conducted to know the complete antibiotic sensitivity and resistance of NFGNB. Further studies will definitely help in better understanding of changes in its antimicrobial resistance pattern. This study can be very helpful in initiating the treatment of such patients thereby reducing the illness.

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ISOLATION AND CHARACTERIZATION OF RHIZOSPHERIC MICROORGANISMS FROM POTATO (*Solanum tuberosum*) PLANT AND EVALUATION OF THEIR PGPR CHARACTERISTICS

SUBMITTED FOR THE PARTIAL FULFILLMENT OF THE AWARD OF M.Sc. IN MICROBIOLOGY

SUBMITTED BY

SUPARNA CHOUDHURY

M.Sc. SEMESTER – IV

ROLL: BNC/MCB-IV NO.: 003

REGISTRATION NO.: 1201721400225 OF 2017-2018

UNDER SUPERVISION OF

Mrs. PARAMA DAS GUPTA

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Date : 14:07:2022

To Whom It May Concern

This is to certify that Ms. Suparna Choudhury of P.G. Department of Microbiology, Bidhannagar College, has successfully completed her project entitled "Isolation and Characterization of Rhizospheric Microorganisms from Potato Plant and Evaluation of their PGPR Characteristics" under my supervision from 1st March to 31st May 2022.

I wish her every success in her future.

Parama Das Gupta.

Parama Das Gupta

Assistant Professor

Department of Microbiology

Bidhannagar College

Kolkata-700064

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Finally, I would like to acknowledge my departmental staff members and my fellow friends who helped me in successful completion of this internship.

Suparna Choudhury

(SUPARNA CHOUDHURY)

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ABSTRACT: ~

Rhizosphere is the narrow zone of soil specifically influenced by the plant root system. A large amount and various types of bacteria generally colonize near the root zone, known as rhizobacteria. Rhizobacteria that benefit plants by stimulating growth and suppressing disease are referred to as Plant Growth Promoting Rhizobacteria (PGPR). These bacteria lodging around the plant roots are more versatile in transforming, mobilizing, solubilizing the nutrients compared to those from non-rhizospheric soils. They have normal plant growth promoting properties and widely recognized mechanisms like fixation of nitrogen, solubilization of phosphate and zinc, production of siderophores, hydrogen cyanide (HCN), ammonia (NH_3), Indole Acetic Acid (IAA) and antifungal activity. Potato (*Solanum tuberosum*) is one of the most widely grown vegetable crop in the world, and particularly prevalent on the Indian sub-continent. The objectives of the present study are to isolate bacterial strains from the potato rhizosphere, to characterize the isolates on the basis of morphological and biochemical attributes and to assess the plant growth promoting effects of them.

Keywords: Rhizosphere, Plant Growth Promoting Rhizobacteria

INTRODUCTION

The rhizosphere is the narrow zone of soil particularly influenced by the root system. The zone of influenced around plant roots harbors a multitude of microorganism that are affected by both abiotic and biotic stresses [1].

Rhizobacteria that benefit plants by restoring growth and retention diseases are referred to as Plant Growth Promoting Rhizobacteria (PGPR) [2]. It has been tested as biocontrol agents for suppression of plants diseases and also as inducers of disease resistance in plants [3]. Also, the rhizobacteria are the dominant decisive forces in recycling the soil nutrients and for soil fertility. The plant growth promoting and biocontrol efficacy of PGPR often depend upon the rhizosphere competence of the microbial inoculums. It refers to the survival and colonization potential of PGPR [4]. The bacteria living around the plant roots are more adaptable in transforming, mobilizing, solubilizing the nutrients compared to those from bulk soils [3]. PGPR are considered to promote plant growth directly or indirectly. The direct promotion of plant growth by PGPR entails either providing the plant with a compound that is synthesized by the bacterium and the indirect promotion of plant growth occur when PGPR lessen. The direct mechanisms involve the production of Indole Acetic Acid (IAA), hydrogen cyanide (HCN), ammonia (NH_3), and siderophore and the indirect mechanisms involve antifungal activity, detection of heavy metal resistance activity [5, 6]. The widely recognized properties and mechanisms of plant growth promotion by PGPR like – solubilization of phosphate and zinc, fixation of nitrogen etc. [7].

The potato (***Solanum tuberosum***) is one of the most common grown vegetable crop in the world and specifically extensive on the Indian sub-continent [1]. The aim of the recent study is to isolate bacterial strains from the potato rhizosphere, to characterize the isolates on the basis of biochemical and morphological attributes, and to evaluate the plant growth promoting out comes of them.

MATERIALS & METHODS: ~

Isolation of the experimental sample –

The experimental site was located at the field Kanaipur, Nabagram Colony, Konnagar, West Bengal - 712246. The location of the site is at 22.41575 N and 88.36861 E.

Potato (*Solanum tuberosum*) plant root samples were collected from the agriculture field of rhizosphere soil. Sample was obtained in sterile plastic container and transported to the laboratory for further analysis within 6-8 hours of collection. The soil still attached to the plant roots was swept with a brush. After that serial dilutions were prepared from the ground soils [4]. 10 grams of soil samples were taken in 0.9% NaCl and shaken at 1000 rpm for 15 minutes. After that samples were settled for 5 minutes and 1 ml of the suspension was serially diluted up to 10^{-6}. 0.1 ml of each dilution was spread on to nutrient agar plates and incubated for 37°C for 24 hours. The growing colonies were observed and again inoculated on to Pikovckaya Agar and Ashby's nitrogen free mannitol agar plates [8]. Colonies surrounded by clear zones on Pikovskaya Agar medium and growth on Ashby's nitrogen free mannitol agar plates were selected. Five colonies from Pikovskaya agar medium and three colonies from Ashby's nitrogen free mannitol agar plates were selected. Finally, morphological and biochemical properties of eight different bacterial colonies are determined by using the standard protocol.

Fig. 1 – Kanaipur, Nabagram Colony, Konnagar: Site of sample collection

❖ Morphological Characteristics

Cell shape, arrangement and gram character were determined by performing gram staining [1]. Production of capsule was determined by negative staining [2]. The motility of each isolates was tested [9].

❖ Biochemical Characteristics

A series of biochemical tests were conducted to characterize the isolated bacteria. Catalase [3], Oxidase [4], IMViC [9], Urease [10], Nitrate reductase [20], TSI agar [1], and Carbohydrate fermentation tests [10] were performed. Production of various hydrolytic enzymes like Amylase [2], Protease [4], Lipase [3] were also detected.

❖ PGPR Characteristics

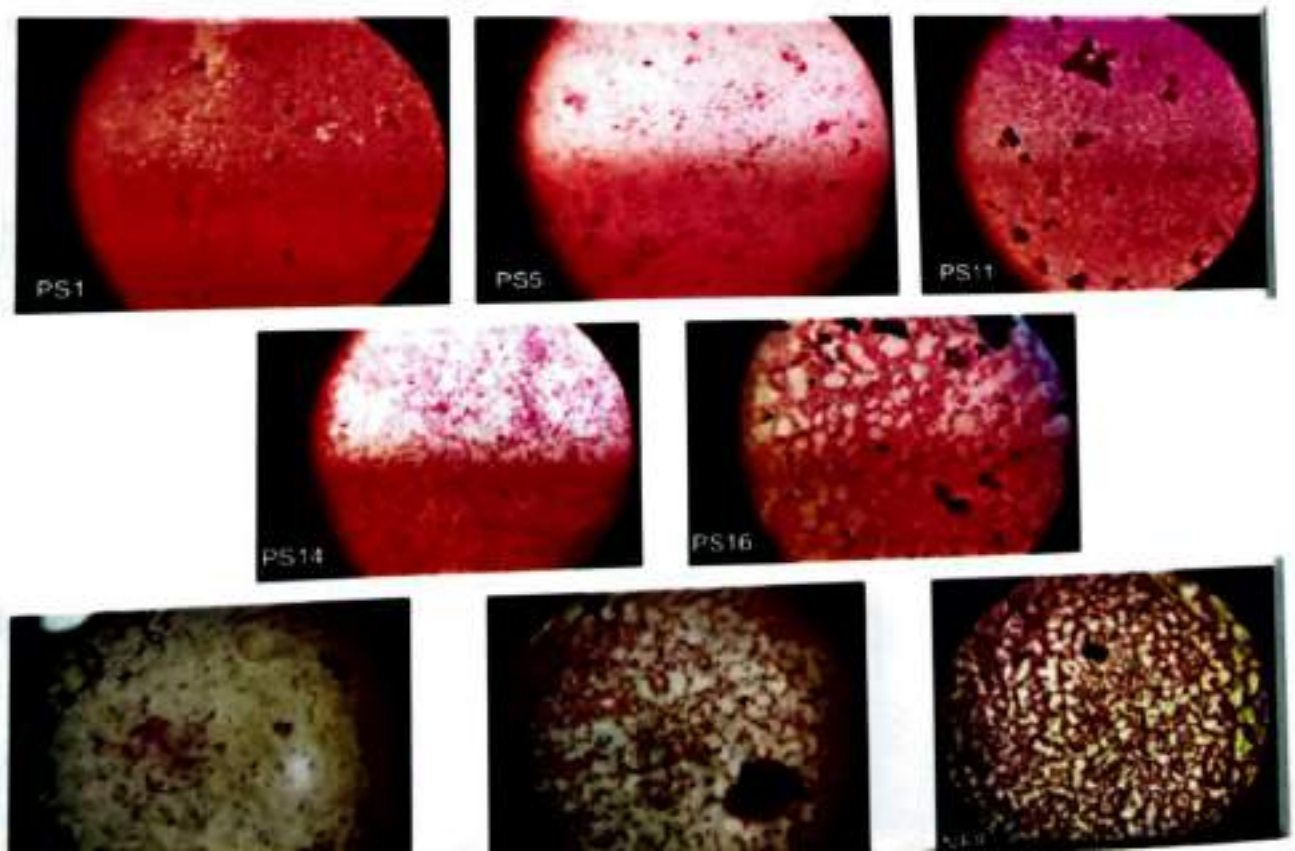
Plant Growth Promoting (PGP) characteristics of the bacterial isolates were performed. Production of Indole Acetic Acid (IAA) [5], Siderophore [15], Hydrogen Cyanide (HCN) [6] and Ammonia (NH_3) [22], solubilization of Phosphate [7, 8] and Zinc [11, 13], fixation of atmospheric Nitrogen [22], Heavy metal tolerance [19] and Anti-fungal activity [20] were detected.

More than 20 different rhizobacterial strains from the soil of potato roots are isolated. These strains are purified and coded as PS and NF. 8 isolated strains are selected for further experimentations.

❖ **Morphological characteristics of samples:**

- Gram Staining:

SAMPLE	SHAPE	ARRANGEMENT	GRAM CHARACTER
PS1	Coccus	Cluster	Negative (-ve)
PS5	Rod	Single	Negative (-ve)
PS11	Coccus	Cluster	Negative (-ve)
PS14	Rod	Single	Negative (-ve)
PS16	Coccus	Chain	Negative (-ve)
NF1	Rod	Single	Negative (-ve)
NF4	Coccus	Chain	Negative (-ve)
NF8	Coccus	Chain	Negative (-ve)



- Capsule Negative (-ve)

SAMPLE	CAPSULE FORMATION
PS1	Negative (-ve)
PS5	Negative (-ve)
PS11	Negative (-ve)
PS14	Negative (-ve)
PS16	Negative (-ve)
NF1	Negative (-ve)
NF4	Negative (-ve)
NF8	Negative (-ve)

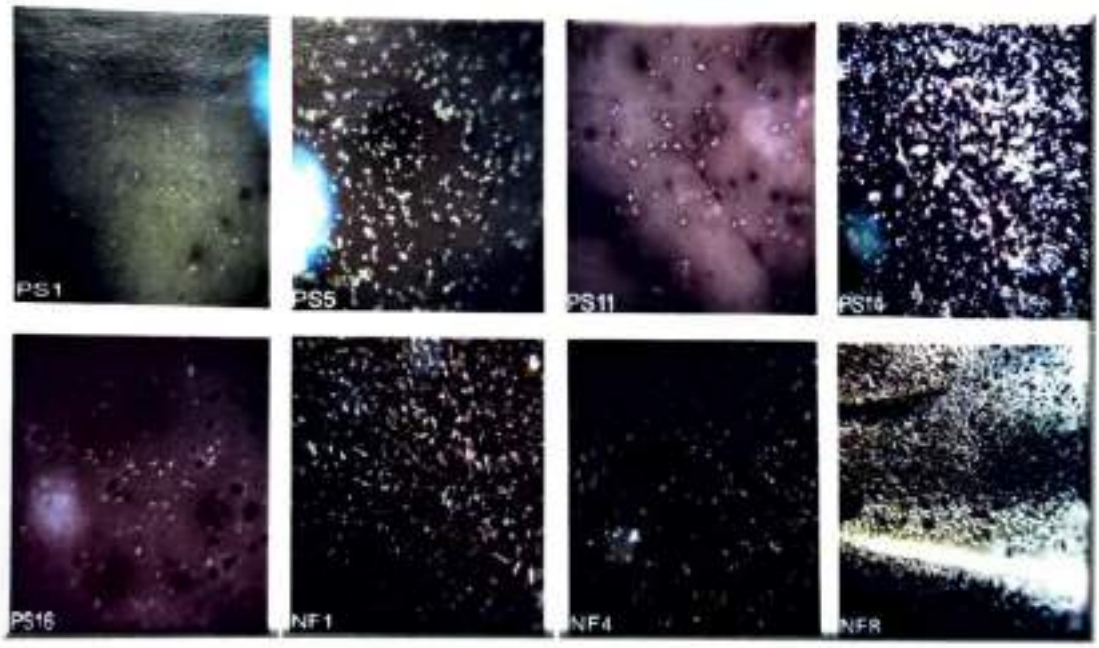


Fig. 3 shows Capsule Staining: No isolates form any capsule.

- Motility Test-

SAMPLE	MOTILITY
PS1	Non-Motile
PS5	Non-Motile
PS11	Non-Motile
PS14	Non-Motile
PS16	Non-Motile
NF1	Non-Motile
NF4	Non-Motile
NF8	Non-Motile

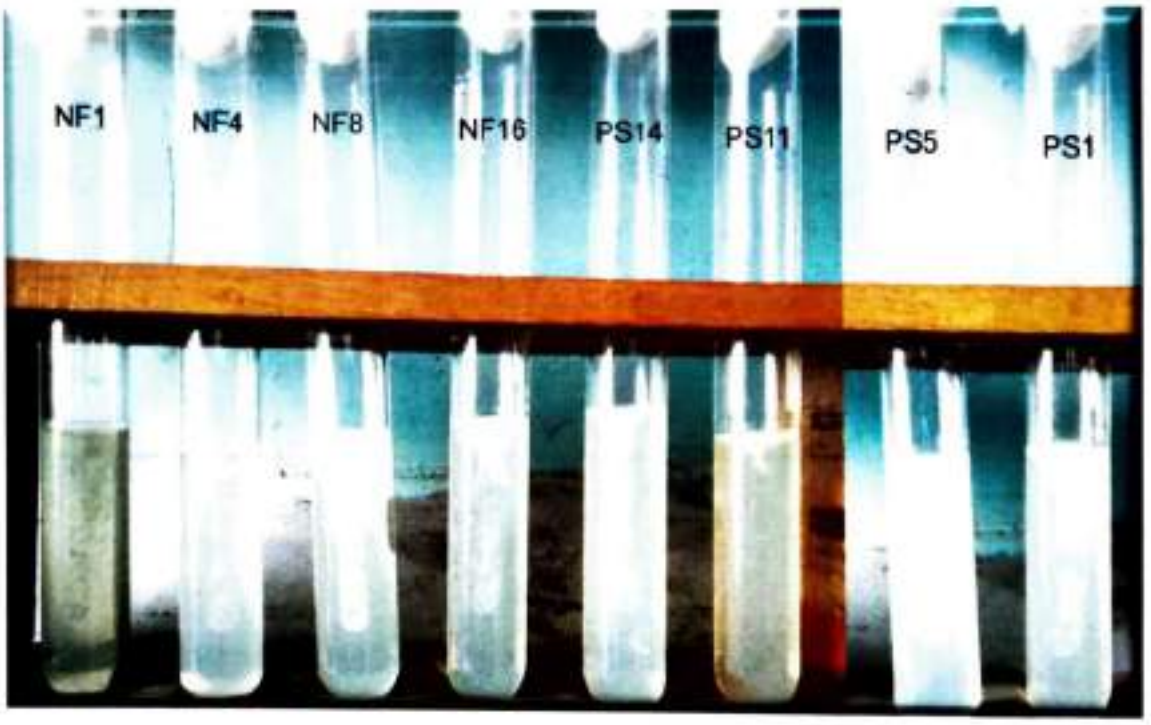


Fig. 4 shows Motility Test: All isolates are non-motile in nature.

❖ **Biochemical Characteristics of samples:**

- Catalase Test

SAMPLE	RESULTS
PS1	Positive (+++ve)
PS2	Positive (+++ve)
PS3	Positive (+++ve)
PS4	Positive (+ve)
PS6	Positive (+++ve)
NF1	Positive (+ve)
NF4	Positive (+++ve)
NF8	Positive (+++ve)

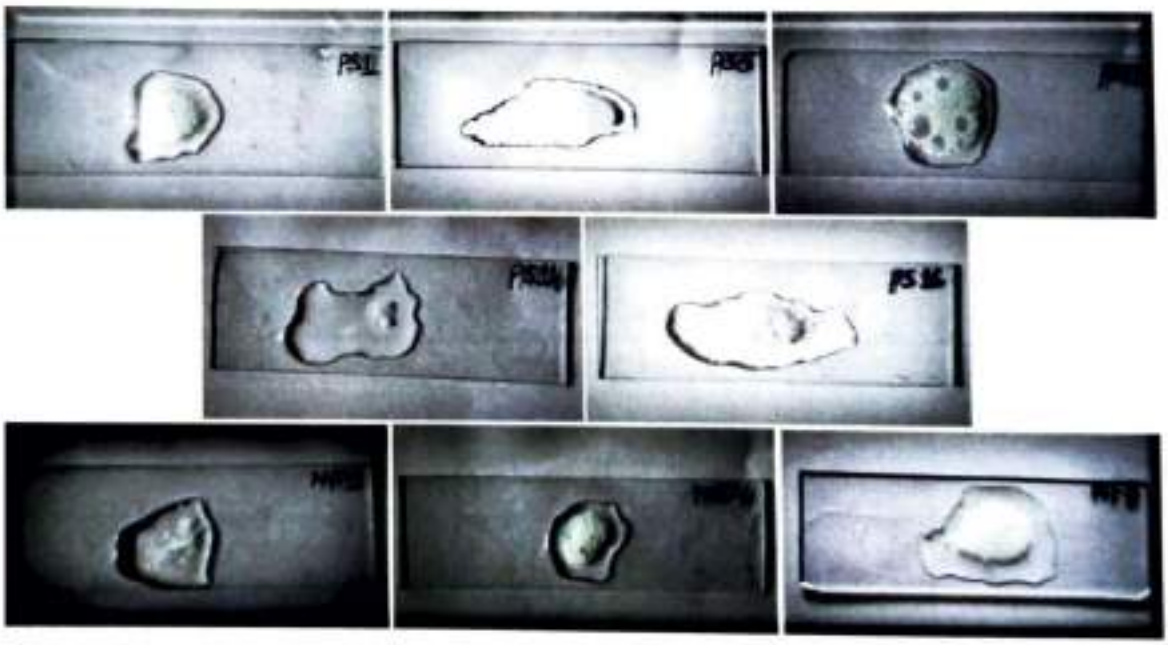


Fig. 5 shows Catalase Test: All isolates are catalase positive in nature.

