**ISOLATION OF PSEUDOMONAS FROM WATER AND ITS ANTAGONISTIC / SYMBIOTIC ACTIVITY**

DISSERTATION

Submitted for the award of M. Sc. degree of

BARKATULLAH UNIVERSITY

in the

FACULTY OF SCIENCE

BY

ANUJA JATAV



Under the Supervision of

Dr. R. K. Tenguriya Ph.D.

Head Microbiology, Govt. MVM, College, Bhopal

DEPARTMENT OF MICROBIOLOGY

BARKATULLAH UNIVERSITY, BHOPAL, MP STATE, INDIA

YEAR 2023

**ACKNOWLEDGEMENT**

This thesis becomes a reality with the kind support and help of many individuals, I would like to extend my sincere thanks to all of them.

Foremost, I want to offer this endeavor to our GOD almighty for the wisdom he bestowed upon me, the strength peace of my mind and good heath in order to finish this research.

I would like to express my venerated gratitude to **Dr. R. K. Tenguriya, Head Microbiology** for giving me the opportunity to do the project from Govt. Motilal Vigyan Mahavidyalaya, Bhopal. I pray to god for his good health in future.   
  
To begin with, I want to express my profound gratefulness to **Dr. Yogesh Vyas** and colleagues for imparting his knowledge and expertise in this study and enhancing curiosity in the field of Microbiology and giving me opportunity to do project at MICROBIQ LABS, Indore.

Last but not the least, my heartfelt thanks to my parents, without their blessing nothing would have worked out.

ANUJA JATAV   
M. Sc. Microbiology IV Semester, Year 2023  
Govt. M.V.M. Collage, Bhopal

DECLARATION

I hereby declare that the work incorporated in the present dissertation entitled “ISOLATION OF PSEUDOMONAS FROM WATER AND ITS ANTAGONISTIC / SYMBIOTIC ACTIVITY STUDY” is my own work and is original.

This work (in part or in full) has not been submitted to any University for the award of a Degree or a Diploma.

ANUJA JATAV   
M. Sc. Microbiology IV Semester, Year 2023  
Govt. M.V.M. Collage, Bhopal

****



**GOVT. MOTILAL VIGYAN MAHAVIDYALAYA, BHOPAL**

**Dr. R.K. Tenguria Professor &H.O.D**

M.Sc, M.Phil, Ph.D (Microbiology) FASEADivision

of Microbiology

Department of Botany

Govt. M.V.M. Bhopal – 462008

Date : -

This is to certify Miss Anuja Jatav M.Sc. IV th Sem Microbiology

Govt. M.V.M. Bhopal has completed her three months Dissertation

Work entitled Isolation Of Pseudomonas From Different Water

Bodies And Its Antagonistic / Symbiotic Activities

Under the guidance of Dr.R.K.Tenguria Professor and Head for

The partial fulfillment of the requirement of the award of the

Of the award of degree of Master of Science in Microbiology

Residence : E-109/32, Shivaji Nagar, Bhopal 462016 (M.P.) India

Phone : (R) 0755-2572420, (O)0755-2551460, M : 9907225768

E-Mail : tenguria\_raj@yahoo.co.in

**AFFIDAVIT**

I miss anuja jatav class m. sc. IV semester microbiology collage govt. motilal vigyan mahavidyalaya Bhopal (M. P.) state that i personally attended the institute and received training and the project is prepared by me and is original work of mine .

**ABBREVIATIONS**

µg/mcg : Microgram

µl : Microlitre

BOD : Biological oxygen demand

CFU : Colony forming unit

cm : Centimeter

DW : Distilled water

g/gm : Gram

hrs : Hours

l : Litre

MD : Mean differences

mg : Milligram

min : Minute

ml : Milliliter

mm : Millimeter

n : Subject number

nm : Nanometer

r : Replicates

Rf : Relative fraction

SD : Standard deviation

SCDA : Soyabean Casein Digest Agar

SDW : Sterile distilled water

sec : Seconds

UV : Ultraviolet

**TABLE OF CONTENTS**

|  |  |  |
| --- | --- | --- |
| **S. No.** | **Title** | **Page No.** |
| 1.0 | Introduction |  |
| 2.0 | Objectives |  |
| 3.0 | Material & Methods |  |
| 4.0 | Results |  |
| 5.0 | Summary & Conclusions |  |
| 6.0 | References |  |

**INTRODUCTION**

Surface water play a key role in transmission of pathogenic agent discharged through human and animal stool. These agents may find their way into water via domestic waste water / surface runoff from agriculture land and posture during rainfall or by direct deposition of waste matter with access to steam channels. They can be transferred to human by various roots like recreation, irrigation of crops drinking. Thus, quality of water needs evaluation to generate base line data for welfare of society microbial investigation of water is required frequently to know the status of water body in term of microbial pollution.

*Pseudomonas* is one of the aerobic, heterogenous and ecologically important group of bacteria for assessment of water. *Pseudomonas* is gram negative, motile, rods shaped and 1-2 micrometer long and 0.5 -1.0 micrometer wide in size, monoflagellated bacterium.

*Pseudomonas* has incredible nutritional versatility and found ubiquitous in environment, soil, water, human, animal, plants, sewage, marine water, hospital. the pathogen grows best at temperature between 37oC to 42oC. *Pseudomonas* can break down polycyclic aromatic hydrocarbons in soil. *Pseudomonas* is detected in water reservoirs polluted by animals and human, such as sewage and sinks inside and outside of hospitals. *Pseudomonas* is a potent infectious agent and one of the most frequently identified pathogens associated with waterborne outbreaks of dermatitis.

*Pseudomonas* is often resistant to many classes of antibiotics and therapeutic agents, and this makes it problematic during infection as it can be difficult to treat. *Pseudomonas* is often termed an ‘opportunistic ‘pathogen because it rarely infects healthy individuals. Clinically, the primary risk is for patients with compromised immune systems including those with cystic fibrosis (CF), Cancer, AIDS, indwelling Medical Devices, Burn and Eye injuries and non-healing diabetic wounds.

Prasad et al. 2017 carried out Bacterial screening of river Kshipra water at Ujjain after Kumbh (Religious Fair) and assessed *Pseudomonas* distributions, contamination, and occurrence. Bhasin *et al.* 2020 have carried out another study for Bacterial diversity of river Kshipra with relation to human health and found that its role in contribution of natural fresh water and drinking water, as a route of *Pseudomonas* infection and the study show that most of the site of Kshipra River are heavily, contaminated with *P. aeruginosa* that constantly increased WHO limits for portability. *P. aeruginosa* population is found to be high during summer and onset of rain.

Based on review of literature, comparative isolation of *Pseudomonas* from high bioburden and low bioburden and its association has not been reported anywhere. Therefore, present study was under taken to study comparative *Pseudomonas* isolation and its association antagonistic or symbiotic with microorganisms.

Present study was proposed to study Isolation of *Pseudomonas* from water such as Municipal Supply Drinking Water (Treated Water), Pond Water (Talawali Pond), RO Water (Purified Water), Potable Water (Matka – Stored Public Drinking Water), Kshipra River Sangam Water and *Pseudomonas* Antagonistic / Symbiotic Activity against microorganisms.

**Clinical Manifestations : -**  
  
pseudomonas aeruginosa and p maltophilia account for 80 percent of opportunistic infections by pseudomonads . pseudomonas aeruginosa infection is serious problem in patients hospitalized with cancer , cystic fibrosis , and burns , the case fatality is 50 percent . other infections caused by pseudomonas species include endocarditis , pneumonia , and infections of the urinary tract , central nervous system , wounds , eyes, ears , skin , and musculoskeletal system .   
  
