Tuijin Jishu/Journal of Propulsion Technology
ISSN: 1001-4055
Vol. 46 No. 2 (2025)

Isolation of Gram -ve Bacteria from the Sewage Water and Urine Samples, Synthesis & Characterization of Sodium Nitrate Nano Particles using UV- Visible Spectroscopy and Determination of UV Decimal Death Time of Gram -ve Bacteria *P. vulgaris, P.mirabilis* and *K.pneumoniae*

Eswari Beeram [1*], M.D. Sihan [2], Vansh Nandanwar [3], Shaik Thasleem Bhanu [4]

[1] *Assistant Professor, Department of Biological and Chemical Sciences, School of Liberal Arts and Sciences, Mohan Babu University, Andhra Pradesh, India*

[2, 3, 4] *B.Sc. MiBiC, Department of Chemical Sciences, Sree Vidyanikethan Degree College, Andhra Pradesh, India*

Abstract:- We have isolated gram -ve bacteria *Enterobacter aerogenes* and *Pseudomonas aeruginosa* successfully from sewage and urine samples and the bacteria shown to possess swarming motility. Both gram negative bacteria *Enterobacter aerogenes* and *Pseudomonas aeruginosa* has shown negative tests for Indole test and methyl red test and positive for citrate utilisation test. Studies on antimicrobial activity using *Enterobacter aerogenes* proved to possess antifungal properties and inhibit the growth of fungus strain *C.tropicalis* isolated from mouth washings. *Klebsiella pneumoniae* forms combined biofilms with *Candida tropicalis* and responsible for fungal abundance appearance on surface of agar plate. *C.tropicalis* can be able to grow on chromogenic substrates like PNPP and bile pigments due to presence of enzyme alkaline phosphatase and can survive in stools of humans. Sodium nitrate nano particles are synthesized and characterized using U.V visible spectrophotometer through a scan with range of 260nm to 360nm and the decimal death rate of bacteria *P. vulgaris, P.mirabilis* and *K.pneumoniae* were calculated accordingly and graphically represented with 7 minutes in case of *P. mirabilis* and *K. pneumoniae* and 5 minutes for *P.vulgaris*.

Keywords*: Enterobacter aerogenes, Pseudomonas aeruginosa, C.tropicalis, Decimal death rate, Fungal abundance, biofilm formation.*

1. Introduction

E. aerogenes is newly named as *K.aerogenes* and it is the pathogen associated with the poor clinical outcome. *Enterobacter genus* possess total of 983 genes in which around 300 is identified to be genes coding for virulence factors (Wesevich. A et al., (2020), Gu et al., (2022)). characterized using pan genome analysis carried on bacteria recently by the scientists. *K.aerogenes* is recognized as one of the pathogen associated with multi drug resistance compared to other strains and identified as bacterium that can cause blood borne infections. The pathogenicity and poor clinical outcome of the bacteria is majorly attributed due to its virulence factors it possess.

P. aeruginosa is a gram negative opportunistic pathogen that causes chronic obstructive pulmonary disease and infects patients with severe burns, wounds and cystic fibrosis (Qin.s et al., (2022). *P.aeruginosa* is one of the bacteria most oftenly studied as a model organism in the microbial population. Even though so many advances through the studies on this model organism due to lack of proper understanding and research findings like how the immune system of host responds to the pathogen through the signaling mechanisms and what are the host immune system responses to the pathogen it is difficult even now to develop novel drugs that can treat the infections caused by the pathogen. So, studies on isolation of *E.aerogenes* and *P.aeruginosa* from the biological samples can create a platform in creating some novel drugs to treat the infections caused by these pathogens because of the associated virulence.

2. Methodology

Serial Dilution:

1ml of the Urine and sewage water samples is diluted serially from 10^{-1}-10^{-9} dilution range and 0.1ml of the diluted sample from 10^{-7} dilution is used for plating on Macconkey agar using spread plate technique.

Subculturing of Microbes on MacConkey Agar w/o CV, NaCl w/ 0.5% Sodium Taurocholate:

0.1 ml of serially diluted sample is plated on the Macconkey agar and the colonies grown on macconkey agar medium is used for sub culturing on MacConkey Agar w/o CV, NaCl w/ 0.5% Sodium Taurocholate to restrict the swarming motility of the *Proteus spp*. Strains like *P.aeruginosa* and *E.aerogenes* can able to grow on 0.5% sodium taurocholate and the microbes cultured are observed and preliminary identification is done based on their colony morphology and lactose utilisation.