- Oxidase Test-

SAMPLE	RESULTS
PS1	Positive (+++ve)
PS5	Positive (+++ve)
PS11	Negative (-ve)
PS14	Positive (+ve)
PS16	Positive (++ve)
NF1	Positive (+ve)
NF4	Positive (++ve)
NF8	Positive (+++ve)

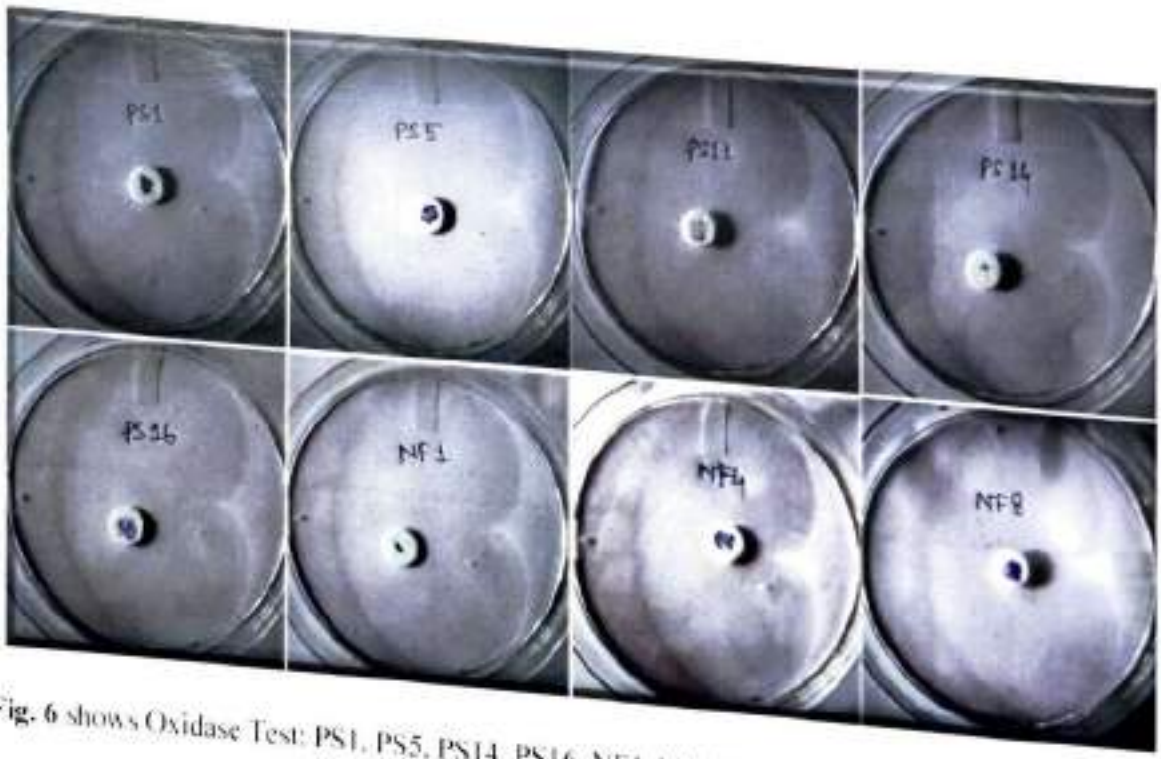


Fig. 6 shows Oxidase Test: PS1, PS5, PS14, PS16, NF1, NF4 & NF8 are oxidase positive and PS11 is oxidase negative.

- IMViC Test-

- **Indole (I) Test----**

SAMPLE	RESULTS
PS1	Negative (-ve)
PS5	Negative (-ve)
PS11	Negative (-ve)
PS14	Negative (-ve)
PS16	Negative (-ve)
NF1	Negative (-ve)
NF4	Positive (+ve)
NF8	Positive (+ve)

Fig. 7 shows Indole Test: NF4 & NF8 are indole positive and PS1, PS5, PS11, PS14, PS16 & NF1 are indole negative.

- **Methyl Red (MR) Test**

SAMPLE	RESULTS
PS1	Negative (-ve)
PS5	Negative (-ve)
PS11	Negative (-ve)
PS14	Positive (+ve)
PS16	Negative (-ve)
NF1	Negative (-ve)
NF4	Positive (+ve)
NF8	Negative (-ve)

Fig. 8 shows Methyl Red Test: PS14 & NF4 are MR positive and PS1, PS5, PS11, PS16, NF1 & NF8 are MR negative.

- Voges Proskauer (VP) test

SAMPLE	RESULTS
PS1	Negative (-ve)
PS5	Negative (-ve)
PS11	Negative (-ve)
PS14	Negative (-ve)
PS16	Negative (-ve)
NF1	Positive (+ve)
NF4	Positive (+ve)
NF8	Positive (+ve)

Fig. 9 shows Voges Proskauer Test: NF1, NF4 & NF8 are VP positive and PS1, PS5, PS11, PS14 & PS16 are VP negative.

SAMPLE	RESULTS
PS1	Positive (+ve)
PS5	Positive (+ve)
PS11	Positive (+ve)
PS14	Negative (-ve)
PS16	Positive (+ve)
NF1	Negative (-ve)
NF4	Positive (+ve)
NF8	Positive (+ve)

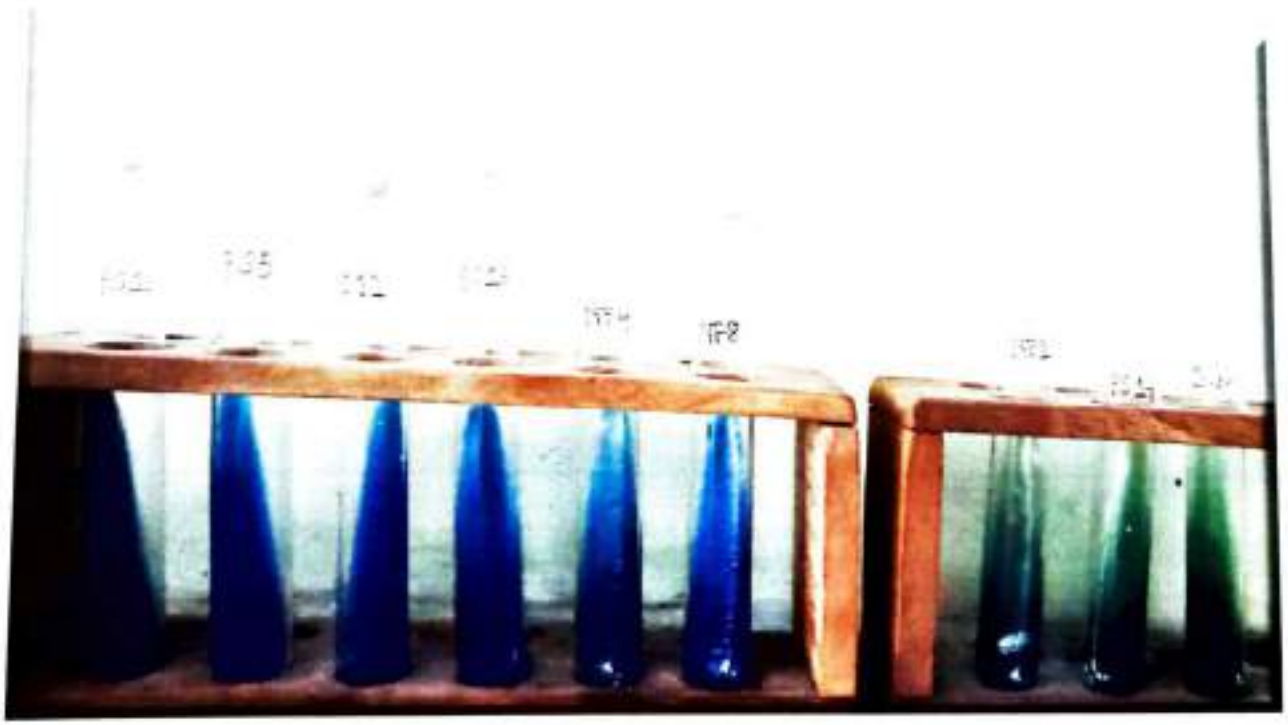


Fig. 10 shows Citrate Test: PS1, PS5, PS11, PS16, NF4 & NF8 are citrate positive and PS14 & NF1 are citrate negative.

- Amylase Test-

SAMPLE	RESULTS
PS1	Negative (-ve)
PS5	Negative (-ve)
PS11	Negative (-ve)
PS14	Negative (-ve)
PS16	Negative (-ve)
NF1	Positive (+ve)
NF4	Negative (-ve)
NF8	Negative (-ve)

Fig. 11 shows Amylase Test: NF1 is amylase positive and PS1, PS5, PS11, PS14, PS16, NF4 & NF8 are amylase negative.

- Protease Test-

SAMPLE	RESULTS
PS1	Negative (-ve)
PS5	Negative (-ve)
PS11	Negative (-ve)
PS14	Positive (+ve)
PS16	Negative (-ve)
NF1	Positive (+++ve)
NF4	Negative (-ve)
NF8	Negative (-ve)

Fig. 12 shows Protease Test: PS14 & NF1 are protease positive and PS1, PS5, PS11, PS16, NF4 & NF8 are protease negative.

- Nitrate Reduction

SAMPLE	RESULTS
[illegible]	Positive (++ve)
[illegible]	Positive (++ve)
[illegible]	Positive (+ve)
[illegible]	Positive (+++ve)
[illegible]	Positive (+ve)
[illegible]	Positive (+++ve)
[illegible]	Positive (+ve)
[illegible]	Positive (+ve)

Fig. 13 shows Nitrogen Reduction Test: All isolates are nitrate reductase positive in nature.

- Triple Sugar Iron (TSI)

SAMPLE	RESULTS
PS1	Yellow butt and red slant
PS5	Yellow butt and red slant
PS11	Yellow butt and red slant
PS14	Red butt and slant
PS16	Yellow butt and red slant
NF1	Yellow butt and red slant
NF4	Yellow butt and red slant
NF8	Yellow butt and slant

Fig. 14 shows Triple Sugar Iron Test: PS1, PS5, PS11, PS16, NF1 & NF4 show yellow butt & red slant, PS14 shows both red butt & slant and NF8 shows both yellow butt & slant.

- Carbohydrate Fermentation Test

- **Glucose Test----**

SAMPLE	ACID PRODUCTION	GAS FORMATION
PS1	Negative (-ve)	Negative (-ve)
PS5	Negative (-ve)	Negative (-ve)
PS11	Negative (-ve)	Negative (-ve)
PS14	Negative (-ve)	Negative (-ve)
PS16	Negative (-ve)	Negative (-ve)
NF1	Negative (-ve)	Negative (-ve)
NF4	Negative (-ve)	Negative (-ve)
NF8	Negative (-ve)	Negative (-ve)

Fig. 15 shows Glucose Fermentation Test: No isolates produce any acid and can't form any gas.

SAMPLE	ACID PRODUCTION	GAS FORMATION
PS1	Negative (-ve)	Negative (-ve)
PS5	Negative (-ve)	Negative (-ve)
PS11	Negative (-ve)	Negative (-ve)
PS14	Negative (-ve)	Negative (-ve)
PS16	Negative (-ve)	Negative (-ve)
NF1	Negative (-ve)	Negative (-ve)
NF4	Negative (-ve)	Negative (-ve)
NF8	Negative (-ve)	Negative (-ve)

Fig. 16 shows Lactose Fermentation Test: No isolates produce any acid and can't form any gas.

- **Sucrose Test----**

SAMPLE	ACID PRODUCTION	GAS FORMATION
PS1	Negative (-ve)	Negative (-ve)
PS5	Positive (+ve)	Positive (+ve)
PS11	Positive (+ve)	Positive (+ve)
PS14	Negative (-ve)	Negative (-ve)
PS16	Positive (+ve)	Positive (+ve)
NF1	Negative (-ve)	Negative (-ve)
NF4	Positive (+ve)	Positive (+ve)
NF8	Negative (-ve)	Negative (-ve)

Fig. 17 shows Sucrose Fermentation Test: PS5, PS11, PS16 & NF4 are producing acid and can form gas but PS1, PS14, NF1 & NF8 can't produce any acid and form any gas.

- **Mannitol Test---**

SAMPLE	ACID PRODUCTION	GAS FORMATION
PS1	Negative (-ve)	Negative (-ve)
PS5	Negative (-ve)	Negative (-ve)
PS11	Negative (-ve)	Negative (-ve)
PS14	Negative (-ve)	Negative (-ve)
PS16	Negative (-ve)	Negative (-ve)
NF1	Negative (-ve)	Negative (-ve)
NF4	Negative (-ve)	Negative (-ve)
NF8	Negative (-ve)	Negative (-ve)

Fig. 18 shows Mannitol Fermentation Test: No isolates produce any acid and can't form any gas.

- Urease Test-

SAMPLE	RESULTS
PS1	Negative (-ve)
PS5	Negative (-ve)
PS11	Negative (-ve)
PS14	Negative (-ve)
PS16	Negative (-ve)
NF1	Negative (-ve)
NF4	Negative (-ve)
NF8	Negative (-ve)

Fig. 19 shows Urease Test: All isolates are urease negative in nature.

SAMPLE	RESULTS
PS1	Positive (+ve)
PS5	Positive (+ve)
PS11	Positive (+ve)
PS14	Positive (++ve)
PS16	Negative (-ve)
NF1	Negative (-ve)
NF4	Negative (-ve)
NF8	Negative (-ve)

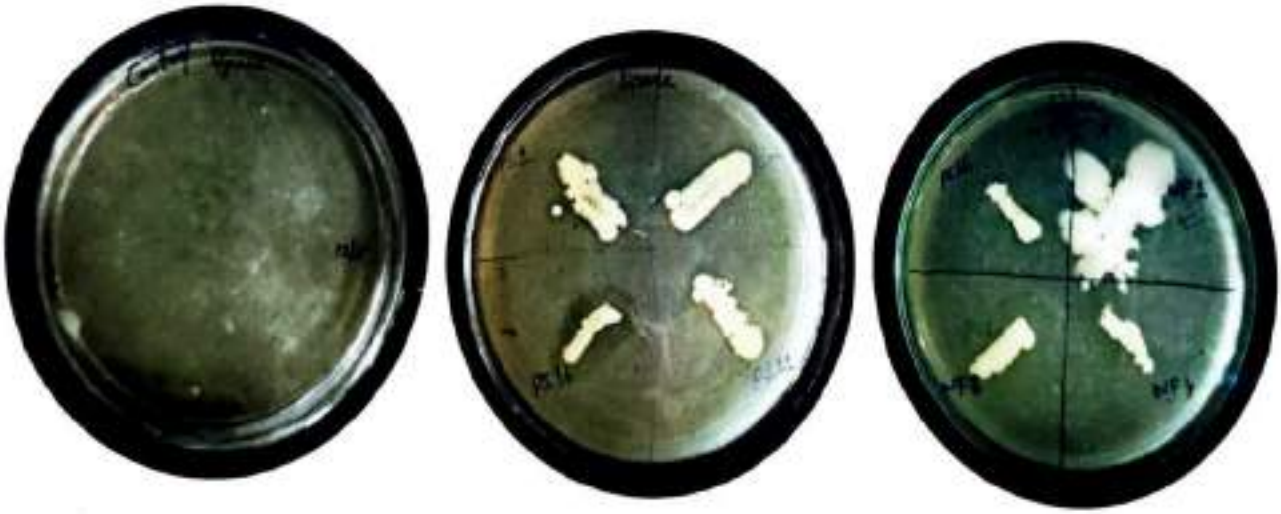
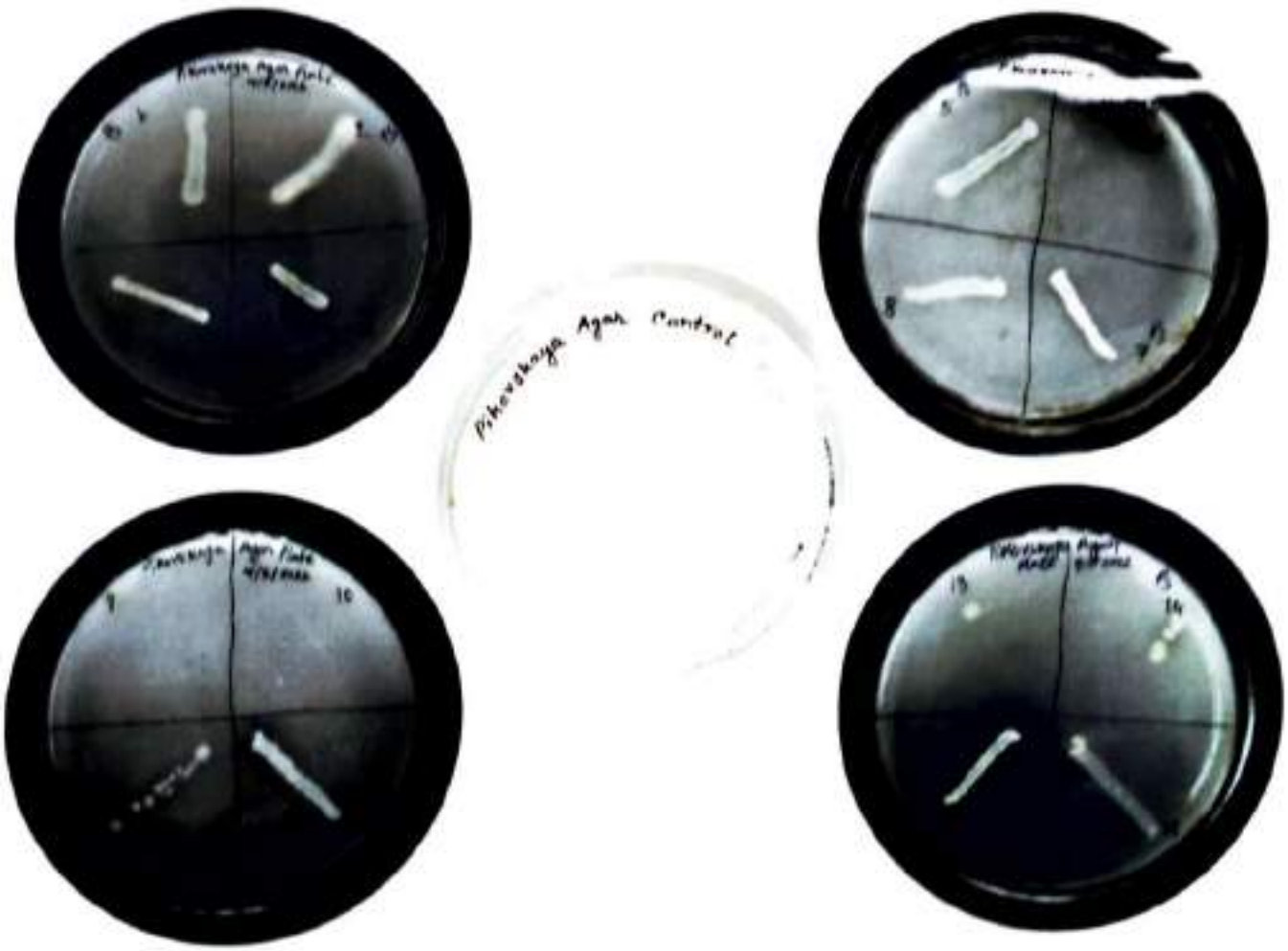


Fig. 20 shows Lipase Test: PS1, PS5, PS11 & PS14 are lipase positive and PS16, NF1, NF4 & NF8 are lipase negative in nature.

- Phosphate (P) Solubilizing Assay

SAMPLE	RESULTS
PS1	Positive (+ve)
PS5	Positive (+ve)
PS11	Positive (+ve)
PS14	Positive (+ve)
PS16	Positive (+ve)
NF1	Positive (+ve)
NF4	Positive (+ve)
NF8	Positive (+ve)



- Zinc (Zn) Solubilizing Activity

SAMPLE	RESULTS
PS1	Negative (-ve)
PS5	Negative (-ve)
PS11	Negative (-ve)
PS14	Negative (-ve)
PS16	Negative (-ve)
NF1	Negative (-ve)
NF4	Negative (-ve)
NF8	Negative (-ve)

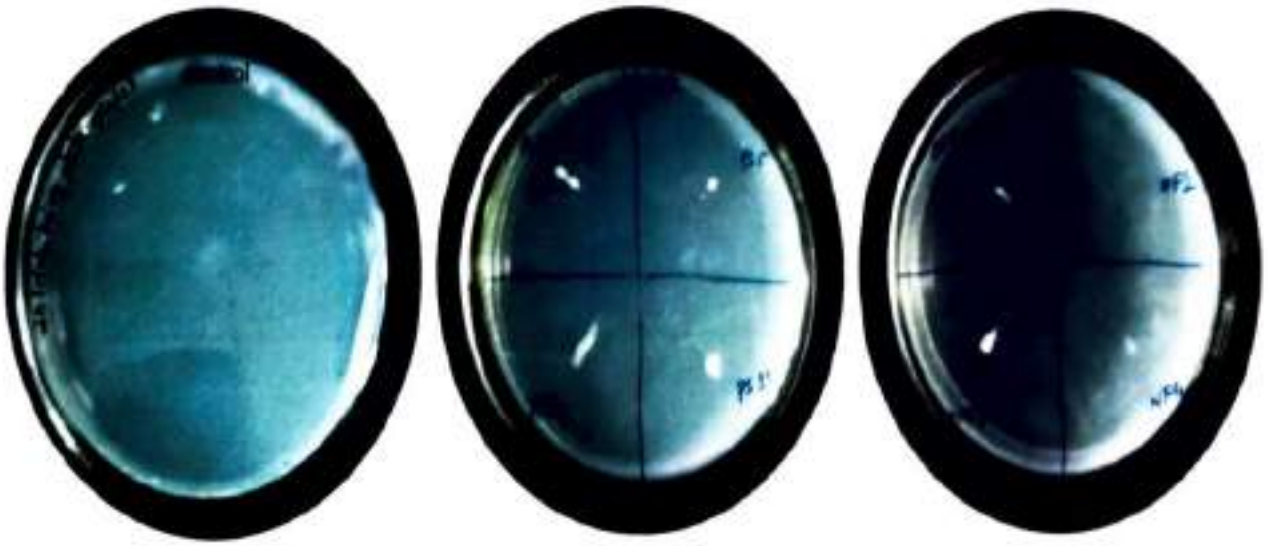


Fig. 22 shows Zinc Solubilizing Ability: All isolates can't solubilize zinc.