*Pseudomonas aeruginosa* causes various diseases . Localized infection following surgery or burns commonly results in a generalized and frequently fatal bacteremia. Urinary tract infections following introduction of *P aeruginosa* on catheters or in irrigating solutions are not uncommon. Furthermore, most cystic fibrosis patients are chronically colonized with *P aeruginosa*. Interestingly, cystic fibrosis patients rarely have *P aeruginosa* bacteremia, probably because of high levels of circulating *P aeruginosa* antibodies. However, most cystic fibrosis patients ultimately die of localized *P aeruginosa* infections. Necrotizing P aeruginosa pneumonia may occur in other patients following the use of contaminated respirators. *Pseudomonas* aeruginosa can cause severe corneal infections following eye surgery or injury. It is found in pure culture, especially in children with middle ear infections. It occasionally causes meningitis following lumbar puncture and endocarditis following cardiac surgery. It has been associated with some diarrheal disease episodes. Since the first reported case of P aeruginosa infection in 1890, the organism has been increasingly associated with bacteremia and currently accounts for 15 percent of cases of Gram-negative bacteremia. The overall mortality associated with P aeruginosa bacteremia is about 50 percent. Some infections (e.g., eye and ear infections) remain localized; others, such as wound and burn infections and infections in leukemia and lymphoma patients, result in sepsis. The difference is most probably due to altered host defenses.

*Pseudomonas maltophilia* is the second most frequently isolated pseudomonad species in clinical laboratories. In nature, *P maltophilia* is found in water and in both raw and pasteurized milk. It has been associated with a variety of opportunistic infections in humans, including pneumonia, endocarditis, urinary tract infections, wound infections, septicemia, and meningitis. *Pseudomonas cepacia*, although primarily a plant pathogen (onion bulb rot), also is an opportunist. Most human infections caused by *P cepacia* are nosocomial and include endocarditis, necrotizing vasculitis, pneumonia, wound infections, and urinary tract infections. *Pseudomonas cepacia* causes chronic lung infections in cystic fibrosis patients. These infections differ from those caused by *P aeruginosa*in that *P cepacia* has become systemic in a number of cystic fibrosis patients, whereas *P aeruginosa*infections remain confined to the lungs. *Pseudomonas cepacia* is highly resistant to aminoglycosides and other antibiotics, making it very difficult to control.

Unlike most pseudomonads, *P mallei* and *P pseudomallei* can cause disease in otherwise healthy individuals. *Pseudomonas mallei* is the agent of glanders, a disease primarily of equines. Humans generally become infected by inhalation or by direct contract through abraded skin. These infections are frequently fatal within 2 weeks of onset, although chronic infections also have been reported. Today, *P mallei* infections of equines are controlled andare rarely encounteredin the western world. Similarly, melioidosis, an endemic glanderslikedisease of animals and a human pulmonary infection caused by *P pseudomallei*, is rare inthe western hemisphere. Melioidosis is still found in Southeast Asia, and travellers returning from that area are sometimes infected.

**Structure, Classification, and Antigenic Types : -**

*Pseudomonas aeruginosa* is a Gram-negative rod measuring 0.5 to 0.8 μm by 1.5 to 3.0 μm. Almost all strains are motile by means of a single polar flagellum, and some strains have two or three flagella . The flagella yield heat-labile antigens (H antigen). The significance of antibody directed against these antigens, aside from its value in serologic classification, is unknown. Clinical isolates usually have pili, which may be antiphagocytic and probably aids in bacterial attachment, thereby promoting colonization. Species are distinguished by biochemical and DNA hybridization tests . antisera to lipopolysaccharide and outer membrane proteins show cross – reactivity among serovars.

The cell envelope of *P aeruginosa*, which is similar to that of other Gram-negative bacteria, consists of three layers: the inner or cytoplasmic membrane, the peptidoglycan layer, and the outer membrane. The outer membrane is composed of phospholipid, protein, and lipopolysaccharide (LPS). The LPS of *P aeruginosa* is less toxic than that of other Gram-negative rods. The LPS of most strains of *P aeruginosa* contains heptose, 2-keto-3-deoxyoctonic acid, and hydroxy fatty acids, in addition to side-chain and core polysaccharides. Recent evidence suggests that the LPS of a large percentage of strains isolated from patients with cystic fibrosis may have little or no polysaccharide side chain (O antigen), and that this finding correlates with the polyagglutinability of these strains with typing sera.

Studies of isolated outer membranes suggest strong conservation of many of the outer membrane proteins of *P aeruginosa*. Although numerous serologic types exist (based on evaluations of O-specific antigens), many of the outer membrane proteins from these strains are antigenically crossreactive.

*Pseudomonas aeruginosa* is a nonfermentative aerobe that derives its energy from oxidation rather than fermentation of carbohydrates. Although able to use more than 75 different organic compounds, it can grow on media supplying only acetate for carbon and ammonium sulfate for nitrogen. Furthermore, although an aerobe, it can grow anaerobically, using nitrate as an electron acceptor. This organism grows well at 25° C to 37° C, but can grow slowly or at least survive at higher and lower temperatures. Indeed, the ability to grow at 42° C distinguishes it from many other *Pseudomonas* species. In addition to its nutritional versatility, *P aeruginosa* resists high concentrations of salt, dyes, weak antiseptics, and many commonly used antibiotics. These properties help explain its ubiquitous nature and contribute to its preeminence as a cause of nosocomial infections.

**Pathogenesis : -**

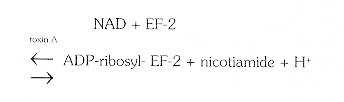
Some strains of *P aeruginosa* produce large amounts of extracellular polysaccharide. These mucoid strains usually are isolated only from patients with cystic fibrosis. The role of these polysaccharides in the pathogenesis of *P aeruginosa* chronic lung infections is unknown, but they may impede phagocytosis and impair diffusion of antibiotics and thus facilitate colonization and persistence. Interestingly, mucoid strains are frequently deficient in production of elastase, toxin A, and flagella, and their LPS lacks long polysaccharide side chains.

Neutropenia in cancer patients and others receiving immunosuppressive drugs contributes to infection . pseudomonas aeruginosa has several virulence factors , but their role in pathogenesis are unclear . an alginate is antiphagocytic , and most strains isolated produce toxin A , diphtheria - toxin – like exotoxins . all strains have endotoxins , which is major virulence factor in bacteremia and septic shock .   
  
 Most strains of *P aeruginosa* also produce one or more pigments, the most common being pyocyanin (a phenazine pigment) and fluorescein. These pigments are nontoxic in animals. Pyocyanin, however, retards the growth of some other bacteria and thus may facilitate colonization by *P aeruginosa*. One or more of these pigments appear to function in iron acquisition by *P aeruginosa*. Additional work is needed to clarify the role of these pigments in P aeruginosa infections.

Approximately 90 percent of *P aeruginosa* strains produce extracellular protease. Three separate proteases have been purified that differ in pH optimum, isoelectric point, and substrate specificity. Although all are capable of digesting casein, one of them, protease II, also digests elastin. When injected into the skin of animals, purified *P aeruginosa* proteases induce formation of hemorrhagic lesions, which become necrotic within 24 hours. These proteases also cause rapid tissue destruction when injected into the cornea of animal eyes or into rabbit lungs; they also probably contribute to the tissue destruction that accompanies *P aeruginosa* eye or lung infections and may aid bacteria in tissue invasion. Their effects, however, appear to be localized, and they are not highly toxic to animals (LD50 = approximately 200 μg/mouse)

Toxin A

Toxin A, the most toxic known extracellular protein of *P aeruginosa*, is produced by 90 percent of all strains. The median lethal dose of pure toxin A is about 0.2 μg/mouse. Its toxicity has been attributed to its ability to inhibit protein synthesis in susceptible cells. It achieves this by catalyzing the transfer of the ADP-ribosyl moiety of nicotinamide adenine dinucleotide (NAD) onto elongation factor 2 (EF-2) according to the following reaction:



The resultant ADP-ribosyl-EF-2 complex is inactive in protein synthesis. This intracellular mechanism of action of toxin A is identical to that of diphtheria toxin fragment A . Also like diphtheria toxin, *Pseudomonas* toxin A is released by *P aeruginosa* as a proenzyme. Toxin A is toxic to animals and cultured cells, but the proenzyme has little or no enzymatic activity. the relationship between the various forms of toxin A and their enzymatic activity and mouse toxicity. Evidence suggesting that toxin A may be a major virulence factor of *P aeruginosa* includes observations that toxin A-deficient mutants are less virulent in several animal models than their toxin A-producingparental strains, as well as the observation that most patients surviving *P aeruginosa* sepsis have elevated levels of antitoxin A antibody or are infected with strains that produce little or no detectable toxin A in vitro. These studies need to be expanded before firm conclusions can be reached.