Gram Staining:

Macconkey agar plates and MacConkey Agar w/o CV, NaCl w/ 0.5% Sodium Taurocholate agar plates were observed for identified microbial growth and the bacteria is differentiated in to either Gram positive or negative using Gram staining Procedure, which in brief explained below.

Thin smear of bacterial culture is made on the glass slide using inoculation loop and subjected to heat fix. Heat fixed slides were flooded with crystal violet for 1 min. After 1min the slide is washed under running tap water and flooded with Iodine solution and allowed to stand for 1min and similarly with acetone or ethanol for 15-30 sec. Finally the slides are counter stained with saffranin for 1 min, washed under running tap water, air dried and observed over 20X objective lens using bright field microscope and the images are captured.

IMVIC Tests:

The isolated strains are biochemically characterized using Indole test, Methyl red and Citrate utilization tests for identification of the genus of the strain.

Isolation and Identification of Candida Tropicalis:

10 ml of drinking water is taken in the mouth and allowed for mouth goggling for few minutes and the sample collected is used for serial dilution. 0.1ml of serially diluted sample is used for isolation and cultivation of Candida on potato dextrose agar. Confirmation of the fungal strain was carried through staining by Lactophenol cotton blue stain and examination over the bright feild microscope. Isolated fungal culture is used for study of antigonistic and synergistic effect on growth of *P. vulgaris, P.mirabilis* and *K.pneumoniae on C.tropicalis* are studied using agar well plating method.

Chromogenic Substrate Utilization by *C.tropicalis*:

Nitrophenol phosphate is used as chromogenic substrate for the C.tropicalis due to presence of enzyme alkaline phosphatase. 0.1 g of PNPP is weighed and mixed in the sterilised agar in the biosafety chamber under sterile conditions and poured in to petriplates and allowed to solidify. 0.1ml of the fungal culture grown overnight in czapexdox broth is spread using L- rod on the agar plate. Wells are made using sterile tips after spreading and the

bacterial strain used for the study is added to the wells and allowed for incubation for 48 hrs and the results are recorded.

Synthesis of Sodium Nitrate Nano Particles:

Concentration of 1mM, 2mM and 3mM solutions of Sodium hydrogen phosphate is prepared and same concentration of potassium Nitrate is weighed and added to previous solution with magnetic stirring to synthesize the sodium nitrate nano particles and Polyethylene glycol is used as a reducing agent. All the three solutions are allowed to stand for 5 to 15 days and the synthesis of sodium nitrate nano particles are monitored on 5^{th} day and 12^{th} day using UV - Visible spectrophotometer.

Antagonistic/ Antimicrobial Effect of Proteus on E.aerogenes:

Pure culture of *Proteus mirabilis* and *P.vulgaris* and *K. pneumoniae* isolated from the root extracts of *Kalanchoe pinnata, Euphorbia tithymaloides* and *Murray koeingii* are tested for their anti microbial effect on *E.aerogenes* by using well plating method. Respective strain used for testing is taken in the well.

3. Results

The bacterial strain isolated from sewage sample can be confirmed as *Enterobacter aerogenes* based on the colony morphology, lactose utilization on the macconkey agar, and positive biochemical IMVIC tests shown by the bacterial strain. *E.aerogenes* is a lactose fermenter and forms pinkish colonies on the macconkey agar and the strain is positive for citrate utilisation test and negative for Indole test and Methyl red test procedures.