SAMPLE	RESULTS
PS1	Positive (+ve)
PS5	Positive (+ve)
PS11	Positive (+ve)
PS14	Positive (+ve)
PS16	Positive (+ve)
NF1	Positive (++ve)
NF4	Positive (++ve)
NF8	Positive (++ve)

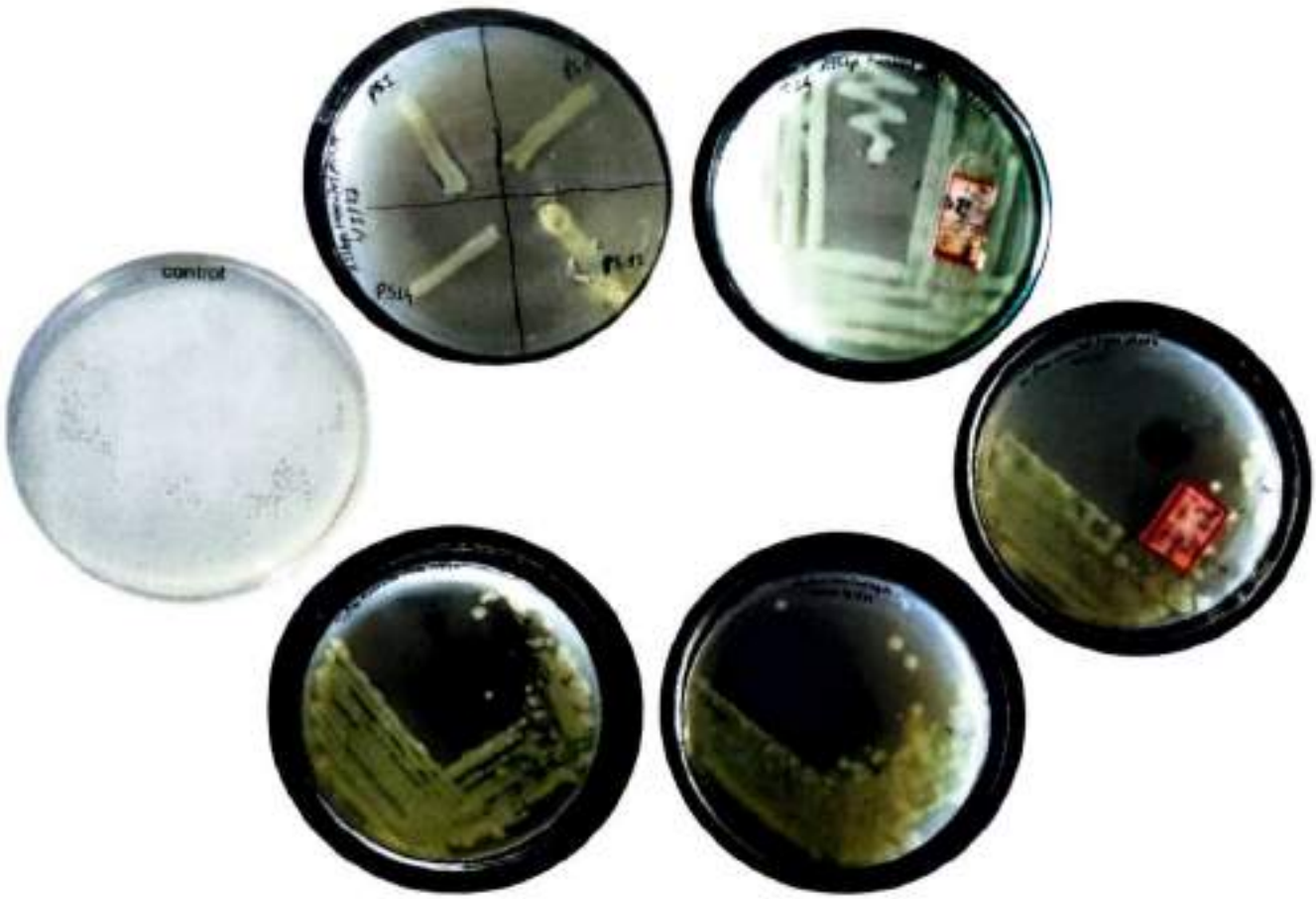


Fig. 23 shows Nitrogen Fixation Ability: All isolates are able to fix nitrogen.

- **Indole Acetic Acid (IAA) Production Test-**

SAMPLE	RESULTS
PS1	Positive (+++ve)
PS5	Positive (+++ve)
PS11	Positive (+++ve)
PS14	Negative (-ve)
PS16	Positive (+++ve)
NF1	Negative (-ve)
NF4	Positive (+++ve)
NF8	Positive (++ve)

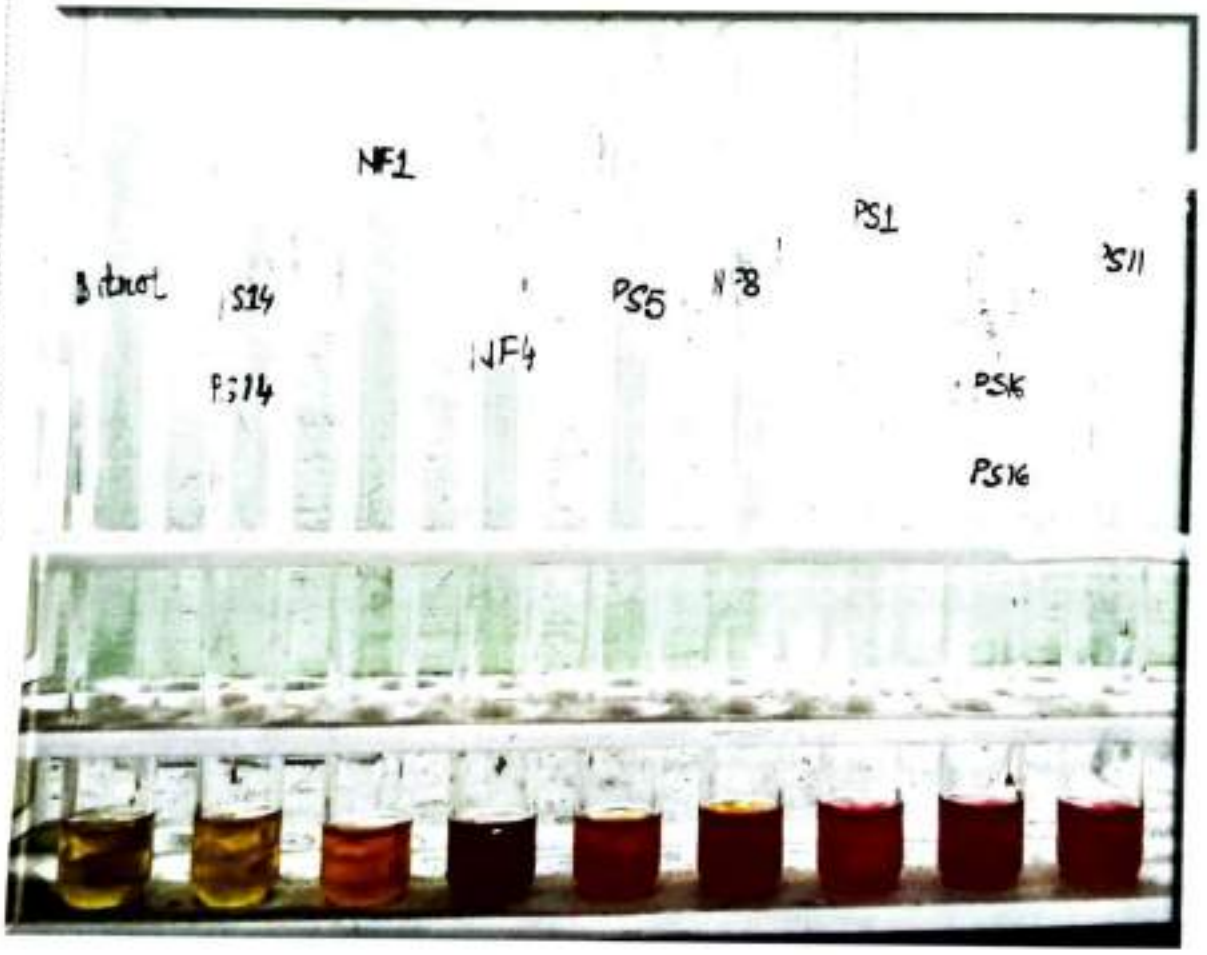


Fig. 24 shows Indole Acetic Acid Production Test: PS1, PS5, PS11, PS16, NF4 & NF8 are IAA positive and PS14 & NF1 are IAA negative.

- **Ammonia (NH_3) Production Test**

SAMPLE	RESULTS
PS1	Positive (+ve)
PS5	Positive (+ve)
PS11	Positive (+ve)
PS14	Positive (+ve)
PS16	Positive (+ve)
NF1	Positive (+ve)
NF4	Positive (+ve)
NF8	Positive (+ve)

Fig. 25 shows Ammonia Production Test: All isolates are able to produce ammonia.

- Hydrogen Cyanide (HCN) Formation Test

SAMPLE	RESULTS
PS1	Negative (-ve)
PS5	Negative (-ve)
PS11	Positive (+ve)
PS14	Positive (+ve)
PS16	Negative (-ve)
NF1	Negative (-ve)
NF4	Positive (+ve)
NF8	Negative (-ve)

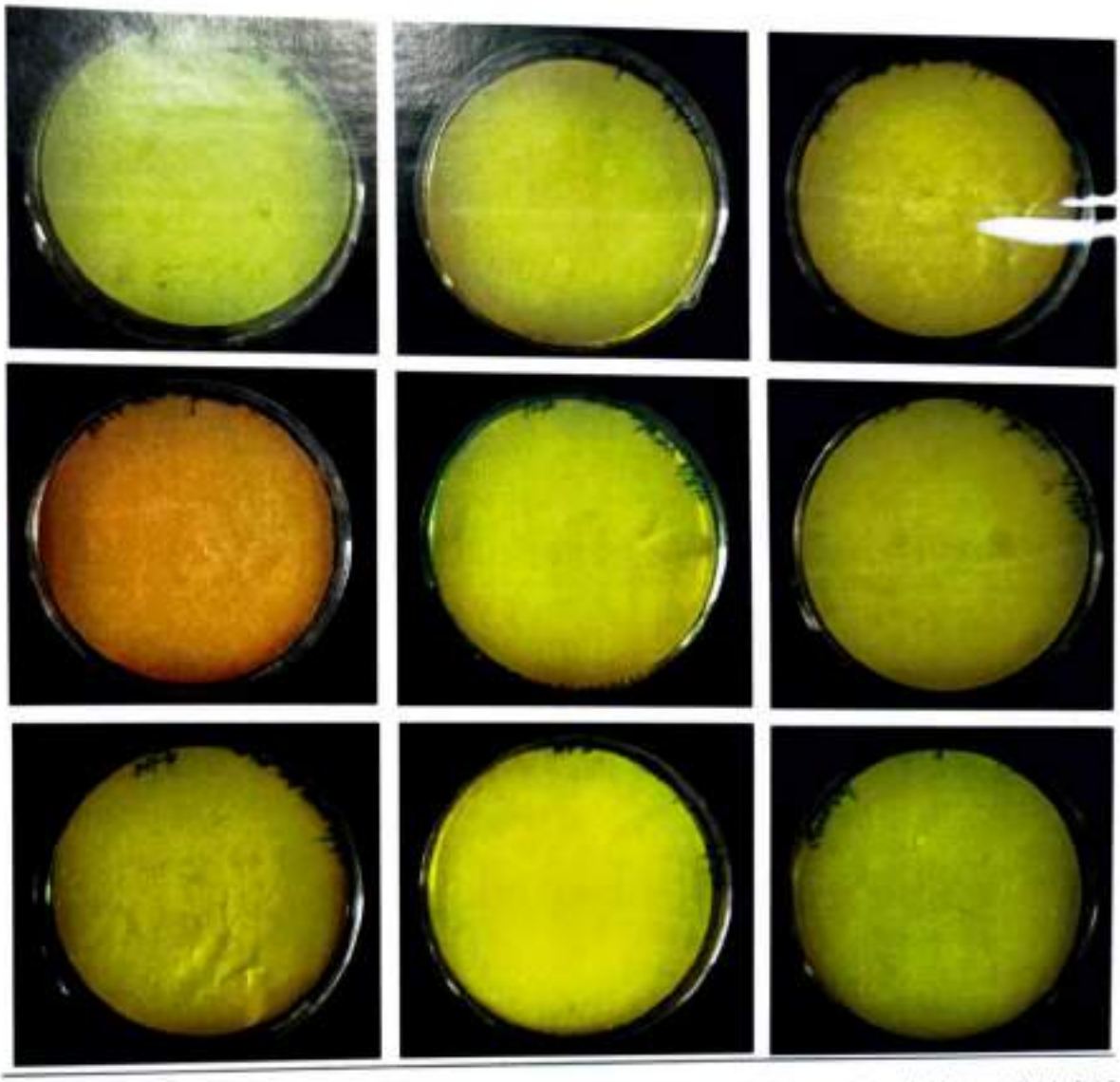


Fig. 26 shows Hydrogen Cyanide Formation Test: PS11, PS14 & NF4 can form HCN and PS1, PS5, PS16, NF1 & NF8 can't form HCN.

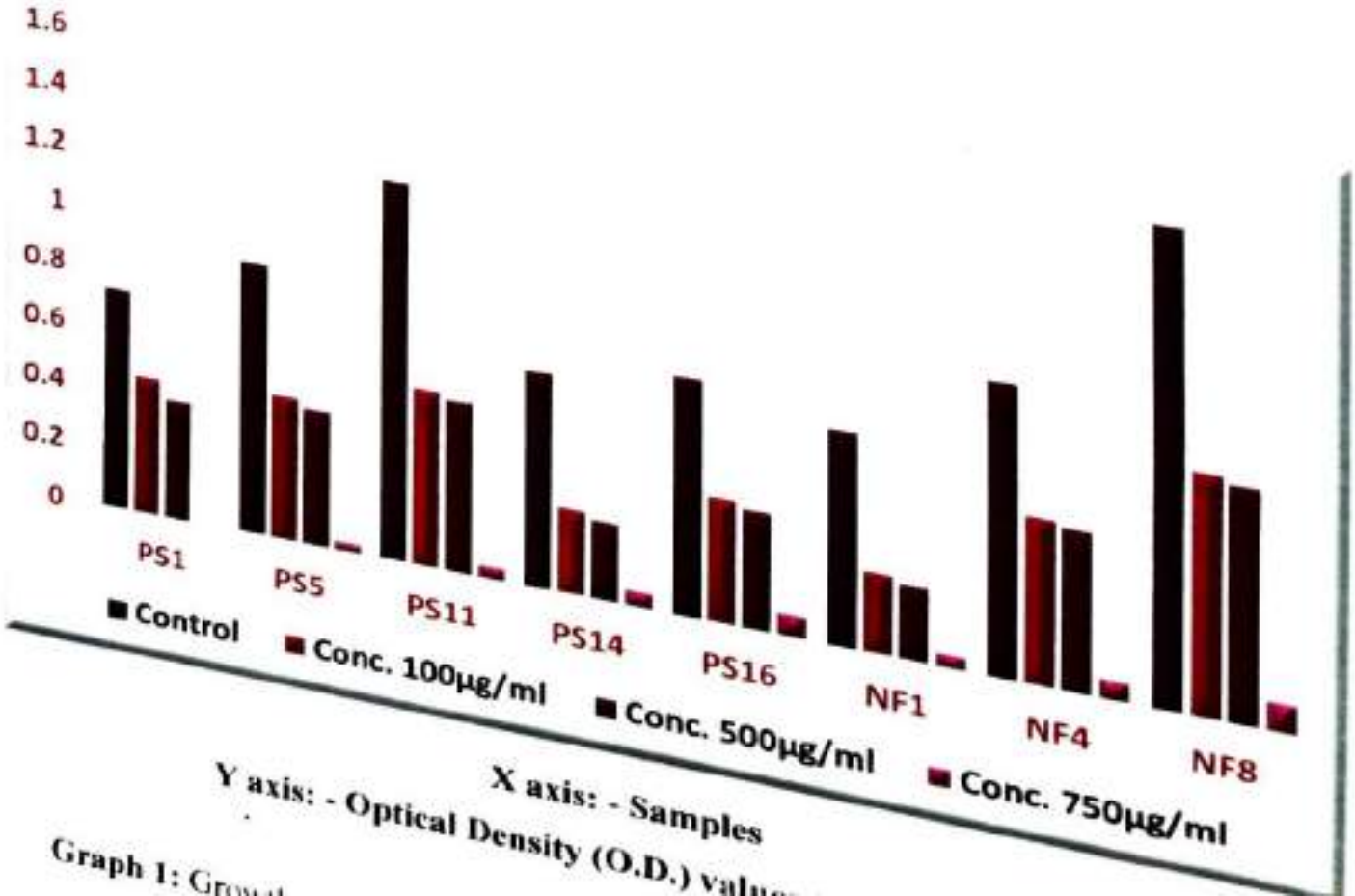
SAMPLE	RESULTS
PS1	Positive (+ve)
PS5	Negative (-ve)
PS11	Negative (-ve)
PS14	Negative (-ve)
PS16	Positive (+ve)
NF1	Positive (++ve)
NF4	Negative (-ve)
NF8	Positive (+ve)

Fig. 27 shows Siderophore Formation Test: PS1, PS16, NF1 & NF8 can form siderophore and PS5, PS11, PS14 & NF4 can't form siderophore.

- Heavy Metal Resistance (HMR) Capacity -

- Lead Nitrate [Pb $(NO_3)_2$] ----

SAMPLE	O.D. VALUE WITHOUT HEAVY METAL	O.D. VALUE IN 100µg/ml	O.D. VALUE IN 500µg/ml	O.D. VALUE IN 750µg/ml
PS1	0.73	0.45	0.39	0
PS5	0.89	0.47	0.44	0.03
PS11	1.23	0.57	0.55	0.04
PS14	0.69	0.27	0.25	0.05
PS16	0.75	0.39	0.37	0.07
NF1	0.67	0.25	0.23	0.04
NF4	0.90	0.51	0.49	0.06
NF8	1.45	0.73	0.71	0.09



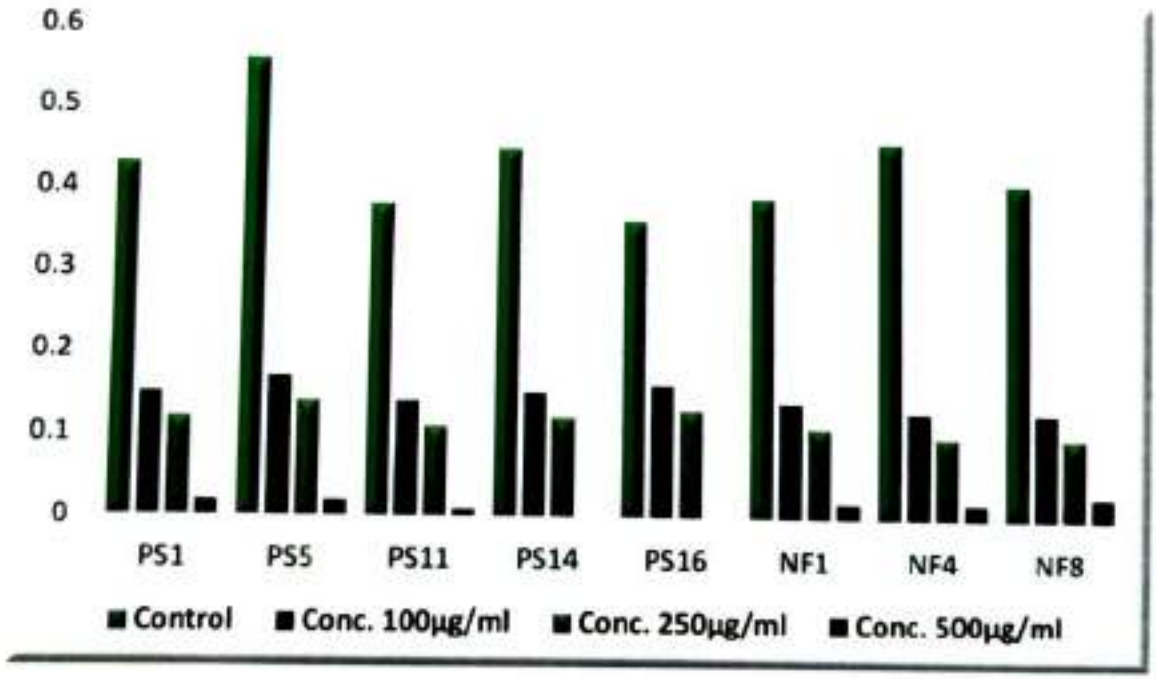
X axis: - Samples

Y axis: - Optical Density (O.D.) value

Graph 1: Growth

- **Cadmium Sulphate [$CdSO_4$] ----**

SAMPLE	O.D. VALUE WITHOUT HEAVY METAL	O.D. VALUE IN 100µg/ml	O.D. VALUE IN 250µg/ml	O.D. VALUE IN 500µg/ml
PS1	0.43	0.15	0.12	0.02
PS5	0.56	0.17	0.14	0.02
PS11	0.38	0.14	0.11	0.01
PS14	0.45	0.15	0.12	0
PS16	0.36	0.16	0.13	0
NF1	0.39	0.14	0.11	0.02
NF4	0.46	0.13	0.10	0.02
NF8	0.41	0.13	0.10	0.03



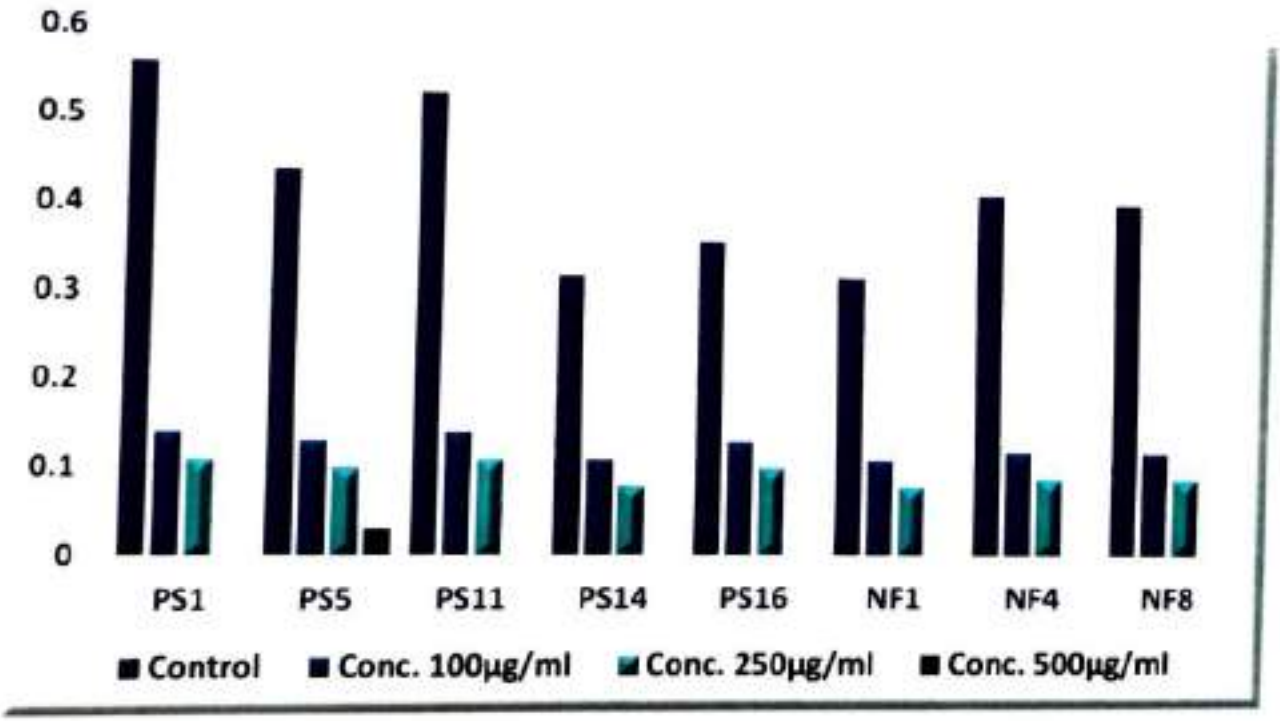
X axis: - Samples

Y axis: - Optical Density (O.D.) values taken in 600nm

Graph 2: Growth of isolates in presence of different concentration of cadmium sulphate.