Exoenzyme S

A second ADP-ribosyltransferase, exoenzyme S, has been described. Exoenzyme S catalyzes the transfer of ADP-ribose onto a number of GTP-binding proteins, including the product of the proto-oncogene c-H-*ras* (p2lC-H-*ras*); however, it does not modify elongation factor 2. Exoenzyme S is produced by about 90 percent of clinical isolates of *P aeruginosa*. Transposon-induced S-deficient mutants are less virulent in several animal models than is their S-producing parental strain; thus, exoenzyme S may be involved in the pathogenesis of some *P aeruginosa* infections.

**Host Defenses** : -  
  
phagocytosis by polymorphonuclear leukocytes is important in resistance to pseudomonas infections. Antibodies to somatic antigens and exotoxins also contributes to recovery . humoral immunity is normally the primary immune mechanism against pseudomonas infection but does not seem to resolve infection in cystic fibrosis patients despite high levels of circulating antibodies . Although 85 percent of *P aeruginosa* isolates are resistant to serum alone, addition of polymorphonuclear leukocytes results in bacterial killing. Killing is most efficient in the presence of type-specific opsonizing antibodies, directed primarily at the antigenic determinants of LPS. This suggests that phagocytosis is an important defense and that opsonizing antibody is the principal functioning antibody in protecting from *P aeruginosa* infections; however, once a *P aeruginosa*infection is established, other antibodies, such as antitoxin, may be important in preventing death.  
 Although evidence suggests interaction between *P aeruginosa* and the cellular immune system, patients with diseases characterized by impaired cellular immune responses (e.g., Hodgkin's disease) do not have an increased incidence of severe *P aeruginosa* infections. However, patients with diminished antibody responses caused by underlying disease or its associated therapy, have more serious *P aeruginosa* infections. This underscores the importance of the humoral response in controlling *P aeruginosa* infections. Cystic fibrosis is the exception. Most cystic fibrosis patients have high levels of circulating antibodies to many bacterial antigens, but are unable to clear *P aeruginosa* efficiently from their lungs.

**Epidemiology : -**

*Pseudomonas aeruginosa* commonly inhabits soil, water, and vegetation. It is found in the skin of some healthy persons and has been isolated from the throat (5 percent) and stool (3 percent) . they often colonize hospital food , sinks , taps , mops , and respiratory equipment . spread is from patient to patient via contact with fomites or by ingestion of contaminated food and water .   
  
The gastrointestinal carriage rates increase in hospitalized patients to 20 percent within 72 hours of admission. Within the hospital, *P aeruginosa* finds numerous reservoirs: disinfectants, respiratory equipment, food, sinks, taps, and mops. Furthermore, it is constantly reintroduced into the hospital environment on fruits, plants, vegetables, and patients transferred from other facilities. Spread occurs from patient to patient on the hands of hospital personnel, by direct patient contact with contaminated reservoirs, and by the ingestion of contaminated foods and water.

Several different typing systems are available for epidemiologic studies: serologic, phage, pyocin, and DNA fingerprinting. In the pyocin system, pyocins (bacteriocins or aeruginocins) produced by the test strain are assayed for bactericidal activity against a series of indicator strains. A number of different serologic typing systems are used. Some employ combinations of heat-stable and heat-labile antigens, whereas others use only heat-stable antigens. No system is universally accepted. Recently, DNA fingerprinting has identified probes that are useful in typing *P aeruginosa* strains.

**Diagnosis : -**

Pseudomonas can be cultured on most general - purpose media and identified with biochemical media . Diagnosis of *P aeruginosa* depends on its isolation and laboratory identification. It grows well on most laboratory media and commonly is isolated on blood agar plates or eosin-methylthionine blue agar. It is identified on the basis of its Gram morphology, inability to ferment lactose, a positive oxidase reaction, its fruity odor, and its ability to grow at 4 2° C . Fluorescence under ultraviolet radiation helps in early identification of *P aeruginosa* colonies and also is useful in suggesting its presence in wounds. Other pseudomonads are identified by specific laboratory tests.

**Control : -**

The spread of *Pseudomonas* is best controlled by cleaning and disinfecting medical equipment. In burn patients, topical therapy of the burn with antimicrobial agents such as silver sulfadiazine, coupled with surgical debridement, has markedly reduced sepsis. Antibiotic susceptibility testing of clinical isolates is mandatory because of multiple antibiotic resistance; however, the combination of gentamicin and carbenicillin can be very effective in patients with acute *P aeruginosa* infections.  
The genus *Pseudomonas* contains more than 140 species, most of which are saprophytic. More than 25 species are associated with humans. Most pseudomonads known tocause disease in humans are associated with opportunistic infections.  
  
  
 These include *P aeruginosa*, *P fluorescens*, *P putida*, *P cepacia*, *P stutzeri*, *P maltophilia*, and *P putrefaciens*. Only two species, *P mallei* and *P pseudomallei*, produce specific human diseases: glanders and melioidosis. *Pseudomonas aeruginosa*and *P maltophilia* account for approximately 80 percent of pseudomonads recovered from clinical specimens. Because of the frequency with which it is involvedin humandisease, *P aeruginosa* has received the most attention. It isa ubiquitous free-living bacterium and isfound in most moistenvironments. Although it seldom causes disease in healthy individuals, it is a major threat to hospitalized patients, particularly those with serious underlying diseases such as cancerand burns.The high mortality associated with these infections is due to a combination of weakenedhost defenses, bacterial resistance to antibiotics, and the production of extracellular bacterial enzymes and toxins.

**OBJECTIVES**

1. Isolation of *Pseudomonas* from water.

Water sampling

* Municipal Supply Drinking Water (Treated Water)
* RO Water (Purified Water)
* Pond Water (Talawli Pond)
* Potable Water (Mataka – Stored Public Drinking Water)
* Kshipra River Sangam Water

Isolation from water samples.

Preliminary identification of *Pseudomonas*.

1. Study of Pseudomonas antagonistic or symbiotic against microorganisms.

**MATERIAL & METHODS**

**Water Samples : -**

A sampling was carried out in one dayfrom different places for isolation of micro-orgasnism.

Bactarial sample was collected aseptically using 500ml sterile bottles and were kept in a low temprature and they were transported to the base laboratory within few hours .

|  |  |
| --- | --- |
| **Sample Type** | **S.No** |
| Municipal Supply Drinking Water (Treated Water) | 01 |
| Pond Water (Talawali Pond) | 01 |
| RO Water (Purified Water) | 01 |
| Potable Water (Mataka – Stored Public Drinking Water) | 01 |
| Kshipra River Sangam Water | 01 |



**Water Samples in Sterile Poly propylene Bottles**

|  |  |
| --- | --- |
| *Area*  *Sample Collection* |  |

**Portable Water (Matka) – Stored in Public**

**(Drinking-Water)**



*Area*

*Sample Collection*

**Narmada And Shipra River Sangam**



Sample Collection

**Talawali Chanda Pond Water**

1. **Culture media**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Culture Media / Diluent** | **Manufacturer** | **Code No.** | **Lot No.** | **Expiry** | **Purpose** |
| Soybean Casein Digest Agar | Himedia | MH290 | 0000522076 | JAN-2027 | Bacterial growth |
| Cetrimide Agar | Himedia | MH024 | 0000532055 | MAR-2027 | Pseudomonas isolation |

**Casein Soyabean Digest Agar (Soyabean Casein Digest Agar)**

**(Tryptone Soya Agar) (DM247)**

**Intended Use**

Casein Soyabean Digest Agar (Soyabean Casein Digest Agar) (Tryptone Soya Agar) (DM247) is recommended for cultivation of a wide variety of microorganisms.