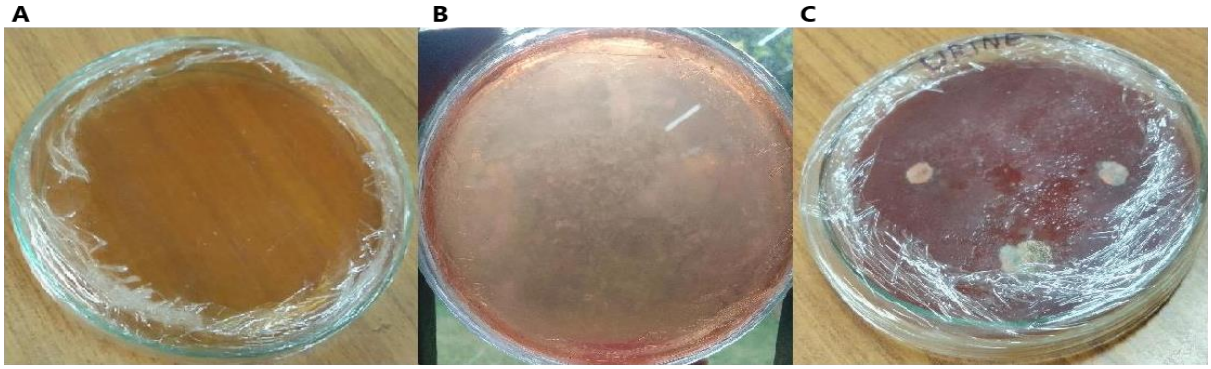


Figure: 1 Colony Morphology of *Enterobacter aerogenes* and *Pseudomonas aeruginosa* on Macconkey agar isolated from the sewage and urine samples (A) control (B) Colonies of *E.aerogenes* on Macconkey agar (C) *P.aeruginosa* Colonies on Macconkey agar.

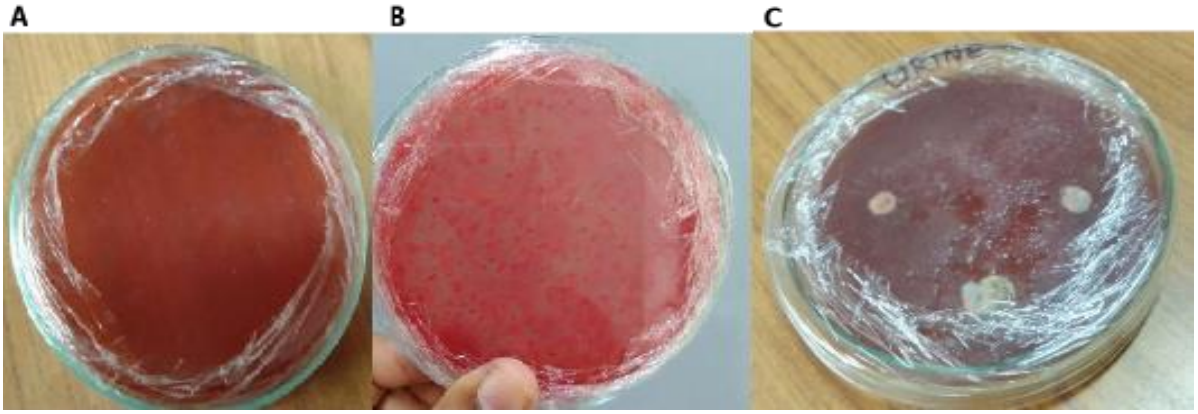


Figure: 2 Colony morphology of *Enterobacter aerogenes* and *Pseudomonas aeruginosa* isolated from sewage and urine samples on MacConkey Agar w/o CV, NaCl w/ 0.5% Sodium Taurocholate. (A) Control (B) Colonies of *Enterobacter aerogenes* on agar plate (C) Colonies of *P.aeruginosa* on Macconkey agar plate

Pseudomonas aeruginosa is a non lactose fermenter and form white colonies on the macconkey agar and it contains unipolar flagella identified through gram staining and confirmed to be gram negative. *P.aeruginosa* is isolated from urine sample of the human and identified by positive biochemical tests for citrate utilisation test (+ve), Indole test (-ve) and methyl red test (-ve) .

E.aerogenes is identified as gram negative bacteria and showed swarming motility while examination through gram staining and captured. In both the samples control showed no growth. *P.aeruginosa* can grow on MacConkey Agar w/o CV, NaCl w/ 0.5% Sodium Taurocholate due to its ability to grow on 5% NaCl and Sodium Taurocholate. *Enterobacter aerogenes* can grow on MacConkey Agar w/o CV, NaCl w/ 0.5% Sodium Taurocholate because of its ability to grow on sodium taurocholate and low concentrations of NaCl. High salt concentrations like 5% NaCl can cause reduction in growth but cannot inhibit the complete growth of bacteria. From fig.2C we can clearly observe the reduction in *E. aerogenes* growth compared to *P.aerugonisa* (Fig.1C).