- Mercuric Chloride [$HgCl_2$] —

SAMPLE	O.D. VALUE WITHOUT HEAVY METAL	O.D. VALUE IN 100µg/ml	O.D. VALUE IN 250µg/ml	O.D. VALUE IN 500µg/ml
PS1	0.56	0.14	0.11	0
PS5	0.44	0.13	0.10	0.03
PS11	0.53	0.14	0.11	0
PS14	0.32	0.11	0.08	0
PS16	0.36	0.13	0.10	0
NF1	0.32	0.11	0.08	0
NF4	0.42	0.12	0.09	0
NF8	0.41	0.12	0.09	0



X axis: - Samples

Y axis: - Optical Density (O.D.) values taken in 600nm

Graph 3: Growth of isolates in presence of different concentration of mercuric chloride.

Antifungal Activity Determination:

SAMPLE	RESULTS
PS1	Negative (-ve)
PS5	Negative (-ve)
PS11	Negative (-ve)
PS14	Negative (-ve)
PS16	Negative (-ve)
NF1	Positive (+ve)
NF4	Negative (-ve)
NF8	Negative (-ve)

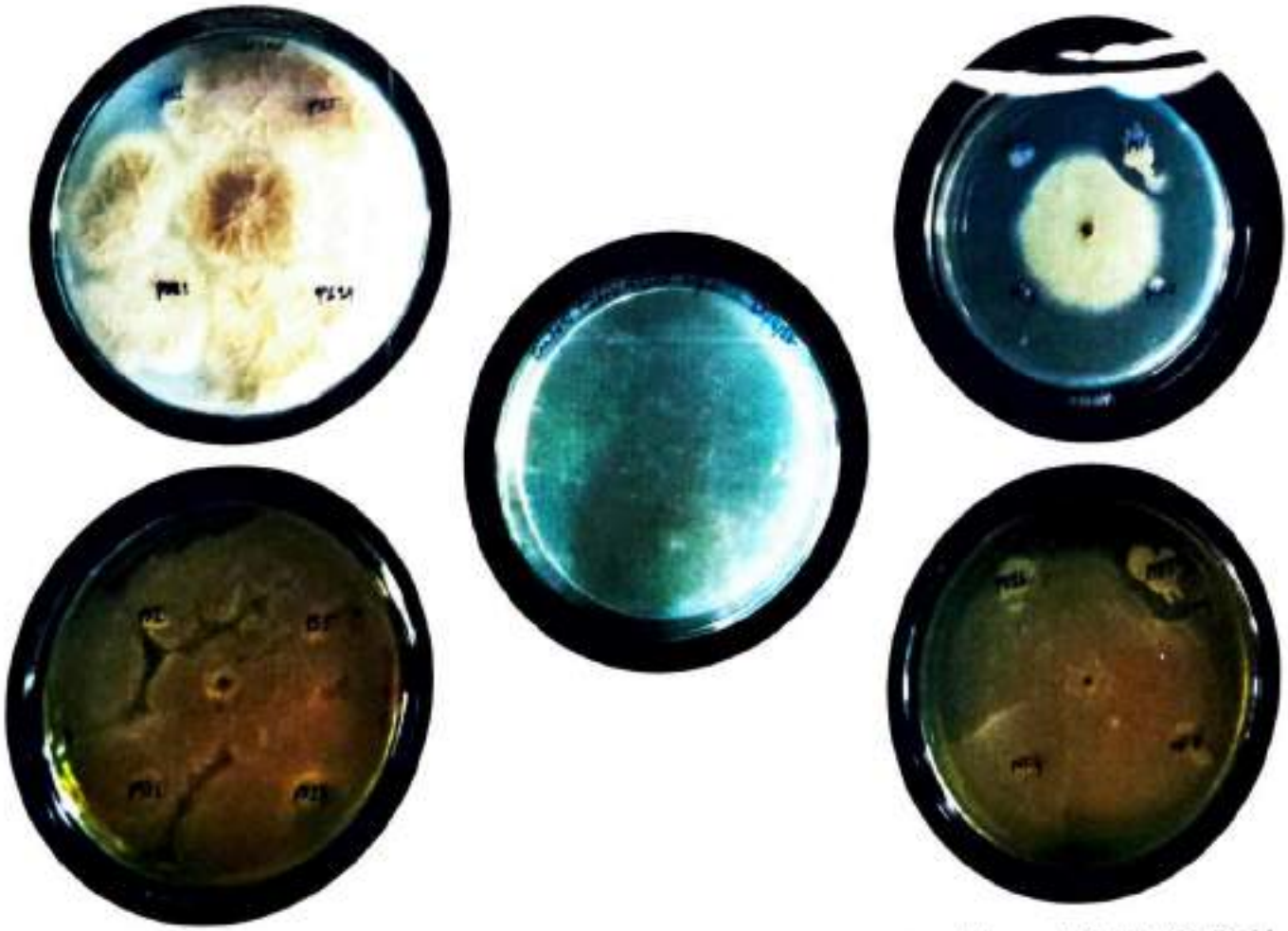


Fig. 28 shows Antifungal Activity Determination: NF1 has antifungal activity and PS1, PS5, PS11, PS14, PS16, NF4 & NF8 don't have any antifungal activity.

DISCUSSION: ~

PGPR colonizing the surface or inner part of roots play important beneficial roles that directly or indirectly influence plant growth and development. In this study, 8 PGPR classified as PS and NF were isolated from the rhizosphere of potato plant. Several different mechanisms have been suggested for similar observations using other PGPR strains. PGPR might indirectly enhance seed germination and vigor index by reducing the incidence of seed microflora, which can be detrimental to plant growth. The amylase hydrolyzes the starch into metabolizable sugars, which provide the energy for growth of roots and shoots in germinating seedlings. All the strains possessed several plant growths promoting traits as well as anti-fungal activity. This reveals the potential of these strains for bio-fertilizer applications and commercial use as bio-control agents in the field. However, from the estimation of PGPR potential to a bio-fertilizer application, it requires a long way of greenhouse experiments with pot filled with different type of soils and finally, field experiments to find out the optimum formulation for the inoculum. Thus, the inoculants can perform close to its optimum potential. [19, 20]

As additional insights are gained regarding Phosphate Solubilizing Bacteria (PSB) and the mechanisms that they use, there is every reason to believe that the use of PSB as bio-fertilizer will likely improve their use, as effective and important components in the establishment of sustainable soil management system. Thus, the employment of PSB as bio-fertilizer is an option that can increase food production without imposing any health hazard, and at the same time conserve the environment. In the present study, a total of 5 PSB were screened from the reclaimed soil. These PSB isolates may possess the potential to be applied in improving soil recovery and crop production. A higher P-solubility capacity of them was observed in the pikovskaya agar medium compare to other bacteria and was therefore chosen from further investigation. It also indicated that isolates achieved an optimal P-solubilizing rate in the following culture condition: 2 days incubation, pH 7 and temperature 30°C. In addition to P-solubilization activity, PSB was reported to secrete phytohormones that might have an influence on root growth. Soluble organic acids could serve as a source of carbon for micro-organisms and subsequently affect the rhizosphere microbial environment, as well as plant growth. The plant root development was affected by the application of PSB. P is an important factor limiting agricultural production. Large amounts of P fertilizer added to soil not only increase the cost of agriculture but also cause environmental problems and are not conducive to the sustainable development of agriculture. PSB have a great ability to transform insoluble P in the soil into an available form and have great application prospects for eco-agriculture. The secretion of organic acids and chelation are major P-solubilization mechanisms of PSB. The presence of many PSB in the soil is an important index of effective promotion of crop growth and sustainable agricultural development. We isolated strains from the rhizosphere of potato plant as a bacterium with a strong potential to solubilize phosphate. Based

on the analysis of the P-solubilization of 5 isolates, the P-solubilization was the highest at 48 h. The content of nitrogen and organic matter also increased, indicating that PSB could improve soil nutrients. In addition, P promotes the development of roots. The ability of PSB survival in the soil is the key to determining its role in the natural environment and is an important basis on which to evaluate PSB colonization ability in the rhizosphere. Indeed, the reason why the number of PSB was higher than that of other microorganisms in soils is that the presence of the host plant may have greatly affected the survival of PSB that were attracted to the rhizosphere of the growing seedlings. There is much research on the colonization of biocontrol bacteria of plants, but little on PSB. However, there has not been a long-term study of its ability to survive in the rhizosphere. These phenomena indicated that isolates are able to enter the plant tissue, and establish interactions with the host to promote plant growth. PSB can turn insoluble P into soluble P and promote plant growth. We selected the 5 strains because, among all the other PSB isolated, they produced the largest halos. Pikovskaya agar media was found the most efficient phosphate solubilizing medium. These bacterial isolates were screened for their ability to produce plant growth regulator, IAA. It seems probable that plant growth substances produced by PSBs improve plant growth by their direct effects on metabolic processes. However, since they induce proliferation of lateral roots and root hairs and thus increase nutrient absorbing surfaces, this may lead to greater rates of nutrient absorption. [16, 17]

Zinc (Zn) is one of the key constituents of plants and is very crucial for their growth and development. Soil bacteria are reported to exert a beneficial effect on plant growth and excellent alternate to chemical fertilizers due to their zinc-solubilizing potential. These bacteria are of great interest as they have been proposed as inoculants for agriculture. In this present study, efforts were made to isolate zinc-solubilizing bacteria. These bacterial strains were screened on Bunt and Rovira medium amended with insoluble zinc salts to check their ability to solubilize zinc [11]. The ability of bacteria to solubilize the 2 inorganic zinc compounds might be interesting as zinc is known to occur in soils in discrete chemical forms varying in their solubility and availability to crop plants. With the advantage of zinc solubilization to make micronutrients availability to plants, plant growth promoting characterization was also necessary to confirm plant growth promotion [13, 14]. This increase in plant growth parameters could be related to ability of Zn-solubilizing bacteria to solubilize the nutrient and make them available to the plants. This was expected as with increase available Zn in soil could result in higher zinc in the plants. This increase in zinc content in root and shoot is also supported in many studies with the use of PGPRs. PGPRs have been reported to overcome nutrient deficiency in many crops. Zinc solubilization by using PGPR is relatively a newer approach and has lots of unexplored efficient zinc-solubilizing strains. The solubilization potential may be vary for different Zn salts. Rhizobacteria play a vital role in environmental cycling processes such as solubilization of metals into soluble forms that are suitable for plant uptake. [18, 21]

Nitrogen (N_2) fixation may also play a role in plant growth promotion. This suggest that while N_2 fixation may be an important mechanism of plant growth promotion, there may also be alternate mechanisms, like hormonal interactions and nutrient uptake or pathogen suppression which might be more pronounced than the contribution of nitrogen fixation. More than 20 different colonies were isolated during the study of rhizosphere associated bacterial species. In continuation of the work towards development of growth promoting inoculants we found that there is a large number of free-living nitrogen fixers associated with the rhizosphere of the potato plant. The nitrogen fixing bacteria were preliminary screened on Ashby's nitrogen free Mannitol agar media. 3 colonies are selected on basis of colony characteristics and marked as NF. Moreover, the isolate was capable of producing siderophore which positively influences the plant growth parameter. Since, iron is one of requisite micronutrients required by number of plants for their proper functioning. Various PGP bacteria have been reported which enhances the planting value parameters in crops.

Ammonia (NH_3) has the highest N – content of any commercial fertilizer and nutrient for plant growth. It can be converted to NO_2^- and NO_3^- by bacteria and then used by plants. NH_3 production by PGPR is one of the essential traits linked to plant growth promotion. It supplies N_2 to the plants and thereby promote root and shoot elongation and their biomass. All of the eight isolates produce ammonia, detected by Nessler's reagent. [22]

One of the most commonly reported mechanism of PGP rhizobacteria is the production of phytohormones such as IAA. Some selected isolates in this study produce IAA. Similar studies have shown that IAA production is very common among PGPR. In fact, many isolates in this study produced higher IAA. This is an important mechanism of plant growth promotion because IAA promotes root development and uptake of nutrients. It has long been proposed that phytohormones act to coordinate demand and acquisition of nitrogen. IAA can also be a contributing factor in increasing plant growth and biomass. IAA production by PGP bacteria, besides increasing cell elongation also encourages the growth of root hair and lateral roots in plants, resulting in availability of ample nutrients and water plant. As an effective plant growth hormone, higher production of IAA is corresponding with enhanced seed germination and seedling growth parameters. IAA in certain quantity is adequate to produce physio-morphological changes in the young seedlings. Our findings of IAA production in PSB isolates agree with those of other researchers. The Salkowski colorimetric assay is traditionally used to detect the bacterial production of IAA. The effect of IAA on plant root development has been well characterized and more recently studies have demonstrated that it could function as a signal molecule between bacteria in a community as well as between bacteria and the plant host. [12]

One of the most well-known groups of PGPR are siderophore producing bacteria. Siderophores are secreted under iron-depleted conditions and their production is inhibited by iron due to suppression of

the siderophore related gene expressions. We found that great amounts of siderophores were synthesized in lack of iron. The siderophore producing bacteria reduced the toxicity of metals and improved the phytoremediation. Siderophore treatment increase the growth of plants in the biological assay, growing on two different soils, one highly contaminated with heavy metals and the second strongly alkaline soil. It expected iron from the host and provide this essential metal nutrient to microbes. The 4 siderophore producing bacterial strains taken in the study. Formation of orange-colored zone around the bacterial colonies were observed which indicated siderophore production by bacterial strains. CAS agar method can only give rough idea and is not a perfect method for quantification of siderophore production [15].

Hydrogen cyanide (HCN) is often act as a biocontrol agent in the agricultural system based on its significant toxicity against phytopathogenic agents to enhance growth production. It is also used in the chelation of metal ions and is indirectly involved in facilitating phosphate availability. Three isolates are able to produce HCN.

Heavy metals generally produce common toxic effects on plants, such as low biomass accumulation, chlorosis, inhibition of growth and photosynthesis, altered water balance and nutrient assimilation which ultimately cause plant death. But some PGPR has able to tolerate these toxic heavy metals and prevent the effects of them and helped in the growth development of plants. Here we use three heavy metals, salts of Pb, Hg and Cd. All the eight isolates are partially tolerant up to 500µg/ml in presence of $Pb(NO_3)_2$. But they are not significantly tolerant to salts of Hg and Cd.

Many fungal species cause different types of infection and disease in host plant but the some PGPR prevent the infection of the fungal species by their own mechanisms. They have some activity against some fungal species which are harmful for the growth development of plant. Here we determine the antifungal activity of all the eight isolates against some fungi isolated from air, bread and orange. Among the eight isolates only NF1 exhibit some antifungal activity against fungi isolated air and bread.

From the above discussion, we understand that all the eight isolates are helped in plant growth promotion in various ways. Some of them can produce phytohormones like IAA, iron chelating proteins like siderophores, various metabolites like NH_3 and HCN, solubilize phosphates and can fix atmospheric nitrogen. Some of them have antifungal activity. So, it can be concluded that these isolates have the potential to use as biofertilizers and biocontrol agents.

CONCLUSION: ~

On the basis of morphological study, it has been concluded that all the eight bacterial isolates are Gram negative bacteria. PS1, PS11, PS16, NF4, NF8 are coccoid and PS5, PS14, NF1 are rod shaped. PS1, PS11 occur in cluster, PS5, majority of PS14, NF1 occur singly and PS16, NF4, NF8 have chain like arrangement. None of them produce capsule as no halo zone is appeared against dark background. All the isolates are non-motile because growth is confined to the stab-line, with sharply defined margins and leaving the surrounding medium clearly transparent.

On the basis of biochemical study, it was noticed that in catalase test, most of the strains (PS1, PS5, PS11, PS16, NF4, NF8) produced high amount of copious bubbles and some (PS14, NF1) are produced lower amount bubbles. In oxidase test, PS1, PS5, and NF8 produced high level; PS16 and NF4 produced moderate level; PS14 and NF1 produced low level of oxidase enzyme respectively because of purple-blue color occurred where as PS11 doesn't produce any oxidase enzyme. In IMViC test, only NF4 and NF8 are indole positive because they produced a cherry red colored ring in the reagent layer on top of the tryptone broth medium within seconds of adding the Kovac's reagent. PS14 and NF4 are MR positive as they produced a distinct red color in glucose-peptone broth upon addition of methyl red. NF1 and NF8 formed a pink-red color in glucose-peptone broth upon addition of Baritt's reagent consequently are VP positive. PS1, PS5, PS11, PS16, NF4 and NF8 utilized citrate be... the Simmon's citrate agar medium changed from green to intense blue. In amylase test, only NF1 produced amylase enzyme because a clear zone around the line of growth after addition of iodine solution. In protease test, NF1 produced high level, PS14 produced moderate level of protease enzyme respectively because of a clear zone around the line of growth. In nitrate reduction test, all the isolates are nitrate reductase positive. In Triple Sugar Iron (TSI) test, some strains (PS1, PS5, PS11, PS16, NF1 and NF4) produce yellow butt and red slant, NF8 gave both yellow butt and slant and PS14 gave both red butt and slant. In carbohydrate fermentation test, none of the bacterial isolates produce acid and gas in glucose, lactose and mannitol but PS5, PS11, PS16 and NF4 produced both acid and gas in sucrose test as the medium turns yellow and gas bubbles appeared in the Durham's tube. In Urease test, none of the isolates produce urease enzyme. In Lipase test, PS14 produced moderate level where as PS1, PS5, PS11 produced low level of lipase enzyme respectively because of a clear zone around the line of growth.

On the basis of PGPR characterization study, it was observed that some isolates (PS1, PS5, PS11, PS14 and PS16) are highly and NF1, NF4 and NF8 are moderately potential to solubilize phosphate because clear zone appeared around the growth in Pikovskaya agar medium. None of the bacterial isolates can solubilize zinc. All of them are able to fix atmospheric nitrogen. In Indole Acetic Acid (IAA) production test, PS1, PS5, PS11, PS16 and NF4 produced high and NF8 produced moderate

amount of IAA respectively because of color changes from a light pink to reddish after addition of Salkowski reagent. In Ammonia production (NH_3) test, all strains are formed ammonia because of the change in color from brown to yellow after addition of the Nessler's reagent. In Hydrogen Cyanide (HCN) formation test, PS13 formed high where as PS11 and NF4 formed moderate amount of HCN respectively because the color of filter paper soaked in picric acid changed from orange to red after incubation. In Heavy Metal Resistance (HMR) determination test, all of the isolates are partially tolerating lead nitrate [$Pb(NO_3)_2$] up to 500µg/ml concentration but not significantly tolerate to cadmium sulphate [$CdSO_4$] and mercuric chloride [$HgCl_2$]. In antifungal activity determination test, only NF1 has this type of property because a zone of inhibition is observed around the fungal colony.