**Product Summary and Explanation**

In 1955, Leavitt et al.(1) discovered that Soyabean Casein Digest Agar facilitated vigorous growth of aerobic and anaerobic microorganisms. Soyabean Casein Digest Agar is a widely used medium, which supports the growth of wide variety of organisms even that of fastidious ones such as Neisseria, Listeria, and Brucella etc. The medium is used in USP Growth Promotion testing and when testing the suitability of counting methods in the presence of product.(2) TSA has a multitude of uses in the clinical laboratory including maintenance of stock cultures, plate counting, isolation of microorganisms from a variety of specimen types and as a base for media containing blood.(3-6) It is also recommended for use in industrial applications when testing water and wastewater,(7) food,(8-13) dairy products,(14) and cosmetics.(9,15) The medium with addition of blood provides perfectly defined haemolysis zones, while preventing the lysis of erythrocytes due to its sodium chloride content. It has been frequently used in the health industry to produce antigens, toxins etc. It’s simple and inhibitor-free composition makes it suitable for the detection of antimicrobial agents in the food and other products.  
  
 Tryptone Soya Agar is recommended by various pharmacopoeias as sterility testing medium. (2, 16) Tryptone Soya Agar conforms as per USP(2) and is used in microbial limit test and antimicrobial preservative - effective test. Gunn et al(17) used this medium for the growth of fastidious organisms and study of haemolytic reaction after addition of 5% v/v blood. Soyabean Casein Digest Agar does not contains X and V growth factors. It can be conveniently used in determining the requirements of these growth factors by isolates of Haemophilus by the addition of X-factor (ID007), V-factor (ID008), and X+V factor discs (ID009) factor to inoculated TSA plates.

**Principles of the Procedure**soyabean casein digest agar is a widely used medium , which supports the growth of wide variety of organisms even that of fastidious ones such as Neisseria , listeria , and brucella etc. the medium with addition of blood provides perfectly defined haemolysis zones, while preventing the lysis of erythrocytes due to its sodium chloride content . it has been frequently used in the health industry to produce antigens , toxins etc. its simple and inhibitor – free composition makes it suitable for the detection of antimicrobial agents in the food and other products . tryptone soya agar is recommended by various pharmacopoeias as sterility testing medium .   
tryptone soya agar conforms as per UPS and is used in microbial limit test and antimicrobial preservative – effective test . used this medium for the growth of fastidious organisms and study of haemolytic reaction after addition of 5 % v/v blood . the combination of tryptone and soya peptone makes this media nutritious by providing amino acids and long chain peptides for the growth of microorganism . sodium chloride maintains the osmotic balance .   
Soyabean Casein Digest Agar contains pancreatic digest of casein and papaic digest of soyabean which provides amino acids, long chain peptides and essential nutrients required for the growth of microorganisms. Sodium chloride maintains the osmotic balance. Haemophilus species may be differentiated by their requirements for X and V factors. Paper strips impregnated with these factors are placed on the surface of the medium after inoculation with the test organism. Following incubation, a zone of growth around the strip indicates a requirement for the factor(s).

**Formula / Liter**

|  |  |
| --- | --- |
| **Ingredients** | **Gms / Liter** |
| Pancreatic digest of casein | 15.00 |
| Papaic digest of soyabean (soyabean) | 5.00 |
| Sodium Chloride | 5.00 |
| Agar | 15.00 |

Final pH (at 25 °C) 7.3 ± 0.2

**Precautions and Disclaimer**

In vitro diagnostic use only . read the label before opening the container . wear protective gloves/ protective clothing / eye protection / face protection . follow good microbiological lab practices while handling specimens and culture . standard precaution as per established guideline should be followed while handling clinical specimens .

**Directions**

1. Suspend 40 grams of the medium in one liter of distilled water.

2. Heat to boiling, to dissolve the medium completely.

3. Autoclave at 121°C, 15 psi pressure, for 15 minutes / validated cycle.

4. Mix well and pour into sterile petri plates.

**Preparation Instructions**

Suspend 40 grams of Soyabean Casein Digest Agar in 1000 mls of distilled water. Boil to dissolve the medium completely. If desired, aseptically add 5% v/v defibrinated blood.

**Storage**

Store between 10 – 30 . in a tightly closed container and prepared medium at 20 – 30 c use before expiry date on the label . on opening , product should be properly stored dry , after tightly capping the bottle inorder to prevent lump formation due to the hygroscopic nature of the product . improper storage of the product may lead to lump formation . store in dry ventilated area protected from extremes of temperature and sources of ignition seal the container tightly after use . use before expiry date on the label . product performance is best if used within stated expiry period .

**Quality Control Specifications**

|  |  |
| --- | --- |
| Dehydrated Appearance | Cream to yellow homogeneous free flowing powder |
| Prepared Medium | Basal Medium : Light yellow coloured clear to slightly opalescent gel. After addition of 5-7%w/v sterile defibrinated blood : Cherry red coloured opaque gel forms in Petri plates |
| Reaction of 4.0% Solution | pH : 7.3 ± 0.2 at 25oC |
| Gel Strength | Firm, comparable with 1.5% Agar gel |

**Expected Cultural Response:** Cultural characteristics was observed after an incubation for Bacterial at 30-35°C 18-24 hours and for Fungal at 20-25°C <=5days

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| S No | Organisms | Inoculum | Observed Lot Value (CFU) | Recovery | Observed Lot value (CFU) w/blood | Recovery w/blood | Heamolysis |
| 1 | Bacillus subtilis ATCC 6633 | 50 – 100 | 35 -100 | >=70 % | 35 -100 | >=70 % | none |
| 2 | Staphylococcus aureus ATCC 25923 | 50 – 100 | 35 -100 | >=70 % | 35 -100 | >=70 % | beta |
| 3 | Staphylococcus aureus ATCC 6538 | 50 – 100 | 35 -100 | >=70 % | 35 -100 | >=70 % | beta |
| 4 | Escherichia coli ATCC 25922 | 50 - 100 | 35 -100 | >=70 % | 35 -100 | >=70 % | none |
| 5 | Escherichia coli ATCC 8739 | 50 – 100 | 35 -100 | >=70 % | 35 -100 | >=70 % | none |
| 6 | Escherichia coli NCTC 9002 | 50 – 100 | 35 -100 | >=70 % | 35 -100 | >=70 % | none |
| 7 | Pseudomonas aeruginosa ATCC 27853 | 50 - 100 | 35 -100 | >=70 % | 35 -100 | >=70 % | none |
| 8 | Pseudomonas aeruginosa ATCC 9027 | 50 – 100 | 35 -100 | >=70 % | 35 -100 | >=70 % |  |
| 9 | Salmonella Abony NCTC 6017 | 50 – 100 | 35 -100 | >=70 % | 35 -100 | >=70 % |  |
| 10 | Micrococcus luteus ATCC 9341 | 50 – 100 | 35 -100 | >=70 % | 35 -100 | >=70 % |  |
| 11 | Streptococcus pneumonia ATCC 6305 | 50 – 100 | 35 -100 | >=70 % | 35 -100 | >=70 % |  |
| 12 | Salmonella Typhimurium ATCC 14028 | 50 – 100 | 35 -100 | >=70 % | 35 -100 | >=70 % |  |
| 13 | Candida albicans ATCC 10231 | 50 - 100 | 35 -100 | >=70 % | 35 -100 | >=70 % |  |
| 14 | Candida albicans ATCC 2091 | 50 – 100 | 35 -100 | >=70 % | 35 -100 | >=70 % |  |
| 15 | Aspergillus brasiliensis ATCC 16404 | 50 – 100 | 25 -70 | 50-70% |  |  |  |

The organisms listed are the minimum that should be used for quality control testing.

**Test Procedure**

1. For clinical specimens, refer to appropriate standard references for details on testing protocol to obtain isolated colonies from specimens.

2. For water, food, dairy or cosmetic samples, refer to appropriate standard references for details on test methods.

3. For pharmaceutical samples, refer to USP General Chapter for details on the examination of nonsterile products and performing microbial enumeration tests.