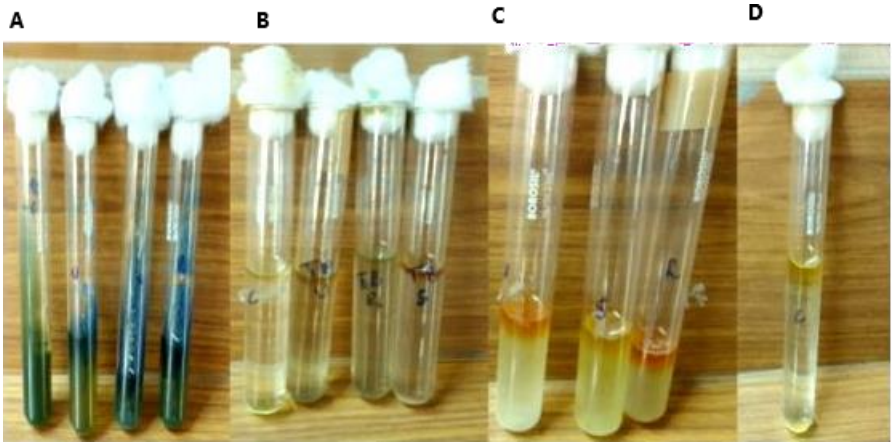


Figure: 3: Biochemical characterization of *E.aerogenes* and *P.aeruginosa* through IMVIC Tests. (A) Citrate utilisation test (B) Indole test (C) Methyl red test (D) Control

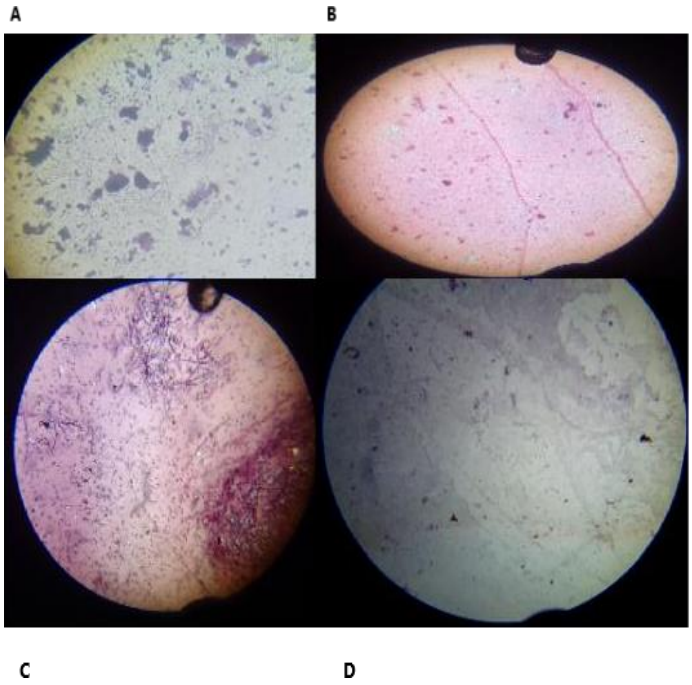


Figure: 4 Microscopic examination and categorization of bacteria in to Gram Positive and Gram Negative through Gram Staining procedure. (A&B) Gram staining of bacteria isolated using Macconkey agar and MacConkey Agar w/o CV, NaCl w/ 0.5% Sodium Taurocholate (sewage sample). (C &D) Gramstaining of bacteria cultured on MacConkey Agar w/o CV, NaCl w/ 0.5% Sodium Taurocholate (urine sample)