So, it can be concluded that these isolates have the potential to use as biofertilizers and biocontrol agents.

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ISOLATION AND CHARACTERIZATION OF PROTEASE PRODUCING BACTERIA FROM SOIL

SUBMITTED FOR THE PARTIAL FULFILMENT of THE AWARD of
M.Sc. IN MICROBIOLOGY

By:

DIPANKAR MONDAL

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Post Graduate Department of Microbiology
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Dated .....

To Whom It May Concern

This is to certify that Mr. Dipankar Mondal of PG Department of Microbiology, Bidhannagar College, has successfully completed his project entitled "**Isolation and characterization of Protease Producing bacteria from soil**" under my supervision from 16th March 2023 to 31st May 2023.

I wish him every success in his future.

Sourav Pakrashi 05.07.2023

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My parents, who supported me throughout my life and provided me with endless motivation for my project work, deserve my gratitude.

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ABSTRACT

The following work aimed to isolate, study and identify protease producing bacteria in soil samples collected from slaughterhouses in South 24 Parganas, West Bengal. Four bacterial isolates with protease producing capability were selected from skimmed milk agar plates. Isolate 1 and 4 were identified as Gram positive, isolate 2 and 3 were Gram negative. Isolate 3 showed highest protease enzyme activity, when cell lysate was incubated with 1% casein solution, whereas Isolate 4 showed lowest activity. Corresponding biochemical assays of the isolates were performed. Isolate 2 and 4 exhibited in-vitro resistance to high concentration of kanamycin, isolate 3 showed resistance against ampicillin. All these isolates were susceptible to tetracycline, polymyxin-B, streptomycin, neomycin and gentamycin. All these isolates displayed high tolerance to nickel and zinc, but all of them were highly sensitive to mercury. Isolate 2 and 4 exhibited tolerances to high concentrations of lead, as high as 500 and 1000 ppm, respectively. Isolate 3 were moderately tolerant against cadmium. These protease producing isolates might be of great potential for application in food, pharmaceutical, agricultural and textile industries.

KEYWORDS: - Bacteria, Protease, Soil, Antibiotic, Enzyme, Metal

1. INTRODUCTION

The cells of all living organisms contain a biochemical substance known as enzymes, which have the ability to catalyse or accelerate biochemical reactions. Enzymes exhibit superior catalytic efficiency and specificity compared to catalysts of chemical origin, making them highly valuable in a range of industries including food, agriculture, and pharmaceuticals **(Pires-Cabral et al. 2010)**. Researchers worldwide have been interested in isolating and purifying enzymes from various environmental sources. Among these enzymes, proteases hold a significant position as they were the first to be produced in large quantities and currently constitute approximately two-thirds of the total enzymes used today **(Raval et al. 2014)**. Proteases, in particular, play a crucial role in the physiological function of nearly all life forms on Earth. While proteases can be produced by various organisms, those derived from microbial sources such as fungi and bacteria are preferred due to their genetic manipulability and adaptability **(Masi et al. 2014; Tiwari et al. 2015)**. Proteases are hydrolytic enzymes that break down proteins into smaller polypeptides or amino acids **(Gupta et al. 2002; Verma et al. 2011)**. Depending upon optimum pH, proteases are of three types- acidic proteases, neutral proteases and alkaline proteases. Most prominent protease-producing bacteria are Pseudomonas sp., Bacillus sp., Aeromonas sp., Staphylococcus sp. **(Saha et al. 2011; Masi et al. 2017b)**.

Antibiotics and resistance

Antibiotics are compounds that can inhibit bacterial growth by killing them or suppressing their growth. Different antibiotics have different target site in a cell such as cell wall, plasma membrane, Metabolism machinery, replication, transcription and translation. Cell wall targeting antibiotics such as penicillin, ampicillin kills mainly gram-positive bacteria, whereas cell membrane targeting antibiotics such as polymyxin-B is effective against gram- negative bacteria. Antibiotics that target cellular machinery such as streptomycin, rifampicin, tetracycline are effective against both gram-positive as well as gram-negative bacteria. But upon prolonged exposure bacteria can develop resistance against certain antibiotics. Antibiotic resistance genes do not exist in bacterial chromosome, they are always integrated by extrachromosal plasmid or by means of phage. Bacteria can develop different resistance mechanisms against antibiotics such as, chemical modification of antibiotics, energy driven efflux pumps, mutation in target proteins, modification in cellular machinery, etc **(Darby et al, 2022).**

Heavy metals and metal tolerance

Heavy metals are metallic elements that occur naturally and have a high atomic weight **(Tchounwou, 2012)**. These metals can interact with cellular components such as cell

membrane, DNA, cellular enzymes, etc **(Wang S, 2001)**. Toxic heavy metals include mercury, arsenic, cadmium, lead, nickel, zinc, chromium, etc. These metals can be highly toxic even at trace amounts. Despite their high toxicity certain bacteria can adapt and thrive in their presence. Five different mechanisms have been identified and studied which imparts the resistance property to the organisms, viz., creation of an extracellular barrier, efflux pumps, extracellular sequestration, intracellular sequestration and reduction of metals from toxic into a non-toxic form. The genes responsible for these attributes can be found in both chromosomal as well as extrachromosomal genetic elements. The process of horizontal gene transfer has been linked with these resistance properties **(Ianeva, 2009)**.

2. METHODOLOGY

There were no ethics or permission requirements for this in-vitro study because it did not include any human subjects or animal data. Specific authorizations were not needed for this work because the little amount of soil sample did not harm the terrestrial environment, wildlife, anyone's personal property or include any endangered or protected species.

2.1 Sample collection:

Soil samples were collected from a slaughterhouse located in Pirnagar, Shyamnagar, South 24 Parganas, West Bengal, India in 20th March, 2023. The temperature was 35°C and humidity was 32%. Soil sample were collected from 5 cm below the soil surface and kept in a sterile sample collection vial and taken to the laboratory as early as possible.

Fig 1. Soil sample in sterile vial

Fig 2. Soil sample isolation site

Fig 3. Sample collection in sterile sample vial

2.2 Isolation of protease producing bacteria:

5 gram of soil sample was dissolved in 45 ml of sterile 0.9% sodium chloride solution. Then serial dilution was made up to 10^{-4}. 0.1 ml from all four dilutions were spread onto the surface of sterile skimmed milk agar plate (**Appendix- A.5)** containing casein as a protein source. The plates were then incubated for 24 hours at 37°C.

2.3 Selection of test isolates and pure culture preparation:

Four bacterial colonies with surrounding prominent hollow zones were selected for further experiments and they were sub cultured on four different sterile skimmed milk agar plates **(Appendix- A.5)**. These plates were considered as pure master plates throughout the experiments and necessary subculture were done as per requirements.

2.4 Morphological characteristics:

The pure plates were thoroughly observed for colony characteristics viz. size, texture, shape, colour, elevation, etc. the morphological characteristics viz. shape, size, appearance, gram character of the isolates was studied using Gram's staining **(Appendix- B.5)**

2.5 Biochemical assays:

2.5.1 Optimization of Carbon and Nitrogen source:

Carbon source optimization assay were performed with a synthetic media **(Appendix- A.2)** contained all growth requirements where the carbon sources were varied accordingly. For carbon source optimization six different carbon sources were used- dextrose, lactose, maltose, sucrose, mannitol and starch.

Nitrogen source optimization assay were performed with a synthetic media **(Appendix- A.3)** contained all growth ingredients where the different nitrogen sources were varied accordingly. For nitrogen source optimization five different nitrogen sources were used- ammonium sulphate, ammonium chloride, ammonium ferrous sulphate, ammonium di-hydrogen phosphate, sodium nitrate.

All four isolates were inoculated in these media and incubated at 37°C for 24 hours. The optical density was measured at 600 nm using a spectrophotometer.

2.5.2 pH optima for growth:

The pH in which the growth of an organism is highest known as optimum pH. To optimize pH isolates were grown in synthetic liquid media **(Appendix- A.4)** having different pH. Nine pH levels were prepared (pH 3-11) with 1N NaOH and 1N HCl. Isolates were inoculated and incubated overnight at 37°C. Optical density was measured at 600 nm using a spectrophotometer.

2.5.3 Growth curve:

Generally, bacterial growth consists of four distinct phases, Lag phase, where bacteria acclimatize to a new environment. Log phase, where bacteria divides and cell number increases

exponentially. This phase is followed by a Stationary phase, where the ratio of mortality and natality is equal, due to the exhaustion of nutrients in the culture media. And finally, Death phase, where the rate of mortality becomes higher than natality. To observe the progression of growth, isolates were inoculated in nutrient broth media **(Appendix-A.1)** inside nephelometric flask. The growth was measured at every one-hour interval by measuring the optical density at 600 nm.

2.5.4 Enzyme assays:

2.5.4.1 Amylase test:

Amylase test or starch hydrolysis test is a biochemical test performed to detect the ability of microorganisms to produce the enzyme α-Amylase (**EC 3.2.1.1).** Starch is a very large molecule so to utilize it as a carbon source microorganism first need to break it down into small molecules. Organisms that produce α-Amylase are able to hydrolyse starch by breaking the glycosidic linkages between the sugar subunits into maltose and glucose. This test is performed by streaking the isolates onto the surface of starch agar medium (**Appendix-A.4)**. Organisms that able to produce amylase, degrade starch present in the medium. Starch degradation was detected by flooding the plates with Iodine solution after 24 hours of incubation at 37℃. Iodine binds with starch forming a complex known as starch iodide, giving the media a brown to blue appearance depending upon the concentration of Iodine. The colonies with hollow zones surrounded by darkened media were considered as a positive result.

2.5.4.2 Catalase test

Catalase test is performed to detect the ability of an organism to produce the enzyme catalase (**EC 1.11.1.6**). Catalase is an enzyme produced by microorganisms to degrade hydrogen peroxide, a reactive oxygen species, produced by microorganisms as metabolic by-products (**Alphonso et al, 2009)**. These products are toxic to microorganisms and might even cause cell lysis if not broken down to neutral products. Catalase contain haem and Mn^{2+} as cofactors. The reaction catalysed by catalase is, $2\ H_2O_2 \rightarrow O_2 + 2\ H_2O$. The production of catalase was detected by adding drops of hydrogen peroxide over bacterial cell smears. A rapid liberation of oxygen bubbles considered as a positive result, whereas no bubbles formation can be depicted as negative result.

2.5.4.3 Lipase test:

Lipase test is performed to detect the ability of a microorganism to produce the enzyme lipase **(EC 3.1.1.3).** Lipase breaks triglycerides into diglycerides then to monoglycerides and simple fatty acids **(Chandra et al, 2020)**. Lipase production was detected by growing the isolates on tributyrin agar media **(Appendix- A.11)** containing glyceryl tributyrate. Microorganism's ability to produce lipase can be detected by the presence of a clear zone around the colony after

overnight incubation at 37℃, regarded as positive result. Whereas the absence of such clear zone considered as negative result.

2.5.4.4 Oxidase test:

Oxidase test is performed to detect the ability of microorganisms to produce the enzyme cytochrome c oxidase **(EC 1.9.3.1)**. Cytochrome c oxidase is the enzyme participate in electron transport chain, where it receives electron from cytochrome c and transfers them to oxygen, producing water **(Michel et al, 1998)**. The presence of cytochrome c oxidase was detected by rubbing 24 hours fresh cultures onto the surface of oxidase disks **(Appendix-B.1)**. These discs were soaked tetramethyl-p-phenylenediamine dihydrochloride, which acts as a substrate for the enzyme and become oxidized to a deep purple compound. The presence of deep purple colour regarded as positive result; no colour change is indication of negative result.

2.5.4.5 Phosphate solubilization test:

This test is performed to detect the production of organic acid by microorganisms to solubilize inorganic phosphate as well as organic phosphate. To detect phosphate solubilization ability bacterial isolates were inoculated over the surface of a sterile Pikovskaya's agar media **(Appendix- A.6)** containing calcium phosphate, which gives a cloudy appearance to the media. Bacteria that able to produce organic acids can solubilize calcium phosphate and thus produce a clear zone around the colony. Bacterial colony without such clear zones around indicates negative result.

2.5.4.6 Triple Sugar Iron test:

In this test bacterial ability to ferment sugars and production of hydrogen sulphide is detected. The name triple sugar means that the medium contains three monosaccharide glucose, lactose and sucrose. Phenol red, sodium thiosulfate and present in the medium serves as indicators of acid production and H_2S production, respectively. Glucose, as simple sugar utilized first by bacteria as a result the entire media become acidic, phenol red shows yellow appearance in acidic condition. In the case of oxidative decarboxylation of peptone, the pH level rises to alkaline condition and the media becomes yellow. If the organism able to ferment lactose and sucrose too, then production of excessive amounts of acid turns the media yellow in colour. The butt of the tube becomes black due to the reduction of sodium thiosulfate to H_2S.

2.5.4.7 Urease test:

This test is performed to detect the production of enzyme urease **(EC 3.5.1.5)** by bacteria. In this test urea agar slants **(Appendix-A.13)** were used containing urea and phenol red as substrate and indicator, respectively. Bacteria that are able to produce urease, degrade urea to

produce ammonia and CO_2. Ammonia presents in the medium react with CO_2 and water to produce ammonium carbonate, which renders the medium alkaline. In alkaline pH phenol red turns deep red in colour, indicates a positive result. Whereas no change of colour indicates a negative result.

2.5.5 IMViC tests:

IMViC tests are an assay of four different tests to study bacterial ability to produce tryptophanase, acids, acetylmethylcarbinol and utilization of citrate by indole, methyl red, voges-proskauer and citrate test, respectively.

2.5.5.1 Indole test:

Indole test was performed to detect the ability of an organism to produce indole from tryptophan by the enzyme tryptophanase **(EC 4.1.99.1)**. Reductive deamination of tryptophan produces indole, pyruvic acid and ammonium. For the test tryptone broth **(Appendix- A.7)** was used containing tryptophan as a substrate. Isolates were inoculated and incubated overnight at 37°C. The presence of indole was detected by adding Kovac's reagent **(Appendix- B.2)**. Indole reacts with p-dimethylaminobenzaldehyde present in Kovac's reagent to form a pink coloured quinoidal compound. The coloured compound forms an insoluble layer on the top of the media due to the presence of amyl alcohol. The presence of pink colour indicates positive result, whereas no colour change indicates negative result.

2.5.5.2 Methyl red test:

Methyl red test was performed to determine the ability of an organism to produce acid end products from the fermentation of glucose. Glucose peptone broth **(Apendix-A.9)** were used in this test. Microorganisms convert glucose to pyruvic acid by glycolysis pathway, but some organism can further metabolize pyruvate via mixed acid fermentation producing lactic acid, acetic acid, formic acid, etc. The production of acid was detected by adding drops of methyl red **(Appendix- B.3)** indicator on the surface of the media. Due to the presence of acids methyl red appears red and the test indicates a positive result, whereas a yellow colouration refers negative result as methyl red appears yellow near neutral pH.

2.5.5.3 Voges-Proskauer test:

Voges-Proskauer test or VP test was performed to detect the ability of the microorganisms to produce neutral end products due to the fermentation of glucose. Glucose peptone broth **(Appendix-A.9)** was used in this test. Organisms produce pyruvic acid from glucose via glycolysis, some organisms can metabolize pyruvic acid to different acidic products via mixed acid fermentation. But some can further metabolize these acidic end products to neutral end

product, acetyl methyl carbinol or acetoin by decarboxylation of acids, which results in rising the pH of the medium towards neutrality. The production of acetoin was detected by using Baritt's reagent **(Appendix- B.4)** containing potassium hydroxide and α-naphthol. In the presence of KOH and α-naphthol, acetoin is converted to diacetyl, which reacts with guanidine groups associated with the amino acid arginine, that forms from the breakdown of peptone, to form a reddish-pink coloured product. The presence of reddish-pink colour indicates positive result, whereas its absence refers a negative result.

2.5.5.4 Citrate utilization test:

This test was performed to demonstrate the ability of an organism to utilize citrate as carbon source. Simmon's citrate agar medium **(Appendix-A.8)** was used in this test which contained citrate as a sole carbon source, ammonium hydrogen phosphate as a nitrogen source and bromothymol blue as pH indicator. The enzyme citrase produced by organisms degrade citrate to produce oxaloacetate and acetate. Oxaloacetate further converted to pyruvate and CO_2. CO_2 then reacts with ammonium salts to form ammonium carbonate, which increase the pH of the medium. As a result, the bromothymol blue present in the medium shifts from green to blue colour, indicates a positive result, where a green colouration of the media indicates negative result.

2.5.6 Protease activity assay:

To compare the activity of protease produced by the isolates, they were grown overnight in protease production media **(Appendix-A.10)**. Being an extracellular enzyme, protease is released into the media. Enzyme's activity was detected by mixing the cell lysate, containing enzyme with 1% casein solution and incubated for 15 minutes. Protease degrade casein to produce free amino acids, Tyrosine. The reaction was stopped by adding trichloroacetic acid. Then Folin-Ciocalteu's reagent was added and incubated. Optical density was measured at 660 nm using a spectrophotometer. The final concentration of tyrosine was determined from a previously prepared standard curve of tyrosine by same method. The activity of the enzyme was expressed as microgram per millilitre per minute ($\mu g.\ ml^{-1}.\ Min^{-1}$)

2.5.7 Antibiotic sensitivity assay:

For this study eight different antibiotics were used, Gentamicin, Chloramphenicol, Kanamycin, Neomycin, Streptomycin, Ampicillin, Tetracycline and Polymyxin-B. Nutrient broth **(Appendix- A.1)** tubes were used for this assay. Stock solutions were prepared at 5 mg/ml for each antibiotic. Different dilutions were made from 2 µg/ml to 125 µg/ml. Isolates were inoculated from freshly cultured nutrient broth media to the antibiotic tubes and incubated overnight at 37℃. Optical density was measured at 600 nm using a spectrophotometer. The

effect of increasing concentration antibiotics on growth was determined by comparing the culture tubes with control tubes without any antibiotics.

2.5.8 Heavy metal tolerance assay:

For this assay five different heavy metals were used, Nickel chloride, Zinc chloride, Lead nitrate, Mercury chloride and Cadmium sulphate. Stock solutions were prepared at 50000 ppm for each metal. Nutrient broth was used for this assay. Different concentrations were made from 25 ppm to 1000 ppm. Cells were inoculated from freshly cultured nutrient broth **(Appendix-A.1)** medium and incubated overnight at 37°C. Optical density was measured at 600 nm using a spectrophotometer. The effect of metals on growth was compared with control tubes without any heavy metal.

3. OBSERVATION AND RESULTS

3.1 Isolation of protease producing bacteria:

Fig 4. Protease producing bacteria with clear zones around the colonies.

Fig 5. Prominent protease producers can be seen in this figure.

3.2 Colony Characteristics:

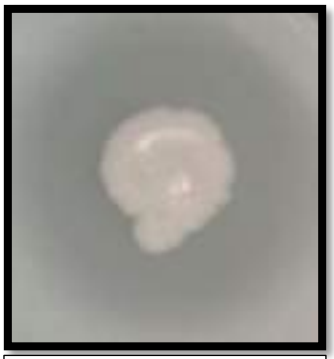


Fig 6. Isolate 1

Fig 7. Isolate 2

Fig 8. Isolate 3

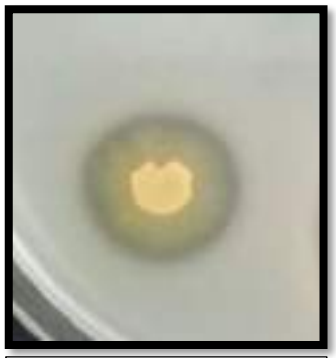


Fig 9. Isolate 4

Isolate	Colony Characteristics
1	White, creamy, undulate margin, slightly raised
2	White, slimy, smooth margin, elevated
3	White, shiny, slimy, lobate margin, convex
4	Yellow "Poached egg" appearance, undulate, flat

Table-1. Colony characteristics of isolates

3.3 Morphological characteristics:

Isolate	Morphology
1	Gram positive, Rod shaped, Singlet appearance
2	Gram negative, Rod shaped, In pair appearance
3	Gram negative, Spherical shaped, Small, Singlet appearance
4	Gram positive, Rod shaped, Small, Singlet appearance

Table- 2. Morphological characteristics of isolates

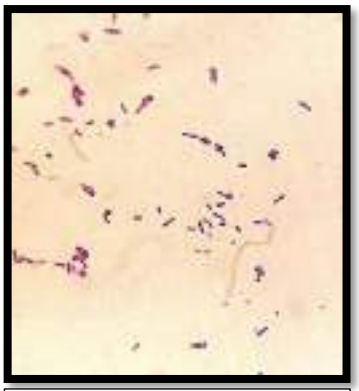


Fig 10. Isolate 1
Gram-Positive, Bacilli

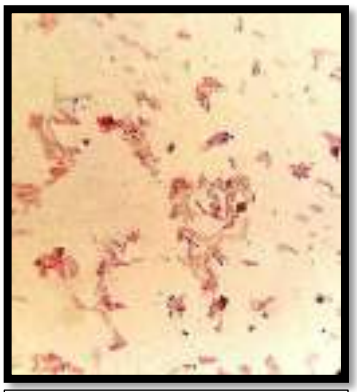


Fig 11. Isolate 2
Gram-Negative, Bacilli

Fig 12. Isolate 3
Gram-Negative, Cocci

Fig 13. Isolate 4
Gram Negative, Bacilli

3.4 Optimization of Carbon and Nitrogen source:

Isolate	Optimal Carbon source	Optimal Nitrogen source
1	Dextrose	Ammonium Chloride
2	Maltose	Ammonium Sulphate
3	Sucrose	Ammonium Chloride
4	Starch	Ammonium Sulphate

Table-3 Optimal Carbon and Nitrogen sources used by isolates for highest growth.