**Results**

After incubation, it is desirable to have isolated colonies of organisms from the original sample. Subculture colonies of interest, so that positive identification can be made by means of biochemical and/or serological testing.

**disposal**

user must ensure safe disposal by autoclaving and / or incineration of used or unusable preparation of this product . follow established laboratory procedures in disposing of infectious materials and materials that come into contact with clinical sample must be decontaminated and disposed of in accordance with current laboratory techniques .   
 **Limitations of the Procedure**

1. For identification, organisms must be in pure culture. Morphological, biochemical and/or serological tests should be performed for final identification.

2. Consult appropriate texts for detailed information and recommended procedures

**Packaging**

**Product Name : Casein Soyabean Digest Agar (Soyabean Casein Digest Agar)**

**(Tryptone Soya Agar)**

**Product Code : DM247 Available**

**Pack sizes : 100gm/ 500gm**

**Cetrimide Agar**

**Intended use**cerimide agar is base is used for the selective isolation of pseudomonas aeruginosa from water and clinical specimens . **Principle and Interpretation:**pseudomonas aeruginosa grows well on all normal laboratory media but specific isolation of the organisms , from environmental sites or for human , animal or plant sources , is best carried out on a medium , which contain a selective agent and also constituents to enhance pigment production . most selective media depend upon the intrinsic resistance of the species to various antibacterial agents . cetrimide inhibits the growth of many microorganisms whilst allowing pseudomonas aeruginosa to develop typical colonies .   
cetrimide is a quaternary ammonium salt , which act as a cationic detergent that reduce surface tension in the point of contact and has precipitant , complexing and denaturing effects on bacterial membrane proteins . it exhibits inhibitory actions on a wide variety of microorganisms including pseudomonas species other than pseudomonas aeruginosa . developed medium A for the enhancement of pyocyanin production by pseudomonas . cetrimide agar developed by lowburry is a modification of tech agar ( medium A) with addition of 0.1% cetrimide for selective isolation of pseudomonas aeruginosa . later . due to the availability of the highly purified certimide , is concentration in the medium was decreased . the incubation was carried out at 37 C for a period of 18 – 24 hours .  
  
pseudomonas aeruginosa can be identified due to their characteristic production of pyocyanin , a blue , water soluble , non- fluorescent phenazine pigment coupled with their colonial morphology and the characteristic grape – like odor of aminoacetophenone . pseudomonas aeruginosa is the only species of pseudomonas or grams – negative rod known to excrete pyocynin . these media are therefore , important in the identification of pseudomonas aeruginosa . this media are used for the examination of cosmetics and clinical specimens for the presence of pseudomonas aeruginosa , as well as for evaluating the efficacy of disinfectant against this organism .   
  
gelatin peptone provide necessary nutrients for pseudomonas aeruginosa . sodium chloride maintains osmotic equilibrium in the medium . magnesium chloride and potassium sulfate stimulates pyocyanin production .   
for the isolation of pseudomonas aeruginisa , plates of cetrimide agar should be inoculated from non – selective medium such as brain heart infusion broth (M210) or soyabean casein digest medium (M011) . if the count is high , the test sample can be directly inoculated onto cetrimide agar . pseudomonas aeruginosa colonies may be appear pigmented blue , blue – green or non – pigmented . colonies exhibiting fluorescent at 250 nm and a blue green pigmentation are considered as presumptive positive . pseudomonas aeruginosa may lose its fluorescence under UV if the culture are left at room temperature for a short time . fluorescence reappears after the plates are re-incubated . type of peptone used in the base may also affect pigment production certain strains of pseudomonas aeruginosa may not produce pyocyanin . other species of pseudomonas do not produce pyocyanin but fluoresce under UV light . most non – pseudomonas species are inhibited on cetrimide agar , and some species of pseudomonas may also be inhibited . some non – fermenters and some aerobic spore formers may exhibit a water – soluble tan to brown pigmentation on this medium . Pancreatic digest of gelatin provide necessary nutrients for *P. aeruginosa such as*nitrogen, vitamins, and carbon.

The addition of magnesium chloride and potassium sulphate stimulates pyocyanin and pyoverdin (fluorescein) production.

Cetyltrimethylammonium bromide (Cetrimide) is the selective agent and inhibits most bacteria by acting as a detergent. When in contact with bacteria, causes the release of nitrogen and phosphorous from the bacterial cell other than *Pseudomonas aeruginosa.*

Glycerol is supplemented as a source of carbon.

Agar is the solidifying agent  
.

**Warning and precautions**in vitro diagnostic use only . read the label before opening the container . wear protective gloves/ protective clothing / eye protection / face protection . follow good microbiological lab practices while handling specimens and culture . standard precautions as per established guidelines should be followed while handling clinical specimens. Safety guidelines may be referred in individual safety data sheets **Composition:**

|  |  |
| --- | --- |
| Ingredients | In gm/Litre |
| Pancreatic Digest of Gelatin | 20.0 gm |
| Potassium Sulfate | 10.0 gm |
| Magnesium Chloride | 1.4 gm |
| Cetyltrimethylammonium Bromide | 0.3 gm |
| Glycerine | 10.0 ml |
| Agar | 13.6 gm |
| Distilled Water | 1000ml |
|  |  |

Final pH 7.2 +/- 0.2 at 25 degrees C

**Uses of Cetrimide Agar**

1. It is primarily used for the selective isolation and presumptive identification of Pseudomonas aeruginosa  
   from clinical and nonclinical specimens.
2. It is also used for determining the ability of an organism to produce fluorescein and pyocyanin (Antibiotica)

## **Preparation of Cetrimide Agar**

1. Add 45.3 gm of the medium in 1 litre of distilled water.
2. Add 10ml of glycerol and boil to dissolve completely.
3. Sterilize by autoclaving at 121°C for 15 minutes.
4. Cool the medium to approximately 50°C and pour into sterile Petri dishes.

## **Interpretation of Results on Cetrimide Agar**

The presence of growth is indicative of a positive reaction. Examine colonies under short wavelength (254nm) ultraviolet light for the presence of fluorescein. Visual examination may also reveal the typical**yellow-green to blue color** which indicates the production of pyocyanin. Both pyocyanin and fluorescein are typically produced by strains of P. aeruginosa.

## **Quality Control on Cetrimide Agar**

**Pseudomonas aeruginosa  ATCC 9027** – Yellow-green to blue colonies.

**Escherichia coli ATCC 8739** – Partial to complete inhibition. No Pigmentations.

## **Limitation of Cetrimide Agar**

1. Occasionally some enterics will exhibit a slight yellowing of the medium; however, this coloration is easily  
   distinguished from fluorescein production because this yellowing does not fluoresce.
2. Some non-fermenters and some aerobic spores formers may exhibit a water-soluble tan to brown pigmentation on this medium. Serratia strains may exhibit a pink pigmentation.
3. Studies of Lowbury and Collins showed P. aeruginosa can lose its fluorescence under UV if the cultures are left at room temperature for a short time. Fluorescence reappears when plates are re-incubated.
4. Further tests are necessary for confirmation of P. aeruginosa.  
     
   **Storage**

Store between 10 – 30 . in a tightly closed container and prepared medium at 20 – 30 c use before expiry date on the label . on opening , product should be properly stored dry , after tightly capping the bottle inorder to prevent lump formation due to the hygroscopic nature of the product . improper storage of the product may lead to lump formation . store in dry ventilated area protected from extremes of temperature and sources of ignition seal the container tightly after use . use before expiry date on the label . product performance is best if used within stated expiry period .

**disposal**

user must ensure safe disposal by autoclaving and / or incineration of used or unusable preparation of this product . follow established laboratory procedures in disposing of infectious materials and materials that come into contact with clinical sample must be decontaminated and disposed of in accordance with current laboratory techniques .   
  