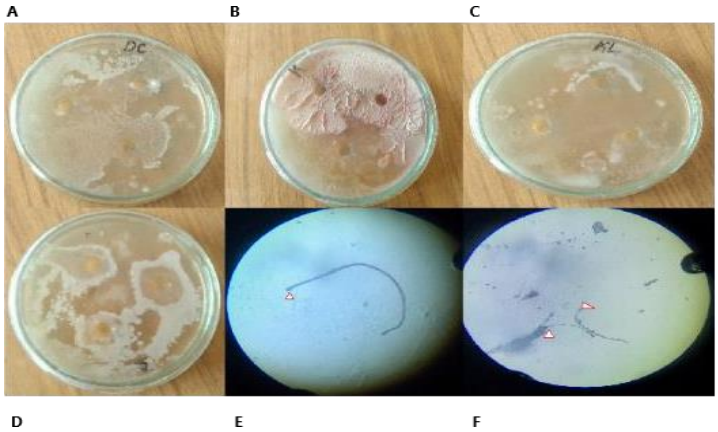


Figure: 5 Examination of Antogonistic and Synergistic effect of *Proteus vulgaris* (A), *Proteus mirabilis* (C), *Klebsiella pneumoniae* (B) and *Enterobacter aerogenes* (D) on *Candida tropicalis* isolated from mouth sample. (E &F) Confirmation of fungal strain as *C.tropicalis* through Lacto phenol cotton blue staining. Arrows point outs the mucor heads.

Inhibition of growth of *Candida tropicalis* can be seen with combinational cultivation of bacterial strains like *Proteus vulgaris, P.mirabilis, Klebsiella pneumoniae* and *E.aerogenes* with the fungus *C.tropicalis*. *C. tropicalis* is isolated from the mouth and the fungal strain is identified as *C.tropicalis* by Lactophenol cotton blue staining (Fig.5F). *E.aerogenes* completely inhibit the growth of *C. tropicalis* observed as by the clearance zones formed on the agar plate in Fig.5D. *Proteus mirabilis* inhibit the growth of *C.tropicalis* clearly identified with the reduced growth observed on the agar plate Fig.5A. *P. vulgaris* inhibit the biofilm formation by *Candida* on the agar plate from Fig.5C. *Klebsiella pneumoniae* and *candida tropicalis* shown to form combine biofilms and responsible for high virulence capacity of the microbes from Fig.5B. *Klebsiella pneumoniae* and *Candida tropicalis* combined fungal abundance is mostly responsible for deadful infections in infected persons.

P. vulgaris , P. mirabilis and Klebsiella pneumoniae was isolated from the root samples of the plants *Euphorbia tithymaloides, Kalanchoe pinnata* and *Murray koenigii* and was used for the study (Eswari Beeram et al., (2024),Chakkour M et al., (2024),Wang et al., (2022)).

Candida tropicalis (Fig. 5F) is characterized for its growth on Para nitro phenol phosphate (PNPP) and bile pigments both individually and in mixed culture and proved to possess the ability to growth on PNPP due to presence of enzyme Alkaline phosphatase (Kadri O etal., (2010)). *C.tropicalis* can able to grow on bile pigments and utilization of bile pigments is responsible for the survival of fungus *Candida* in stool samples.

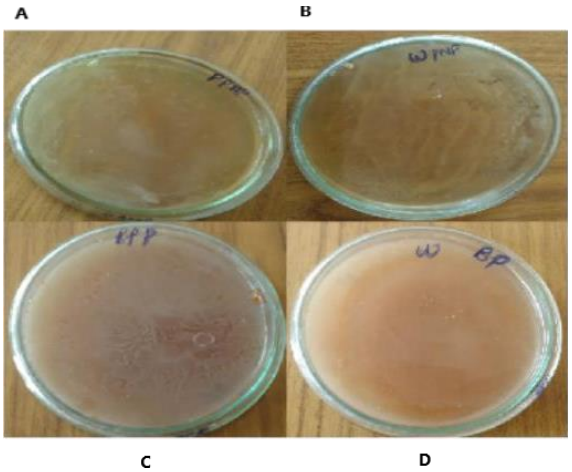


Figure:6 Characterization of *Candida tropicalis* on Chromogenic substrate P- Nitro phenol phosphate and Bile pigments. (A) Control (B) *C.tropicalis* mycelium on agar plate containing PNPP as substrate (C &D) *C. tropicalis* grown on agar plate containing bile pigments.

Proteus mirabilis and P.vulgaris showed to possess antimicrobial activity on *E.aerogenes* clearly identified by the formation of antimicrobial zone as in fig 7B and reduced growth with no zone of inhibition on agar plate Fig.7D.