3.5 Optimization of pH for growth:

Isolate	Optimum pH for growth
1	**7**
2	**7**
3	**7**
4	**8**

Table-4 Optimum pH in which the growth was highest.

3.6 Protease activity assay:

Isolate	Activity of protease (µg/ml/min)
1	1.896
2	3.273
3	2.610
4	1.687

Table-5 Activity of protease produced by isolates. Activity was determined by measuring free tyrosine concentration by Folin assay at 660 nm and using a tyrosine standard curve.

3.7 Biochemical Assays:

Test	Isolate 1	Isolate 2	Isolate 3	Isolate 4
Amylase test	-	+	-	+++
Catalase test	+	-	+	+
Lipase test	-	-	-	-
Oxidase test	-	+++	-	+
Protease production test	+	+	+	+
Phosphate solubilization test	-	-	-	-
Triple Sugar Iron test	+	+	+	+
Urease test	-	-	-	-
Indole test	-	-	-	-
Methyl red test	-	-	++	+
Voges-Proskauer test	-	-	-	-
Citrate utilization test	-	-	+	+

Table-6 Observations of Biochemical assays. ("+" indicates positive results, number of "+" indicates intensity of positive results and "-" indicates negative results.)

3.8 Heavy metal tolerance assay:

Isolate	Nickel (ppm)	Zinc (ppm)	Lead (ppm)	Mercury (ppm)	Cadmium (ppm)
1	200	200	50	-	-
2	200	200	500	-	25
3	200	500	-	-	100
4	200	500	1000	-	50

Table-7 Heavy metal tolerance by isolates. All values were converted to parts per million unit, (1ppm= 1mg/L). Optical density was measured at 600 nm.

3.9 Antibiotic sensitivity assay:

Control for Isolate 1= 1.5

Calculated IC_{50}= 0.75

Antibiotics	Concentration at which IC_{50} obtained
Gentamicin	1 µg/ml
Chloramphenicol	5 µg/ml
Kanamycin	1 µg/ml
Neomycin	1 µg/ml
Streptomycin	2 µg/ml
Ampicillin	1 µg/ml
Tetracycline	1 µg/ml
Polymyxin-B	1 µg/ml

Table-8 Antibiotic sensitivity assay of isolate 1. Control tubes were made without antibiotics. IC_{50}, is the concentration of antibiotic in which growth was reduced to 50% of control tubes.

Control for isolate 2 =1.6

Calculated IC_{50}= 0.8

Antibiotics	Concentration at which IC_{50} obtained
Gentamicin	10 µg/ml
Chloramphenicol	20 µg/ml
Kanamycin	**125 µg/ml**
Neomycin	20 µg/ml
Streptomycin	10 µg/ml
Ampicillin	15 µg/ml
Tetracycline	2 µg/ml
Polymyxin-B	20 µg/ml

Table-9 Antibiotic sensitivity assay of Isolate 2. Control tubes were made without antibiotics. IC_{50}, is the concentration of antibiotic in which the growth was reduced to 50% of control tubes.

Control for Isolate 3 = 1.7

Calculated IC_{50} = 0.85

Antibiotics	Concentration at which IC_{50} obtained
Gentamicin	2 µg/ml
Chloramphenicol	25 µg/ml
Kanamycin	15 µg/ml
Neomycin	5 µg/ml
Streptomycin	2 µg/ml
Ampicillin	**50 µg/ml**
Tetracycline	2 µg/ml
Polymyxin-B	2 µg/ml

Table-10 Antibiotic sensitivity assay of Isolate 3. Control tubes were made without antibiotics. IC_{50}, is the concentration of antibiotic in which the growth was reduced to 50% of control tubes.

Control for Isolate 4 = 1.42

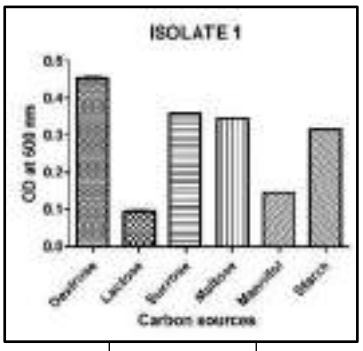
Calculated IC_{50} = 0.71

Antibiotics	Concentration at which IC_{50} obtained
Gentamicin	5 µg/ml
Chloramphenicol	5 µg/ml
Kanamycin	**100 µg/ml**
Neomycin	5 µg/ml
Streptomycin	10 µg/ml
Ampicillin	5 µg/ml
Tetracycline	2 µg/ml
Polymyxin-B	5 µg/ml

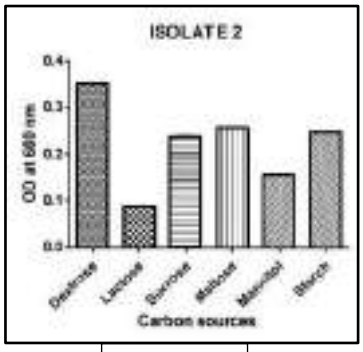
Table- 11 Antibiotic sensitivity assay of Isolate 4. Control tubes were made without antibiotics. IC_{50}, is the concentration of antibiotic in which the growth was reduced to 50% of control tubes

4. EXPERIMENTAL DATA

4.1 Optimization of Carbon and Nitrogen source:



Graph 1A



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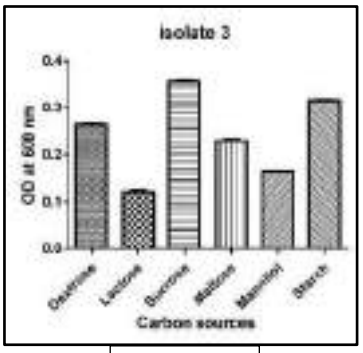
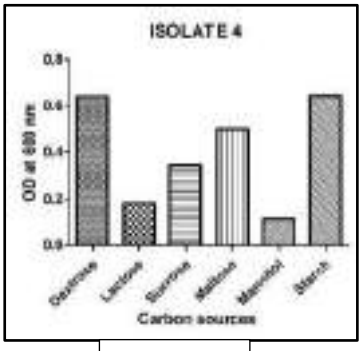


Graph 1C



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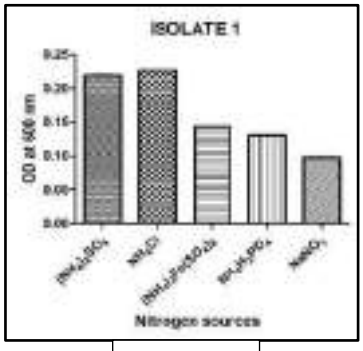
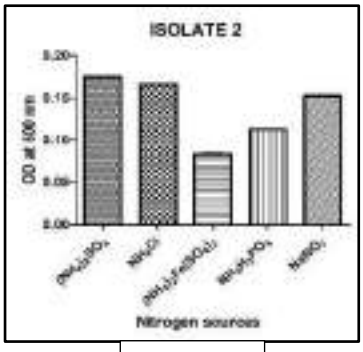
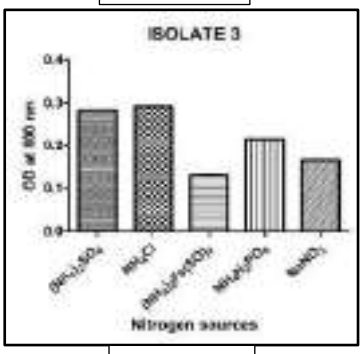


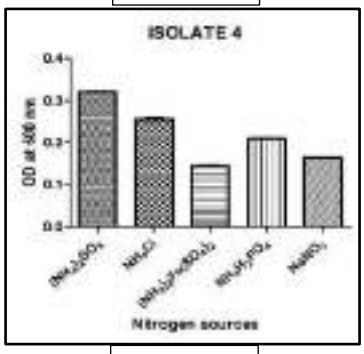
Graph 2A



Graph 2B



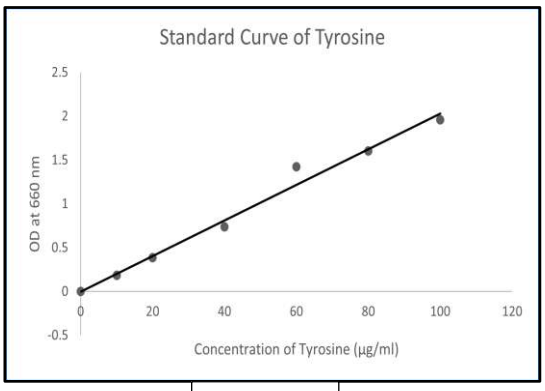
Graph 2C



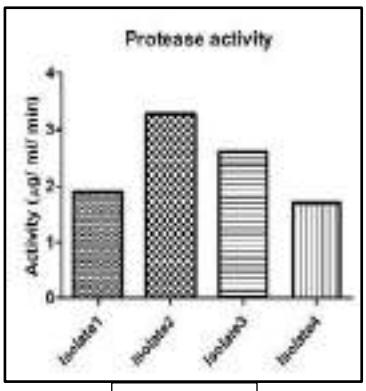
Graph 2D

Graph 1(A-D) depicts the growth of isolate 1,2,3,4 in different carbon sources. Optimum carbon sources dextrose, dextrose, sucrose, starch, respectively. **Graph 2(A-D)** depicts the growth of isolate 1,2,3,4 in different nitrogen sources. Optimum nitrogen sources are NH_4Cl, $(NH_4)_2SO_4$, NH_4Cl, $(NH_4)_2SO_4$, respectively.

4.2 Protease activity assay:



Graph 3A

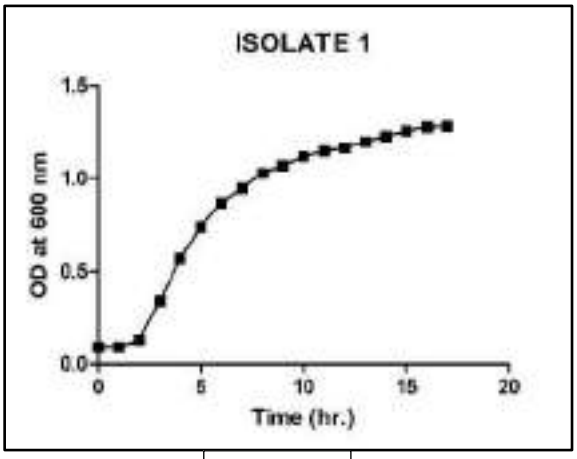


Graph 3B

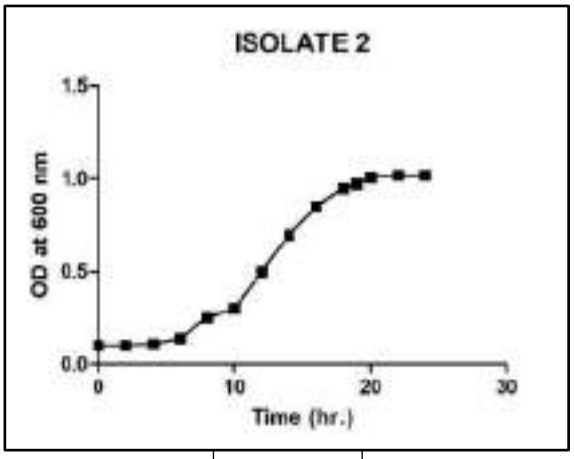
Graph 3A depicts the standard curve of tyrosine.

Graph 3B depicts the comparison of the activity of protease produced by isolates. Activity was expressed as microgram of tyrosine liberated per ml per minute. Isolate 2 exhibited highest activity following isolate 3, 1 and 4.

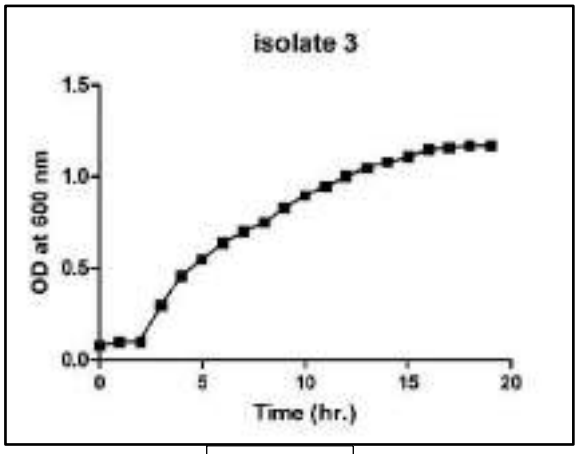
4.3 Growth curve:



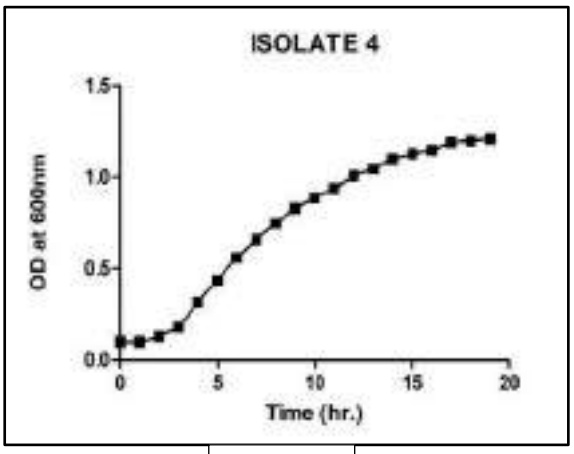
Graph 4A



Graph 4B



Graph 4C



Graph 4D

Graph 4(A-D) depicts the growth curve of isolate 1-4, respectively. Optical density was measured at every one-hour interval.

4.4 Amylase test:

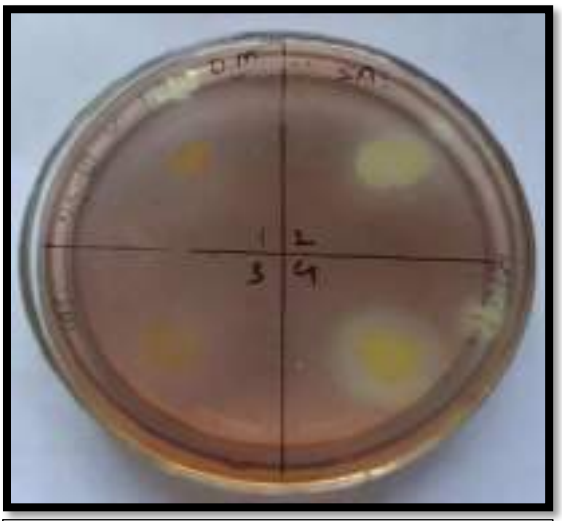


Fig 14. Amylase test. Isolate 2 and 4 are positive, isolate1 and 3 are negative.

4.5 Oxidase test:

Fig 15. Oxidase test. Isolate 2 is strongly positive, 4 is weakly positive, isolate1 and 3are negative.

4.6 Lipase test:

Fig 16. lipase test of isolate 1 and 2, both are lipase negative.

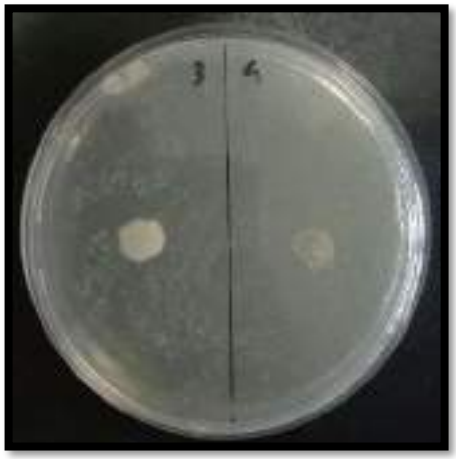


Fig 17. lipase test of isolate 3 and 4, both are lipase negative.

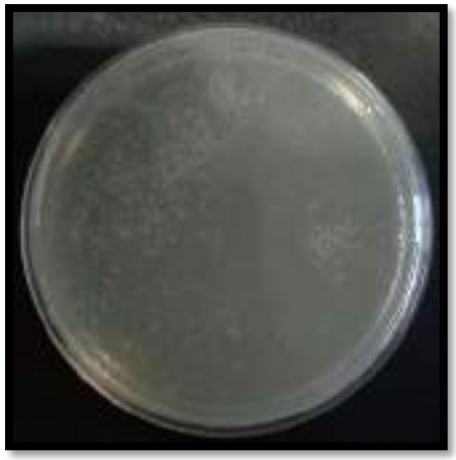


Fig 18. Negative control plate for lipase test (without inoculation).

4.7 Phosphate Solubilization test:

Fig 19. Phosphate solubilization test of isolate 1 and 2. Both of them are negative.

Fig 20. Phosphate solubilization test of isolate 3 and 4. Both of them are negative.

Fig 21. Negative control plate for phosphate solubilization test (without inoculation).

4.8 Triple Sugar Iron test:

Fig 22. TSI test. Negative control tube is on far left. Isolate 1, 2, 3 are did not ferment glucose, isolate 4 was able to ferment glucose. Isolate 2 produced Hydrogen sulfide.

4.9 Urease test:

Fig 23. Urease test. Negative control tube is on far left side. All the isolates are urease negative.

4.10 IMViC tests:

Fig 24. Indole test. Negative control tube is on the far right. All four isolates are Indole negative.

Fig 25. Methyl red test. Negative control tube is on the far left. Isolate 1 and 2 are MR negative. Isolate 3 is strong positive and isolate 4 is weakly positive.

Fig 26. Voges Proskauer test. Negative control tube is on the far right. All four isolates are VP negative.

Fig 27. Citrate utilization test. Negative control tube is on the far left. Isolate 1 and are citrate negative. Isolate 2 and 4 are citrate positive.

4.11 Protease production (Master plate):

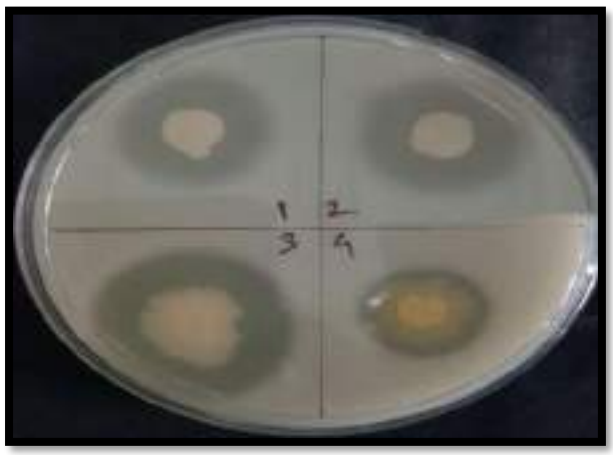
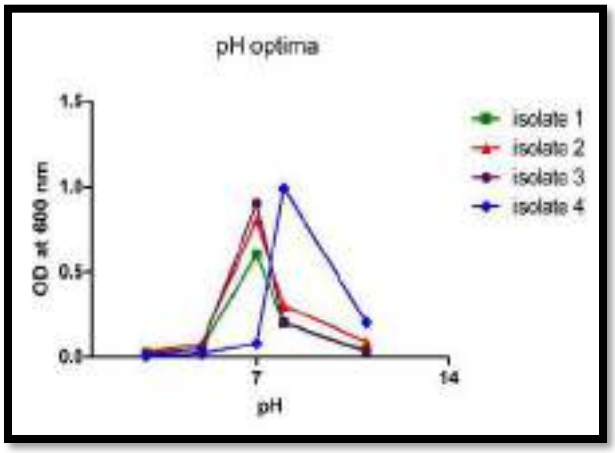


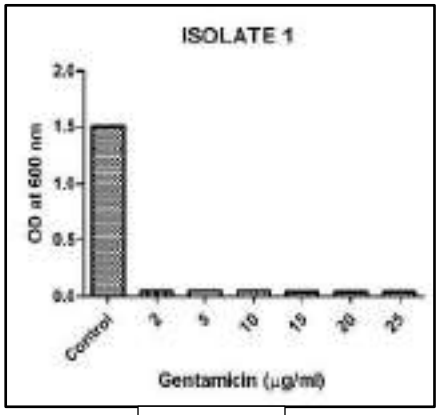
Fig 28. Protease production by isolates on skimmed milk agar plates.