  
  
  
  
 **Equipment / Instruments**

|  |
| --- |
| **Equipment Name** |
| Micropipette 100µL |
| Micropipette 1000µL |
| Weighing Balance |
| pH Meter |
| Autoclave |
| Decontamination Autoclave |
| Bacteriological Incubator 30-35˚C |
| Colony Counter |
| Biosafety Cabinet |
| Vortex Mixer |

**Comments: Above Equipment / Instruments were calibrated/validated state and were within validity period.**

* **Accessories**

|  |  |
| --- | --- |
| Petri Plates 90mm diameter, sterile | Pre-sterilized glass bottles |
| Container | PPEs |
| Micropipette tips 100µL-1000µL | Spreaders, Wire Loops |
| Test Tubes | Membrane filter, Filtration Assembly |

**Comments: Sterilized accessories were used following aseptic technique.**

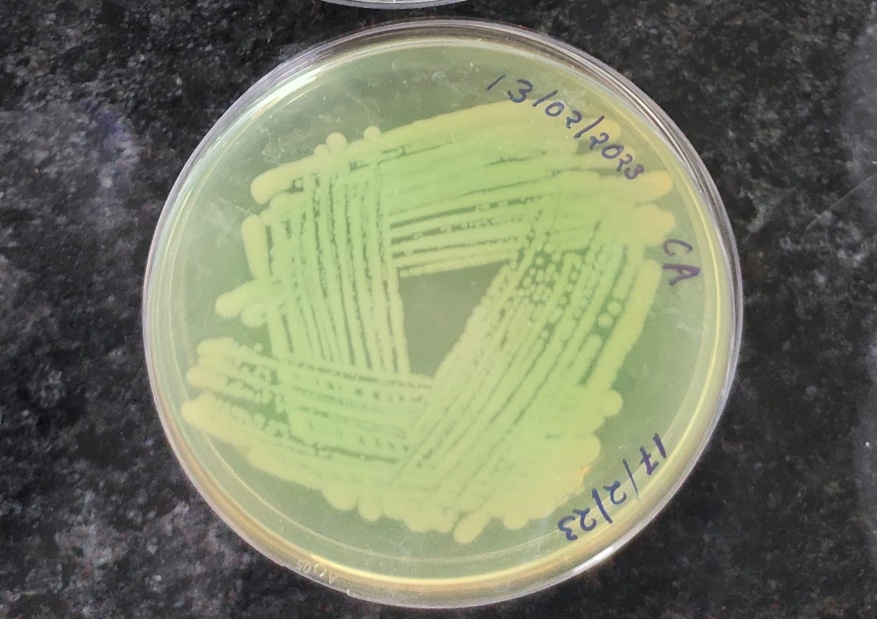
* **Isolation of *Pseudomonas***
* Take loopful of sample, streak it on Cetrimide Agar.
* Incubate the plates at 30-35°C for 72 hours.

1. Allow medium to adjust to room temperature prior to inoculation
2. Streak organism from pure culture or directly from the specimen. For plates, Perform a four quadrant streak to obtain well isolated colonies . if inoculating a tube , streak the surface Of the medium in a fish-tail motion from the bottom up
3. Incubate plates aerobically at 35 ⁰C
4. Examine after 18-24 hours . If no growth is absorbed over reincubate for up to three days before discarding

* **Identification of *Pseudomonas:***

1. **Morphological Identification**

* On the basis of colony, colour, shape & size.
* Gram Staining.
  + 1. Shape – pseudomonas aeruginosa is a slender , rod shape (bacillus) bacterium .
    2. Size – the size of pseudomonas aeruginosa about 1.5 – 3 mm into 0.5mm (micrometer) .
    3. Arrangement of cells – pseudomonas aeruginosa is arranged singly or in pair .
    4. Motility – pseudomonas aeruginosa is actively motile bacterium .



* + 1. Flagella – pseudomonas aeruginosa is a flagellated bacterium with a polar flagellum, Arranged in an amphitrichous manner .
    2. Spores – pseudomonas aeruginosa is a non-sporing bacterium
    3. Capsule – pseudomonas aeruginosa is a non-capsulated bacterium but some strains posses a slime layer .

**Grams staning**

**Introduction**

The Gram staining is one of the most crucial staining techniques in microbiology. It gets its name from the Danish bacteriologist Hans Christian Gram who first introduced it in 1882, mainly to identify organisms causing pneumonia. Often the first test performed, gram staining involves the use of crystal violet or methylene blue as the primary color. The term for organisms that retain the primary color and appear purple-brown under a microscope is Gram-positive organisms. The organisms that do not take up primary stain appear red under a microscope and are Gram-negative organisms.

The first step in gram staining is the use of crystal violet dye for the slide's initial staining. The next step, also known as fixing the dye, involves using iodine to form crystal violet- iodine complex to prevent easy removal of dye. Subsequently, a decolorizer, often solvent of ethanol and acetone, is used to remove the dye. The basic principle of gram staining involves the ability of the bacterial cell wall to retain the crystal violet dye during solvent treatment. Gram-positive microorganisms have higher peptidoglycan content, whereas gram-negative organisms have higher lipid content

Initially, all bacteria take up crystal violet dye; however, with the use of solvent, the lipid layer from gram-negative organisms is dissolved. With the dissolution of the lipid layer, gram negatives lose the primary stain. In contrast, solvent dehydrates the gram-positive cell walls with the closure of pores preventing diffusion of violet-iodine complex, and thus, bacteria remain stained. The length of decolorization is a critical step in gram staining as prolonged exposure to a decolorizing agent can remove all the stains from both types of bacteria

The final step in gram staining is to use basic fuchsin stain to give decolorized gram-negative bacteria pink color for easier identification. It is also known as counterstain. Some laboratories use safranin as a counterstain; however, basic fuchsin stains gram-negative organisms more intensely than safranin. Similarly, *Hemophilus* spp., *Legionella* app, and some anaerobic bacteria stain poorly with safranin.

**Types of equipment needed for Gram staining include:**

* Bunsen burner
* Alcohol-cleaned microscope slide
* Slide rack
* Microscope

**Reagents needed for Gram staining include :**

* Crystal violet (primary stain)
* Gram's iodine solution (the mordant)
* Acetone/ethanol (50:50 v:v) (the decolorizer)
* 0.1% basic fuchsin solution (the counterstain)
* Water

**Procedure**

**1. Preparation of a slide smear:**

* Inoculation loop is used to transfer a drop of suspended culture to the microscope slide.
* If a Petri dish or a slant culture tube has the colony, a drop or a few loopful of water is added to facilitate a minimal amount of colony transfer to the examination slide.
* A minimal amount of culture is required. If culture can be detected visually on an inoculation loop, it indicates the collection of too much culture.
* Culture is spread with an inoculation loop to an even thin film over a circle of 15mm in diameter. A typical slide can contain up to 4 small smears if examining more than one culture.
* The slide can be either air-dried or dried with the help of heat over a gentle flame. The slide should be moved circularly over the flame to prevent overheating or forming of ring patterns in the slide. The heat helps the cell adhesion to the glass slide and prevents the significant loss of culture during rinsing.

**2. Gram staining**

* Crystal violet stain is added over the fixed culture.
* After 10 to 60 seconds, the stain is poured off, and the excess stain is rinsed with water. The goal is to wash off the stain without losing the fixed culture.
* Iodine solution is used to cover the smear for 10 to 60 seconds. This step is known as "fixing the dye." Iodine solution is poured off, and the slide is rinsed with running water. Excess water from the surface is shaken off.[[7]](https://www.ncbi.nlm.nih.gov/books/NBK562156/)
* A few drops of decolorizer is added to the slide. Decolorizers are often the mixed solvent of ethanol and acetone. This step is known as "solvent treatment." The slide is rinsed with water in 5 seconds. To prevent excess decolorization in the gram-positive cells, stop adding decolorizer as soon as the solvent is not colored as it flows over the slide.
* The smear is counterstained with basic fuchsin solution for 40 to 60 seconds. The fuchsin solution is washed off with water, and excess water is blotted with the bibulous paper. The slide can also be air-dried after shaking off excess water.

**3. Microscopic examination of slide:**

* The slide should undergo an examination under a microscope under oil immersion.
* The initial slide examination should use the X40 objective to evaluate the smear distribution, and then they should be examined using the X100 oil immersion objective.
* All areas of the slide require an initial examination. Areas that are only one cell thick should be examined. Thick areas in slides often give variable and incorrect results.
* White blood cells and macrophages stain Gram-negative.
* Squamous epithelial cells stain Gram-positive.