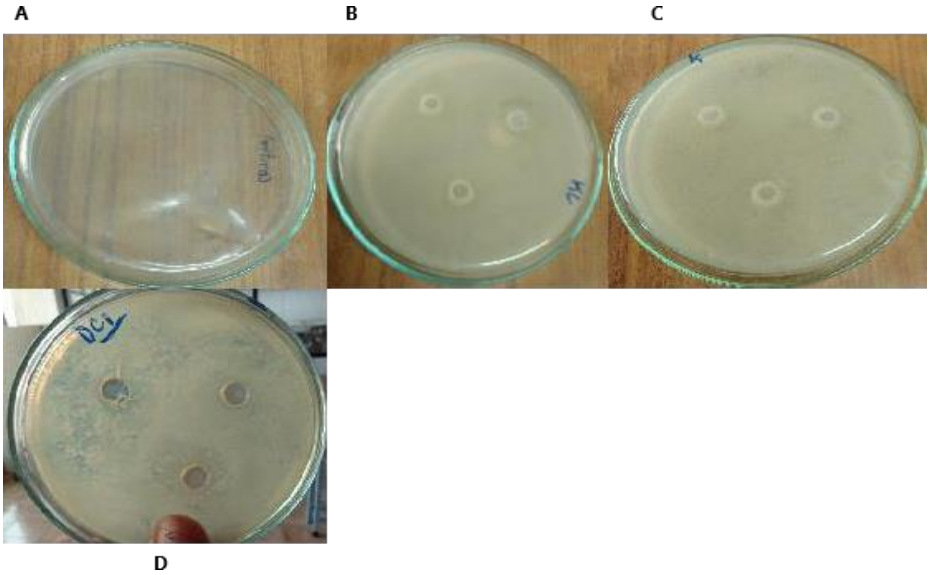


Figure:7 Antagonistic effect of *Proteus* strain on growth of *Enterobacter aerogenes*. (A) Control (B) Antogonistic effect of *P. mirabilis* isolated from *Kalanchoe pinnata* root on *E.aerogenes* growth (c) Negative Effect on growth of *Enterobacter aerogenes* by *K.pneumoniae* isolated from Murray koenigii (D) Antagonistic effect of *P.vulgaris* isolated from *Euphorbia tithymaloides* on *E.aerogenes* isolated from sewage.

Sodium nitrate nano particles are synthesized and characterized using UV- visible spectrophotometer, Shimadzu through scanning range from 260nm to 360 nm. Sodium nitrate usually produces a low absorbance peak in the range of 280nm to 340nm (Gokul P et al., (2021),Nadeem B et al., (2021)). The absorbance is recorded at 5th day and 12th day of synthesis (Fig.8A&B). After characterization the liquid sample is poured in to petriplates and allowed for evaporation at room temperature and the pictures are captured from the evaporated petri dishes Fig.9.

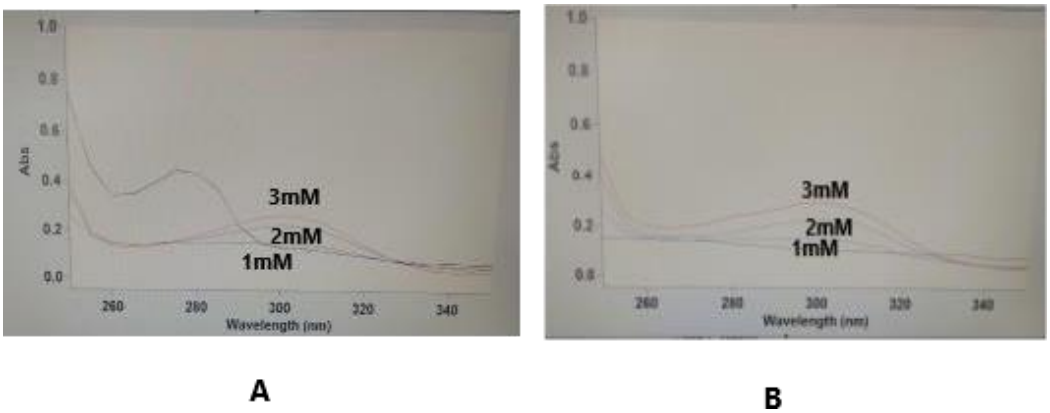


Figure: 8 Characterization of synthesized Sodium Nitrate Nano particles using UV - Visible spectrophotometer at 260nm to 360nm scan. (A&B) at 5th day and 12th day of synthesis.