4.12 pH optima for growth:

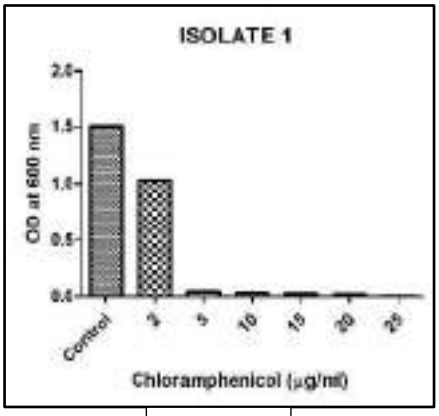


Graph 5. pH optima for growth. Isolate 1,2,3 exhibited optimum pH of 7, while isolate4 showed

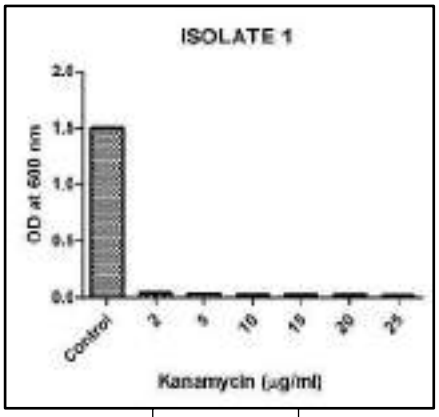
4.13 Antibiotic Sensitivity assay:



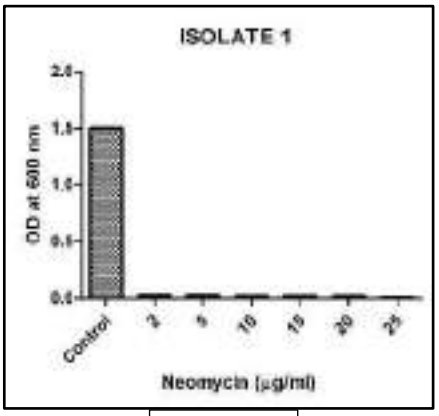
Graph 6A



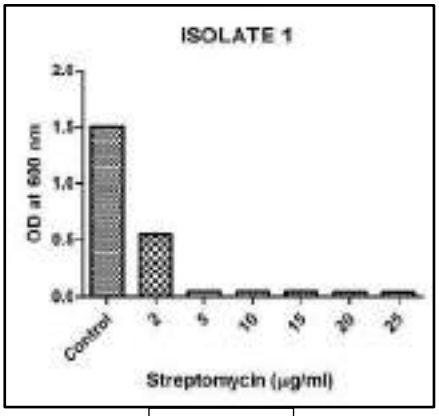
Graph 6B



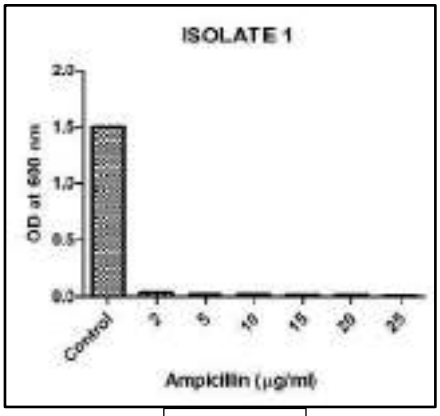
Graph 6C



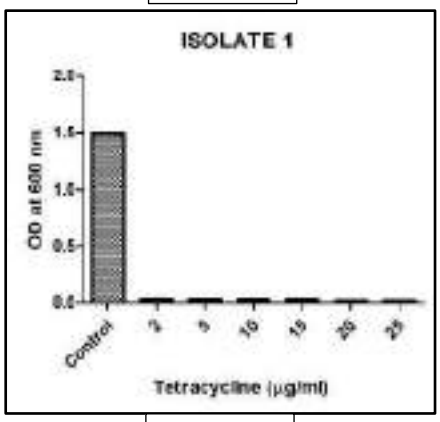
Graph 7D



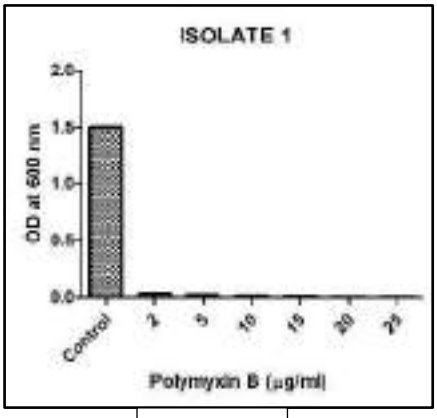
Graph 7E



Graph 7F

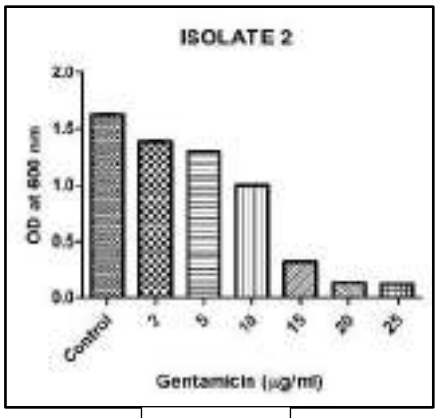


Graph 7G

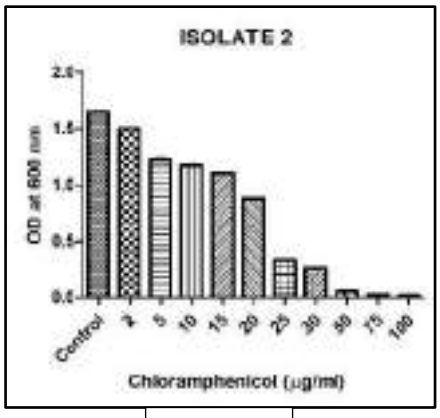


Graph 7H

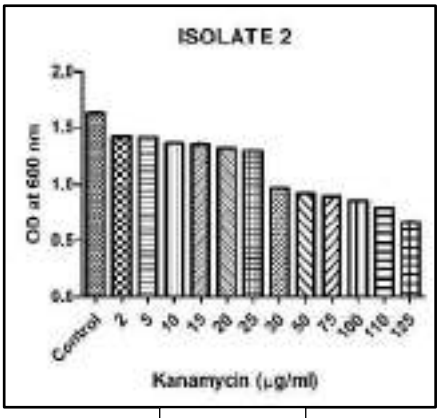
Graph 7(A-H) depicts the growth of isolate 1 in the presence of antibiotics gentamicin, chloramphenicol, kanamycin, neomycin, streptomycin, ampicillin, tetracycline, polymyxin-b, respectively. Isolate 1 exhibited susceptibility towards all the antibiotics tested. In case of chloramphenicol IC_{50} lies between 2-5 µg/ml.



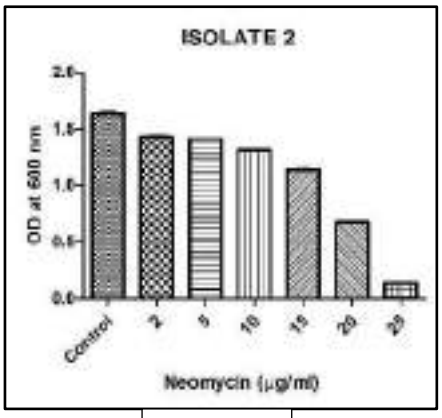
Graph 8A



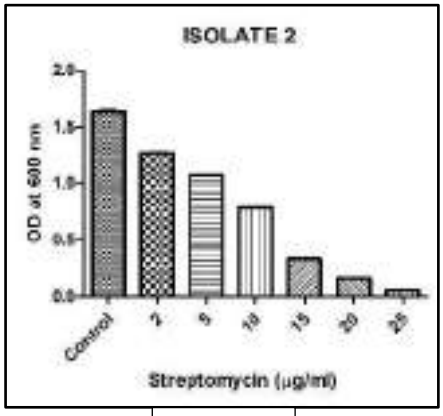
Graph 8B



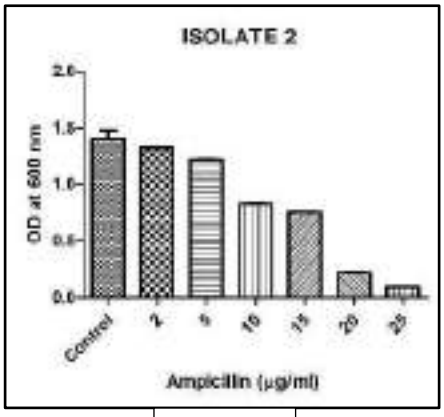
Graph 8C



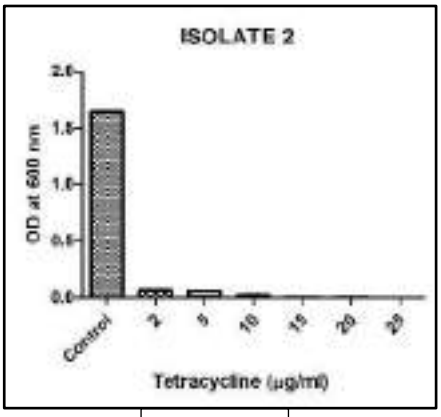
Graph 8D



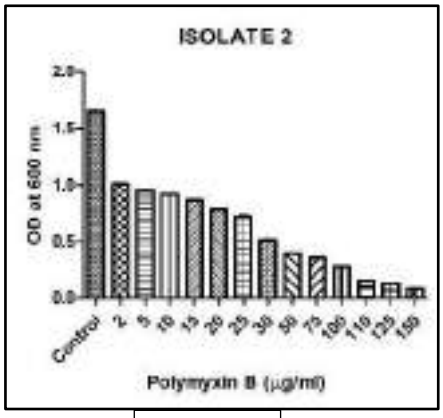
Graph 8E



Graph 8F

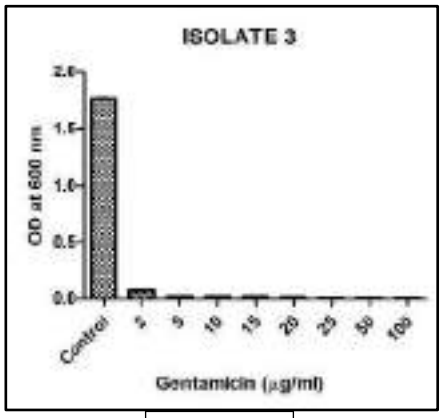


Graph 8G

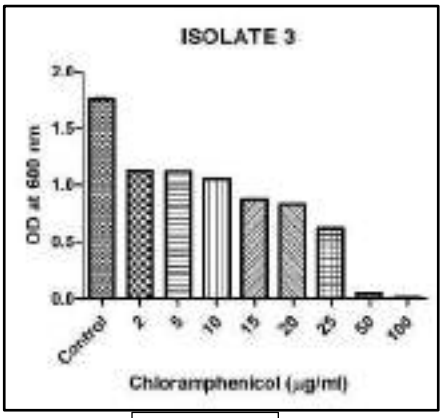


Graph 8H

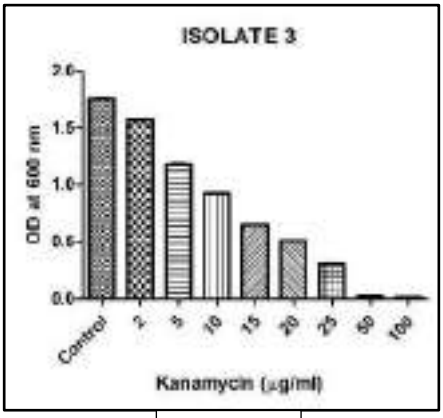
Graph 8(A-F) depicts the growth of isolate 2 in the presence of antibiotics. Isolate 2 exhibited strong resistance against kanamycin and moderate resistance against gentamicin, chloramphenicol, neomycin, polymyxin-b. Isolate 2 is highly susceptible to tetracycline.



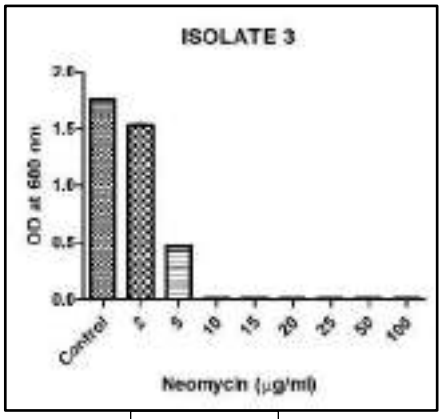
Graph 9A



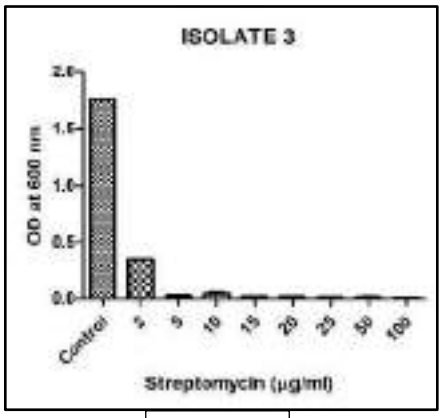
Graph 9B



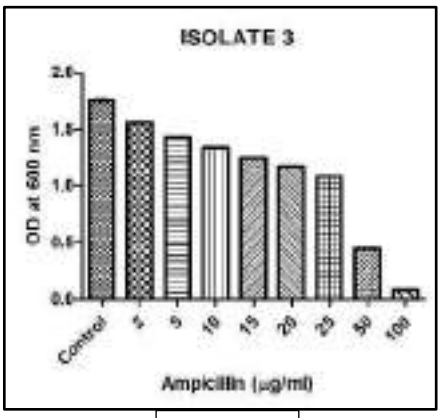
Graph 9C



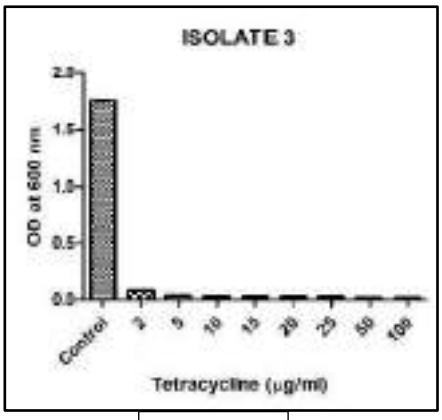
Graph 9D



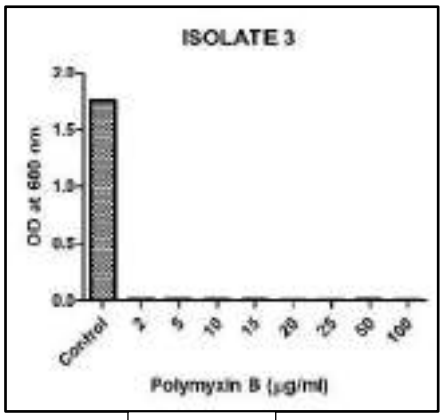
Graph 9E



Graph 9F

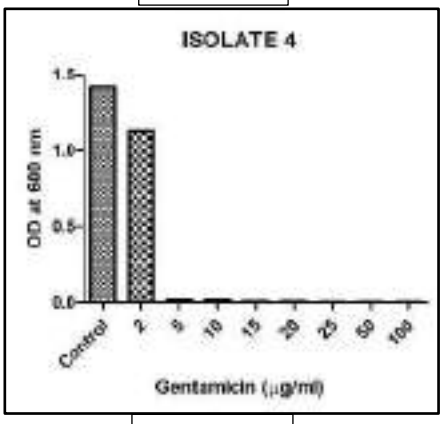


Graph 9G

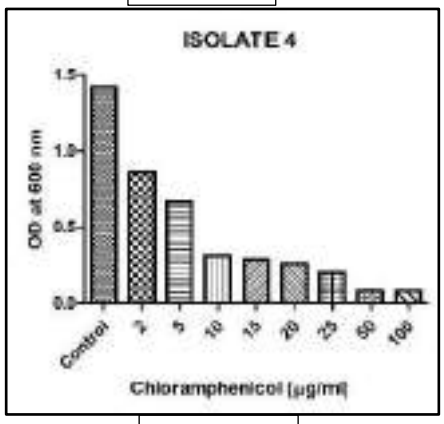


Graph 9H

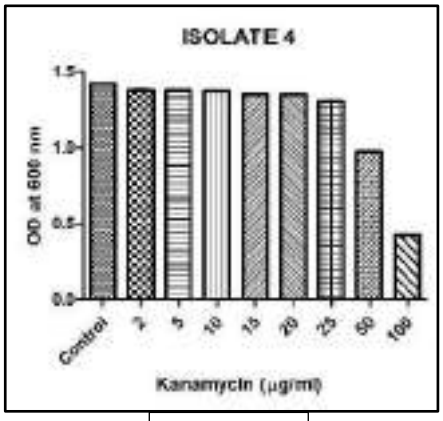
Graph 9(A-H) depicts the growth of isolate 3 in the presence of antibiotics. Isolate 3 is highly susceptible to gentamicin, streptomycin, tetracycline and polymyxin-b, whereas moderately resistant to chloramphenicol, kanamycin and ampicillin.



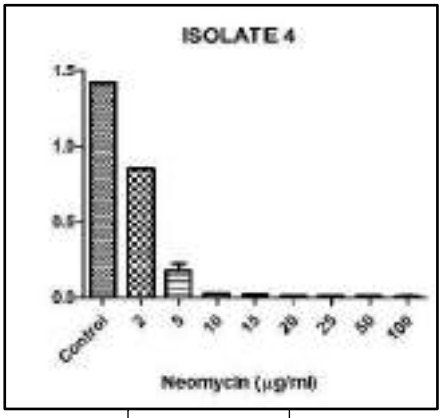
Graph 10A



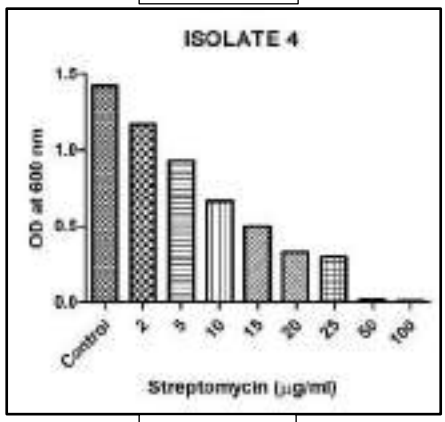
Graph 10B



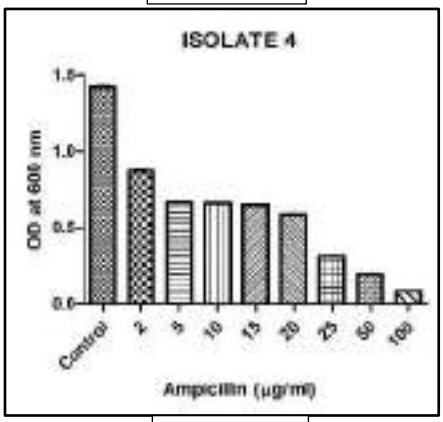
Graph 10C



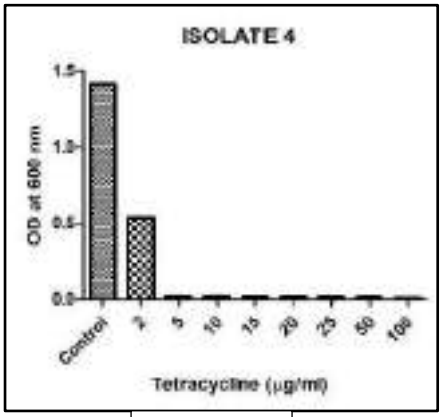
Graph 10D



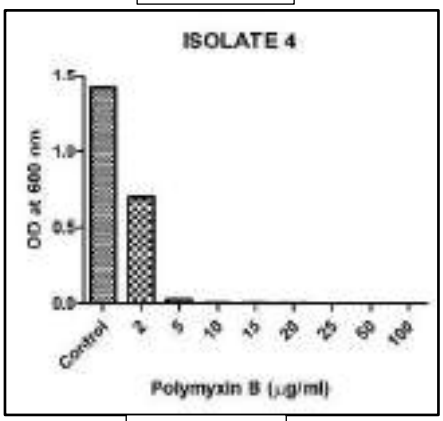
Graph 10E



Graph 10F



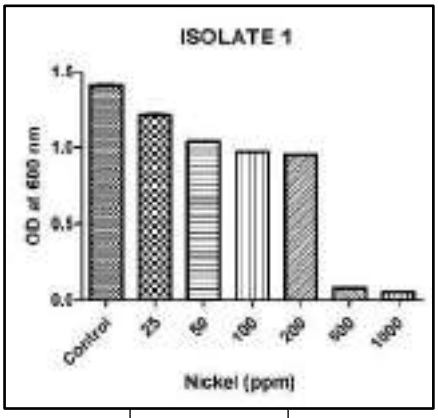
Graph 10G



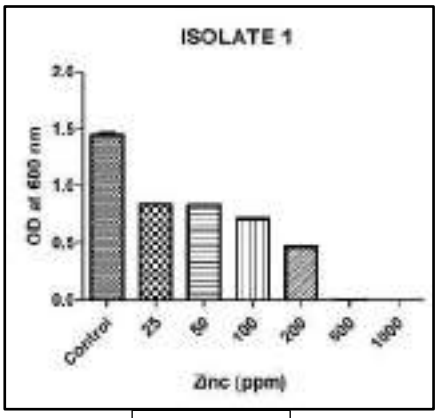
Graph 10H

graph 10(A-H) depicts the growth of isolate 4 in presence of antibiotics. isolate 4 exhibited strong resistance against kanamycin, moderately resistant against ampicillin, whereas susceptible to gentamicin, neomycin, tetracycline and polymyxin-b.