1. **Biochemical Identification**

* Oxidase test by disc method.
* Catalase Test by slide method.

**Oxidase test by disc method**   
  
 **Requirement for oxidase test**   
  
 Culture Media

While performing the oxidase test following either the disc method or filter paper method or swab method or direct plate method, there is no need for culture media. Bacteria grown in any selective medium (or pure colonies from any media) can be used for the test.

However, for the tube method (Gaby and Hadley method) of the oxidase test, nutrient broth medium (or any standard broth medium with low glucose content) is required. (Here, we will use nutrient broth.)

**Composition of Nutrient Broth per 1000 mL**

Peptone- 5.00 grams

HM Peptone B (Beef Extract)- 1.50 grams

Yeast Extract- 1.50 grams

Sodium Chloride- 5.00 grams

Final pH 7.4 ±0.2 at 25°C

**Preparation of Nutrient Broth**

Measure the appropriate amount of nutrient broth powder (or the media components) and mix in the water of the required volume in a conical flask (or glass bottle) according to the instruction of the manufacturing company.

Stir well using a magnetic stirrer or manually and heat to boiling if necessary so that all the components dissolve completely in water.

Dispense 5 mL of broth in each test tube and loosely put on the screw cap (or use a cotton plug to cover the opening).

Autoclave the tubes with nutrient broth at 1210C and 15 lbs pressure for 15 minutes and let it cool to room temperature before inoculation.

**Reagents**

Kovacs’ Oxidase Reagent (for the disc, filter paper, swab, or direct plate method)

1% N, N, N, N-tetramethyl-p-phenylenediamine dihydrochloride

Preparation of Kovacs’ oxidase reagent:

* Dissolve 1.0 grams of N, N, N, N-tetramethyl-p-phenylenediamine dihydrochloride in 100 mL of sterile distilled water and mix well.

Gordon and McLeod Oxidase Reagent (for the disc, filter paper, swab, or direct plate method)

1% dimethyl-p-phenylenediamine dihydrochloride

Preparation of Gordon and McLeod Reagent

* Dissolve 1.0 grams of dimethyl-p-phenylenediamine dihydrochloride in 100 mL of sterile distilled water and mix well.

Gaby-Hadley Reagents (for tube method)

Reagent A (1% α-naphthol)

* Add 1.0 grams of α-naphthol in 100 mL of 98% ethanol.

Reagent B (1% p-amino dimethylaniline oxalate)

* Add 1.0 grams of p-amino dimethylaniline oxalate in 100 mL of distilled water.

**Impregnated Oxidase Disc/Test Strip**

**Equipment**

**A.** Petri plates  
**B**. whatman no. 1 filter paper ( disc or strip )  
**C**. weighing machine   
**D.** autoclave   
**E**. Bunsen burner   
**F.** test tube   
**G.** dropper   
**H.** inoculating loop   
**I.** cotton swab   
**J.** PPE other laboratory material  
  
**Impregnated Disc/Strip (Oxidase Disc) Method**

1. Place the impregnated oxidase disc or strip over a clean petri plate (or glass slide) and moisten it with sterile deionized water. (Some discs may not need to be moistened. Look for the manufacturer’s instructions.)
2. Using a sterile inoculating loop, pick up a well-isolated colony of test bacteria from fresh culture and make a smear on the oxidase disc/strip.

 Observe for color change and note the time required for change in color for up to 60 seconds.  
  
  
  
**precautions during oxidase test**

1. Don’t test bacteria grown on media with dyes (like EMB, MAC medium).
2. Don’t test bacteria grown on a glucose-rich culture medium.
3. Don’t test strict anaerobes.
4. Use freshly made oxidase reagent.
5. While storing the oxidase reagent, store it in a dark place at – 200C.
6. The sample must be taken from well-isolated colonies. Never perform the direct plate method if the culture is mixed culture. (It is recommended to use cultures from selective media to ensure sample purity.)
7. Don’t overflood the plate with an oxidase reagent.
8. Never record the result after 60 seconds while using Kovacs reagent and after 3 minutes while using Gaby-Hadley Reagents.
9. Record time exactly to differentiate rapid oxidase-positive, delayed oxidase-positive, and oxidase-negative bacteria.
10. Do not use nichrome wire loops, as they can give false-positive results.

**catalase test by slide method**  
  
**Principle**

**2H2O2 → 2H2O+ O2 (gas bubbles)**

Catalase mediates the breakdown of hydrogen peroxide (H2O2) into oxygen and water. To find out if a particular bacterial isolate can produce catalase enzyme, a small inoculum of a bacterial isolate is mixed into hydrogen peroxide solution (3%). It is observed for the rapid elaboration of oxygen bubbles. The lack of catalase is evident by a lack of or weak bubble production.

Catalase-positive bacteria include strict aerobes as well as facultative anaerobes. They all can respire using oxygen as a terminal electron acceptor.

Catalase-negative bacteria may be anaerobes or facultative anaerobes that only ferment and do not respire using oxygen as a terminal electron acceptor (i.e. streptococci).

**Percentage of H2O2 used in catalase test**

|  |  |
| --- | --- |
| **Percentage** | **Purpose** |
| 3% H2O2 | Routine testing of aerobes |
| 15% H2O2 | Identification of anaerobic bacteria. |
| 30% H2O2 | In the superoxol catalase (used for the presumptive speciation of specific *Neisseria* sps) |

**procedure**  
  
**slide method** Transfer a small amount of bacterial colony to a surface of a clean, dry glass slide using a loop or sterile wooden stick (be sure the colony is visible to the naked eye on the slide).

Place a drop of 3% H2O2 onto the slide and mix.

A positive result is the rapid evolution of oxygen (within 5-10 seconds), as evidenced by bubbling.

A negative result is no bubbles or only a few scattered bubbles.\*

Dispose of your slide in the biohazard glass disposal container.  
  
  
 **Biochemical Identification**

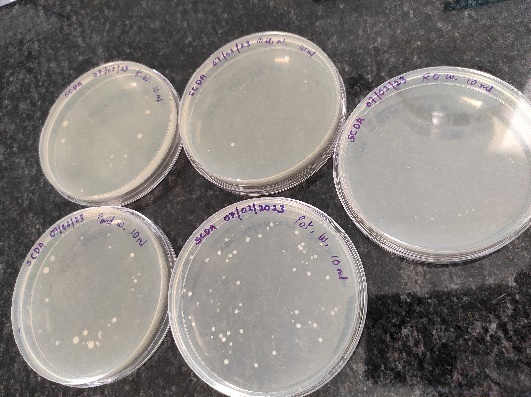
|  |  |  |
| --- | --- | --- |
| **Isolate** | **Oxidase test** | **Catalase test** |
| *Pseudomonas* | + | + |

**Antagonistic/Symbiotic Activity**pseudomonas is a genus of grams – negative bacteria that can be found in various environment , including water. Some strains of pseudomonas can exhibit both antagonistic and symbiotic activities against other micro –organism .  
  
antagonistic activity refers to the ability of pseudomonas to inhibit the growth or activity of other micro-organisms . this can occur through the production of antimicrobial compounds , competition for nutrients , or other mechanisms .  
   
on the other hand , symbiotic activity refers to the ability of pseudomonas to form mutually beneficial relationships with other micro-organisms . this can occur through the exchange of nutrients or other signaling molecules . for example , pseudomonas aeruginosa can form biofilm with other bacteria in water , which can enhance the survival and growth of both species .   
the antagonistic and symbiotic activities of pseudomonas can vary depending on the strain and environmental condition . therefore , its important to study the specific strains of pseudomonas and the micro-organisms they interact with to fully understand their ecological roles in water environments .   
  
Antagonistic testing is a method used to determine the ability of one micro-organisms to inhibit or kill another .   
  