UV decimal death curve was calculated for attenuated strains of *P. Mirabilis, P.vulgaris* and *K.pneumoniae a*nd the death rate was reported at 7th min of exposure to U.V for *P. Mirabilis* and *K. pneumoniae* and at 5th min in case of *P. vulgaris* through concurrent readings. *P.Mirabilis* is proven to resistant to UV and requires greater time period exposures of UV. (Table 1).

U.V exposure decimal death rate data obtained is represented graphically in radar chart for all three bacterial strains *P. Mirabilis, P.vulgaris and K.pneumoniae* (Graph 1).

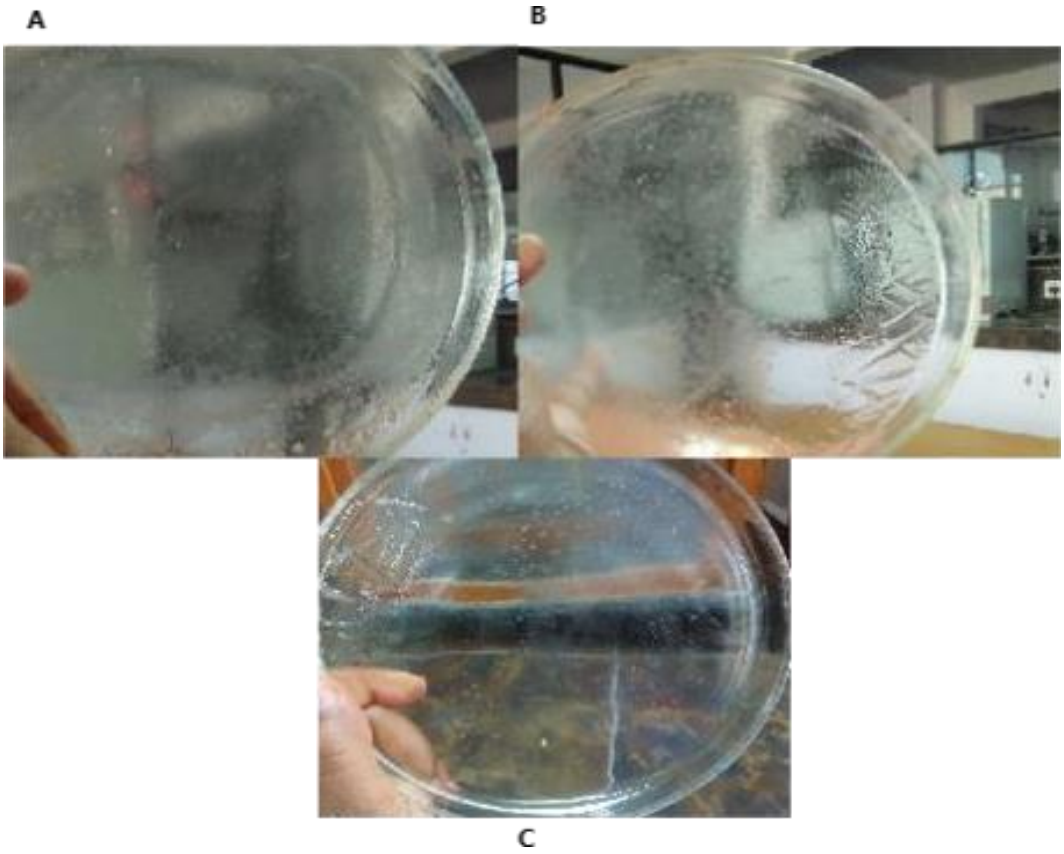
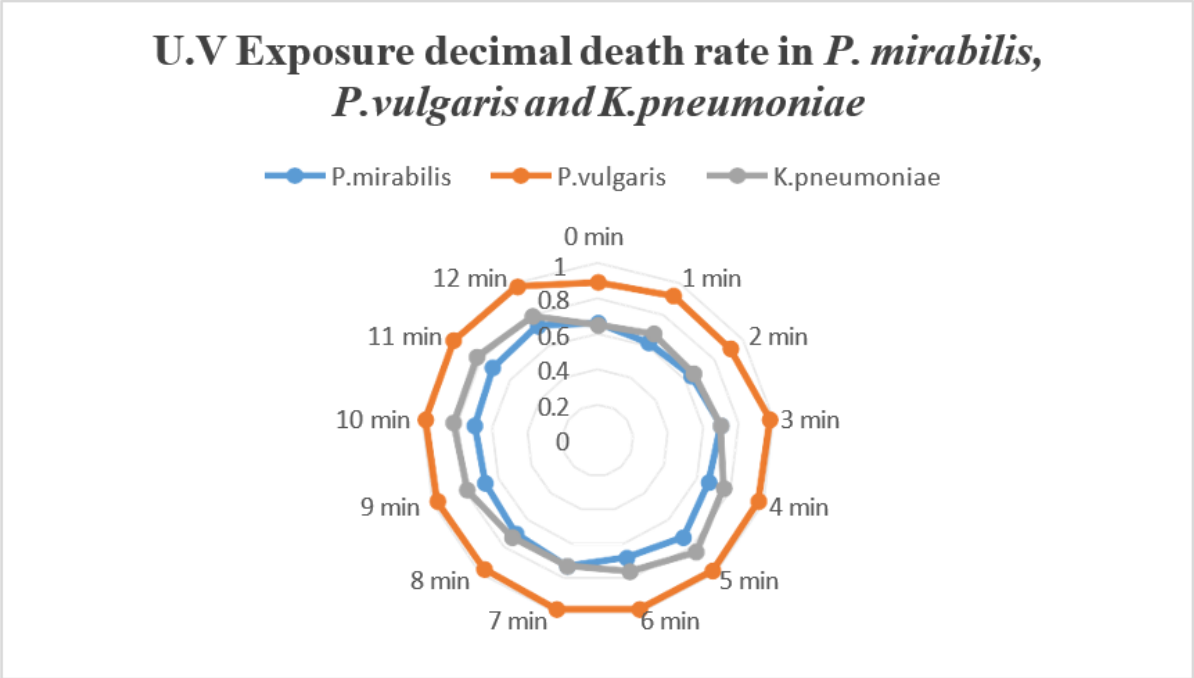