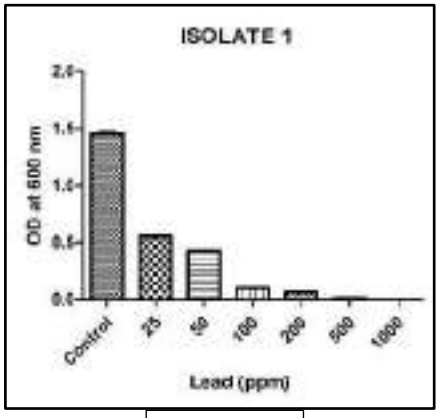
4.14 Heavy metal tolerance assay:



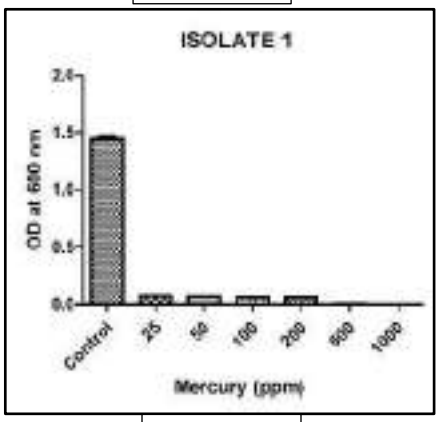
Graph 11A



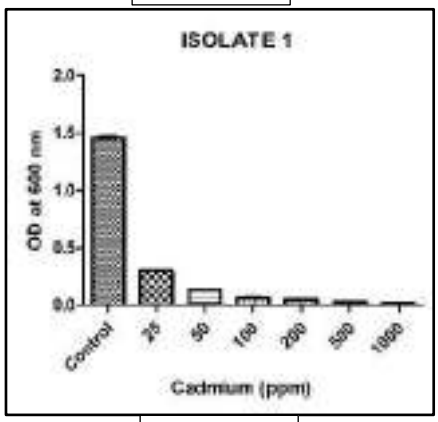
Graph 11B



Graph 11C

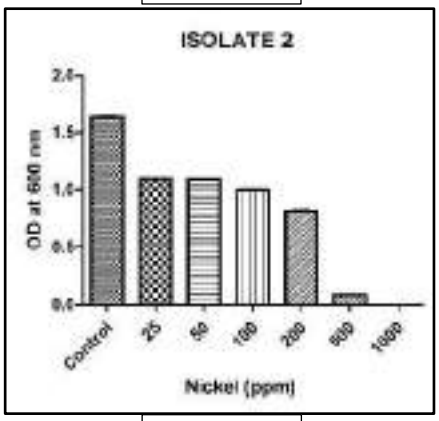


Graph 11D

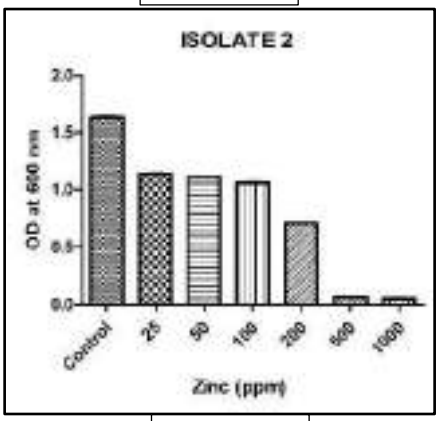


Graph 11E

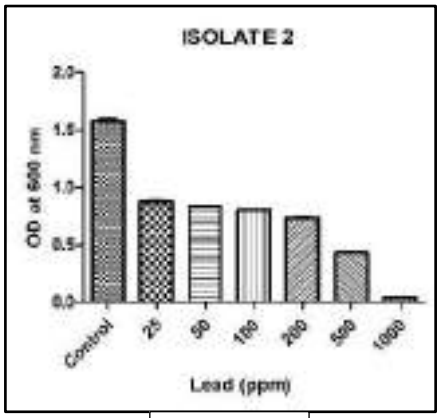
Graph 11(A-E) depicts the growth of isolate 1 in presence of heavy metals. Isolate 1 exhibited high tolerance to Nickel and Zinc, where susceptible to Lead, Mercury and Cadmium.



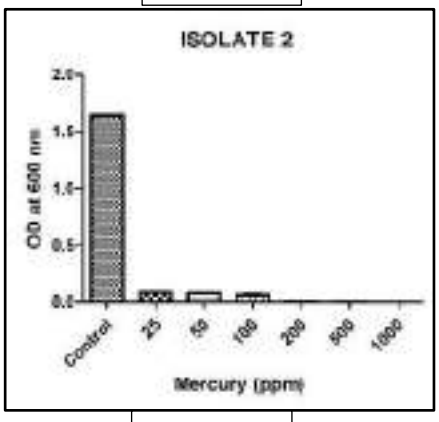
Graph 12A



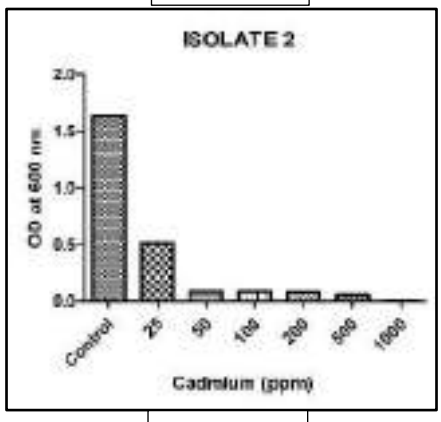
Graph 12B



Graph 12C

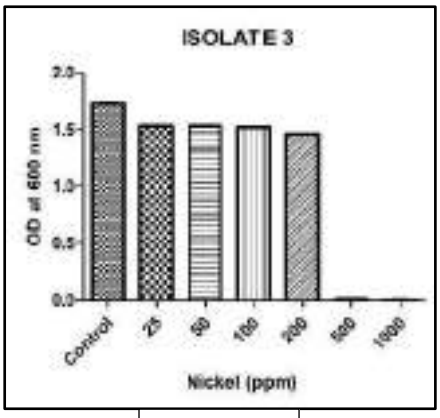


Graph 12D

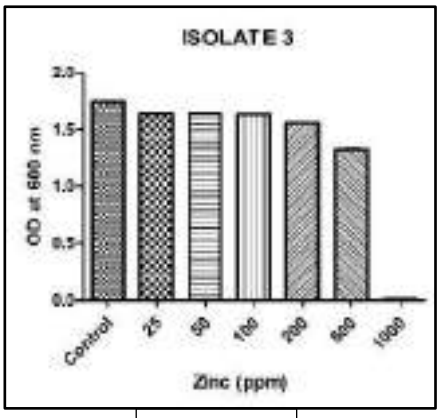


Graph 12E

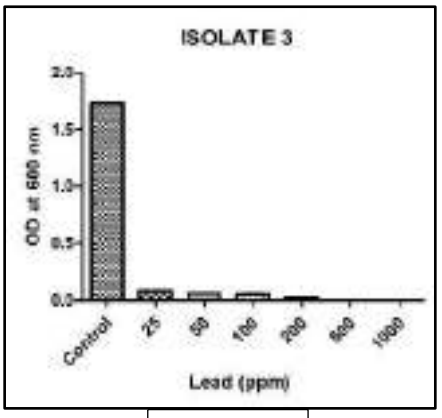
Graph 12(A-E) depicts the growth of isolate 2 in the presence of heavy metals. Isolate 2 exhibited high tolerance to nickel, zinc and lead, whereas very susceptible to mercury and cadmium.



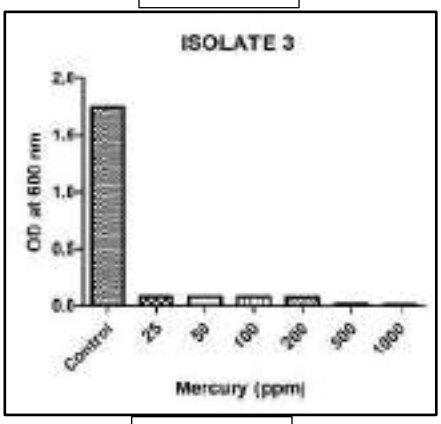
Graph 13A



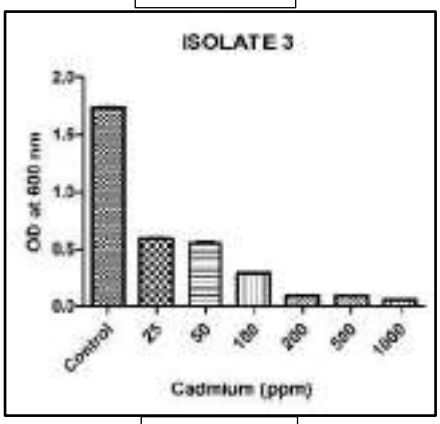
Graph 13B



Graph 13C

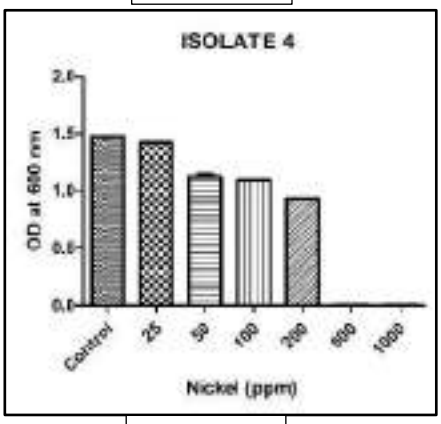


Graph 13D

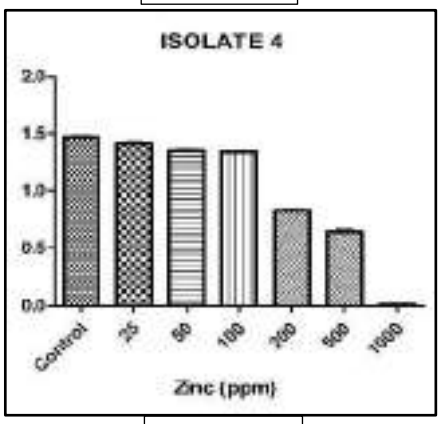


Graph 13E

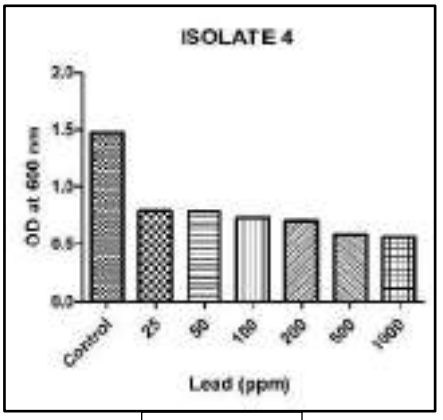
Graph 13(A-E) depicts the growth of isolate 3 in the presence of heavy metals. Isolate 3 exhibited high tolerance against nickel and zinc, moderately tolerant against cadmium, whereas susceptible to lead and mercury.



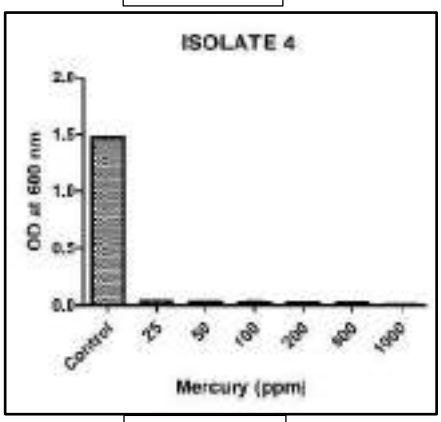
Graph 14A



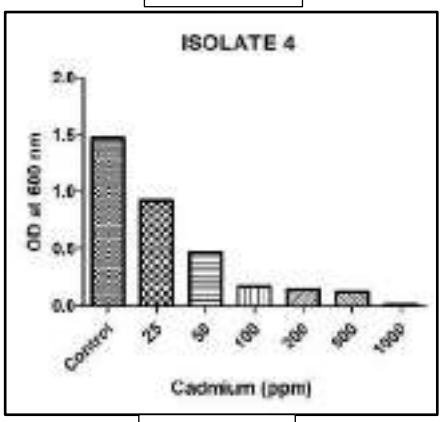
Graph 14B



Graph 14C



Graph 14D



Graph 14E

Graph 14(A-E) depicts the growth of isolate 4 in presence of heavy metals. Isolate 4 exhibited high tolerance against nickel, zinc, lead and moderately tolerant against cadmium, whereas highly susceptible to mercury.

DISCUSSION

The study was conducted to isolate, study and characterize protease producing bacteria form soil sample. For this particular study soil sample from a slaughterhouse were collected. Screening of protease producing isolates were done by enrichment culture technique. Primary identification of isolates was done using gram staining. Isolate 1 and 4 were gram positive and isolate 2 and 3 were gram negative in nature. The optimum pH for growth were 7 for isolate 1,2 and 3, whereas optimum pH for isolate 4 was found to be pH 8. The protease produced by isolate 2 exhibited highest activity and isolate 4 being lowest, when incubated with 1% casein solution.

Isolate 1 was found to be sensitive to common antibiotics. Isolate 2 showed susceptibility to tetracycline, neomycin, gentamicin, ampicillin but was resistant to high concentration of kanamycin. Isolate 3 exhibited resistance to ampicillin and chloramphenicol, whereas very sensitive to polymyxin, tetracycline, streptomycin and gentamycin. Isolate 4 was found to be resistant to kanamycin, while being highly sensitive to tetracycline, neomycin, gentamicin, polymyxin. Antibiotic resistance genes are generally not present in bacterial chromosomes and always confers through plasmid by means of horizontal gene transfer. High resistance levels against antibiotics may be established by certain mechanisms such as energy mediated efflux pumps, ability to chemically modify antibiotics or mutation at the target site of antibiotics.

Heavy metals, which can be lethal to organisms even at trace amounts. But some microorganisms such as the isolates used in this study have been reported to tolerate these toxic heavy metals at very high concentrations. All the isolates were found to be tolerant against high concentration of nickel and zinc as high as 200-500 ppm. Isolate 2 and 4 can tolerate lead at 500 and 1000 ppm respectively. Isolate 3 and 4 showed moderate tolerances against cadmium. All four isolates were found to be highly sensitive to mercury. Tolerance to heavy metals can be demonstrated by the ability of bacteria to chemically change or modify these toxic metals into a non-toxic state.

CONCLUSIONS

Slaughterhouse effluents contain high amount of protein and can be used as efficient protein source by microorganisms. Bacteria need to break down these complex and large molecules in order to uptake and use these as nutrients. For this purpose, they produce and secrete the enzyme, protease, that break down peptide bonds and convert these complex large proteins into simple polypeptide or amino acids. All four isolates used for this study were able to produce protease. Both isolate 2 and 4 were found to produce amylase and oxidase. Isolate 1,3 and 4 were able to produce catalase. Isolate 3 and 4 were able to use citrate as energy source.

Isolate 2 and 4 exhibited high resistance against kanamycin. Isolate 3 showed resistance against ampicillin and chloramphenicol. All isolates were susceptible to tetracycline, streptomycin, gentamicin, polymyxin-B.

All four isolates were able to tolerate high concentration of nickel and zinc (200-500 ppm). Isolate 2 and 4 showed tolerances against high concentration of lead, 500 and 1000 ppm respectively. Isolate 3 and 4 were moderately tolerant against cadmium.

FUTURE PROSPECTS

Optimization of temperature- In general, the temperature of the incubator was 37˚ C. So, the effect of different temperature on growth as well as on protease production may be studied.

Other biochemical assays- other biochemical tests like motility test, coagulase test, carbohydrate fermentation test, ONPG test, nitrate reduction test, etc. may be performed in future.

Staining- For morphological identification only gram staining was performed. Other staining such as negative, endospore, capsule, flagella staining can be performed for more proper morphological characterization.

Antibiotic resistance- The sensitivity to antibiotics like nalidixic acid, rifampicin may be studied.

Assay of protease enzyme- In this study we just detected the activity of the proteases. In future, proper enzyme kinetics, effect of activators, inhibitors can be a topic for study.

Purification of protease- purification of the protease may be performed and the purified enzyme can be subjected to study.

Plasmid isolation- As the attributes of antibiotic resistance and metal tolerance confers with plasmid, so isolation and a proper study on the plasmids can be an area of future aspects.

DNA sequencing and phylogenetic analysis- The isolates selected and used for this study were sent to CSIR-Institute of Genomics and Integrative Biology, Delhi for 16S rDNA sequencing. As soon as the we get the sequencing report, a proper phylogenetic analysis will be performed.

Cloning and Recombinant DNA Technology- The isolates can be subjected to recombinant DNA technology, to increase efficiency and to amplify the production of protease.

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APPENDIX - A

(MEDIA USED)

1. Composition of nutrient broth

Ingredients	Grams/litre
Peptone	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5

2. Composition of carbon optimization media

Ingredients	Grams/Litre
Carbon source (variable)	10.0
$NH_4H_2PO_4$	1.0
K_2HPO_4	2.5
KH_2PO_4	2.5
$MgSO_4.7H_2O$	0.2
$FeSO_4.7H_2O$	0.01
$MnSO_4$	0.007

3. Composition of Nitrogen optimization media

Ingredients	Grams/Litre
Nitrogen source (variable)	1.0
Dextrose	10.0
K_2HPO_4	2.5
KH_2PO_4	2.5
$MgSO_4.7H_2O$	0.2
$FeSO_4.7H_2O$	0.01
$MnSO_4$	0.007

4. Composition of Starch agar media (Amylase test)

Ingredients	Grams/Litre
Starch	20.0
Peptone	5.0
Beef extract	3.0
Agar	20.0

5. Composition of Skimmed milk agar media (Enrichment selection media)

Ingredients	Grams/Litre
Tryptone	5.0
Yeast extract	2.5
Dextrose	1.0
SM powder	28.0
Agar	15.0

6. Composition of Pikovskaya's agar media (Phosphate solubilization test)

Ingredients	Grams/Litre
Yeast extract	0.5
Dextrose	10.0
$Ca_3(PO4)_2$	5.0
$(NH_4)_2SO_4$	0.5
KCl	0.2
$MgSO_4$	0.1
$MnSO_4$	0.0001
$FeSO_4$	0.0001
Agar	12.0

7. Composition of Tryptone broth (Indole test)

Ingredients	Grams/Litre
Casein enzyme hydrolysate	10.0
NaCl	5.0

8. Composition of Simmons citrate agar media (Citrate test)

Ingredients	Grams/Litre
$MgSO_4$	0.2
$NH_4H_2PO_4$	1.0
K_2HPO_4	1.0
$Na_3C_6H_5O_7$	2.0
NaCl	5.0
Bromothymol blue	0.08
Agar	15

9. Composition of MR-VP media (MR and VP test)

Ingredients	Grams/litre
Peptone	7.0
Dextrose	5.0
K_2HPO_4	5.0

10. Composition of Protease production media (Protease activity assay)

Ingredients	Grams/Litre
Dextrose	1.0
Peptone	10.0
Yeast extract	0.2
$CaCl_2$	0.1
$MgSO_4 . 7H_2O$	0.1
K_2HPO_4	0.5

11. Composition for Tributyrin agar media (Lipase test)

Ingredients	Grams/Litre
Peptone	5.0
Yeast extract	3.0
Tributyrin (Glyceryl tributyrate)	10.0
Agar	20

12. Composition of Urea agar base (Christensen) media (Urease test)

Ingredients	Grams/Litre
Peptone	1.0
Dextrose	1.0
NaCl	5.0
Na_2HPO_4	1.2
KH_2PO_4	0.8
Phenol red	0.012
Agar	15

APPENDIX- B

(REAGENTS USED)

1. Kovac's Oxidase Reagent - 1% tetra-methyl-p-phenylenediamine di-hydrochloride, in water.

2. Kovac's Reagent (Indole Test) - p-Dimethyl-aminobenzaldehyde (50g), Hydrochloric Acid, 37% (250mL), Amyl Alcohol (750mL).

3. Methyl Red Indicator - 0.1g methyl red dissolved in 95% ethyl alcohol; volume adjusted up to 500mL using distilled water.

4. Baritt's Reagent (VP test) - Reagent A - α-Naphthol, Reagent B - 40% Potassium Hydroxide.

5. **Gram Staining** - Primary stain – Crystal violet; Mordant - Gram's Iodine; Decolouriser - 95% Ethanol; Counterstain - Safranin.

6. Hydrogen Peroxide for Catalase test.

APPENDIX- C

(METALS USED)

1. Mercuric Chloride:

- Molecular Formulae: $HgCl_2$
- Molar Mass: 271.52 g/mol
- Melting Point: 549 K
- Appearance: White, anhydrous

2. Nickel Chloride:

- Molecular Formulae: $NiCl_2 .6H_2O$
- Molar Mass: 237.69 g/mol
- Melting Point: 413.15 K
- Appearance: Green, Crystalline

3. Cadmium Sulphate:

- Molecular Formulae: CdSO4
- Molar Mass: 208.47 g/mol
- Melting Point: 1273.15 K
- Appearance: White, anhydrous

4. Zinc Chloride:

- Molecular Formulae: $ZnCl_2$
- Molar Mass: 136.286 g/mol
- Melting Point: 563 K
- Appearance: White, Crystalline

5. Lead Acetate

- Molecular Formulae: $Pb(C_2H_3O_2)_2$
- Molar Mass: 325.29 g/mol
- Melting Point: 553 K
- Appearance: White, Crystalline