**materials :-**1. pseudomonas aeruginosa culture   
2. agar plate   
3. Inoculating loop   
4. Other micro-organisms to be tested (optional )  
5. Bunsen burner   
6. Normal saline   
7. IPA ( isopropyl alcohol )  
  
**procedure :-**  
1.prepare the test micro-organisms :  
cultivate the test micro-organisms ( those that will be challenged by pseudomonas ) in nutrient rich media until they reach the stationary phase .   
  
2. Prepare the pseudomonas aeruginosa :  
cultivate the pseudomonas aeruginosa that will be tested for its antagonistic activity in a nutrient rich media until it reaches the stationary phase .  
  
3. Inoculate the pseudomonas aeruginosa :  
inoculate the pseudomonas aeruginosa into an agar plate with a steril swab . use a streaking method to spread the bacteria evenly over the agar surface .   
  
4. Incubate the plate :  
incubate the plate for 24 – 48 hours at the appropriate temperature for pseudomonas .  
   
5. Inoculate the test micro-organisms :  
inoculate the test micro-organisms into the agar plate next to the streak of pseudomonas , using a sterile loop or swab . make sure to label the area where the test micro-organisms are inoculated.  
  
6. Incubate the plate again :  
incubate the plate for 24 – 48 hours .   
  
7. Observe the result :  
check for any inhibition zone around the pseudomonas aeruginosa . if there is no growth or reduced growth of the test micro-organism around the pseudomonas aeruginosa , it indicates that pseudomonas has an antagonistic effect against those micro-organisms.   
  
**note : - it is essential to perform proper controls and replicate the experiment to obtain reliable results . also, always follow appropriate safety measures when working with micro-organisms** .  
  
Against Various Microorganisms:

* *Candida albicans*
* *Escherichia Coli*
* *Staphylococcus aureus*
* *Staphylococcus epidermidis*
* *Micrococcus Luteus*
* *Pseudomonas aeruginosa*
* *Bacillus thuriengensis*
* *Bacillus subtilis*

**RESULTS**

|  |  |
| --- | --- |
|  |  |
| **Fig. 01: Water Sample Testing** | **Fig.02: Pre-Test Sterile Media, Plates in Biosafety Cabinet** |

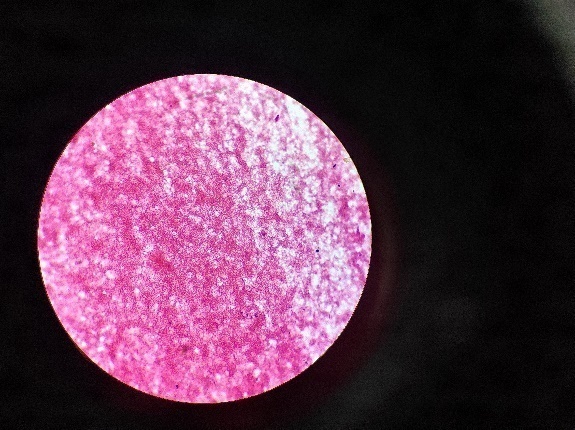


**Fig. 03: Isolation of Pseudomonas from different water samples.**

|  |  |
| --- | --- |
| **Sample Names** | **Presence of *Pseudomonas*** |
| Muncipal Supply Drinking Water (Treated Water) | Present |
| RO Water (Purified Water) | Absent |
| Pond Water (Talawali Pond) | Present |
| Potable Water (Matka Stored Public Drinking Water) | Present |
| Kshipra River Sangam Water | Present |

1. **Morphological Identification:**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Isolate** | **Shape** | **Colony Size** | **Colony Colour** | **Gram staining** |
| *Pseudomonas* | rod-shaped | 3-4 mm | Greenish | Gram negative rods |



**Fig.03: Pink Colour colony seen in Microscopic observation.**

**2. Antagonistic/Symbiotic Activity**



**Fig. 04: Antagonistic/Symbiotic Activity against various microorganisms**

|  |  |  |
| --- | --- | --- |
| **Microorganisms** | **Antagonistic Activity** | **Symbiotic Activity** |
| *Candida albicans* | -ve | +ve |
| *Escherichia Coli* | -ve | +ve |
| *Staphylococcus aureus* | -ve | +ve |
| *Staphylococcus epidermidis* | -ve | +ve |
| *Micrococcus Luteus* | -ve | +ve |
| *Pseudomonas aeruginosa* | -ve | +ve |
| *Bacillus thuriengensis* | -ve | +ve |
| *Bacillus subtilis* | -ve | +ve |

**DISCUSSION**

*Pseudomonads* are a large group of free-living bacteria that live primarily in soil, seawater, and fresh water.Pseudomonas spp. are common inhabitants of aquatic environments, including drinking water.*Pseudomonas* are highly versatile and can adapt to a wide range of habitats, and can even grow in distilled water.

In high bio-burden water samples such as pond, pot water, lake pseudomonasis frequently present, but in case of low bio-burden like RO, municipal water pseudomonasis rarely present. *Pseudomonas* is widely knowns as highly contaminant microbe in pharmaceutical industries, laboratories.

In this study, *Pseudomonas*isolated from different high bioburden water samples as it is frequently present in nature and isolation rate was 4/5 samples.

*Pseudomonas* cause several diseases in human as well as in animals. In normal healthy humans, they are responsible for eye and skin diseases Contamination of recreational waters and tap water has been associated with outbreaks of *Pseudomonas*; however, the relative role water plays in the transmission of this bacterium to humans is still unclear.

On the other hand, *Pseudomonas* shows various beneficial effects against various pathogens. *P. aeruginosa* produces a substance called ‘Pseudocin’ that has a bacteriostatic effect on the growth of *E. coli*, Aerobacter aerogenes, Citrobacter freundii and Klebsiella sp. (Coelho ***et al***. 2010), and can interfere in colorimetric analysis by inhibiting their growth in culture media (Vasconcelos ***et al***. 2006).

Also it shows symbiotic association with some microorganisms

**SUMMARY**

Present study was started on 23/01/2023 and ended on 22/03/2023.

The study objectives were

Isolation of *Pseudomonas* from water.

Study of Pseudomonas antagonistic or symbiotic against microorganisms.

Present study was proposed to study isolation of *Pseudomonas* from water such as municipal supply drinking water (Treated water), pond water (Talawali Pond), RO water (purified water), potable water (matka – stored public drinking water), kshipra river sangam water and *pseudomonas* antagonistic / symbiotic activity against microorganisms.

**SAFETY, HEALTH & ENVIRONMENT**

|  |  |  |
| --- | --- | --- |
| **Hazards** | **Consequences** | **Precautions** |
| Culture Spillage | Contamination/ Infection | Analyst Shall Wear PPEs. |
| Glassware breakage / Hot media falling on skin | Skin injuries | Analyst shall wear PPEs |
| Chemical spillage | Skin irritation | Analyst shall wear PPEs and wash immediate concern skin area with tap water |

**REFERENCES**

Prasad B., Antony R., Malviya J., Kaushal A., and Tiwari S.: Bacterial screening of river Kshipra water at Ujjain after Kumbh (Religious Fair).Indian Journal of Applied Research, 2017; 3(6): 310-312.

Bhasin S., Shukla A. and Shrivastava S., :Bacterial diversity of river Kshipra with relation to Human Health. Environment Conservation Journal, 2020; 21 (1&2): 63-74.

C hardalo , SC Edberg – critical reviews in microbiology , 1997 – taylor and francis

EE geldreich – 2020 – books.google.com

Reynolds, K. A., Mena, K. D., and Gerba , C.P. (2008). Risk of waterborne illness via drinking water in the united states. In D. M. Whitacare (Ed.), Review of environmental contamination and toxicology;vol.192).

E Kumpel, KL Nelson – Environmental science and technology , 2016 – ACS Publication

Wu, jun, et al. “isolation and characterization of pseudomonas aeruginosa from different sources of drinking water in china . “ journal of water and health 12.2(2014):319-327.

Navon –Venezia , Shiri, et al. “Occurance and distribution of pseudomonas aeruginosa in water supplies :a case study in a rehabilitation center.”journal of applied microbiology 105.3 (2008):681-687.