Figure :9 Demonstration of Nitrate Nano particles synthesised on Petriplates. (A) 1mM (B& C) 2mM and 3mM.

Table: 1 Calculation of Decimal death rate of *P.mirabilis, P.vulgaris* and *K.pneumoniae* after UV exposure.

S.No	Time interval of UV exposure	P.mirabilis	P.vulgaris	K.pneumoniae
1.	0 min	0.661	0.890	0.650
2.	1 min	0.621	0.920	0.680
3.	2 min	0.641	0.910	0.660
4.	3 min	0.700	0.980	0.700
5.	4 min	0.670	0.970	0.760
6.	5 min	0.730	0.980	0.840
7.	6 min	0.680	0.980	0.760
8.	7 min	0.730	0.980	0.730
9.	8 min	0.700	0.970	0.730
10.	9 min	0.680	0.970	0.790
11.	10 min	0.70	0.980	0.820
12.	11 min	0.720	0.990	0.830
13.	12 min	0.730	0.980	0.790



Graph:1 Representation of UV decimal death rate of gram -ve bacteria *P.mirabilis, P.vulgaris* and *K.pneumoniae* in radar graphical mode.

4. Discussion and Conclusion

E. aerogenes and *P.aeruginosa* are the gram negative bacteria with high pathogenic potential and mortality rate and poor clinical outcome in hospital patients. *P.aeroginosa* in biofilm forms can survive hypoxic conditions and can resistant to high harsh conditions (Qin.s et al., (2022). Hospital acquired strains of *P.aeruginosa* and *E.aerogenes* can show resistance to the conventionally used antibiotics and can be a major health concern. Exaggerated neutrophil infiltration with inflammation, tissue damage and great bacterial invasion are the characteristics associated with the bacteria is responsible for the exaggerated bacterial infections caused by P.aeruginosa.

Bacteremia including meningitis, septic shock, bleeding diathesis and sclerema are the most common complications caused by the *E.aerogenes* and can be transmitted through health workers and abuse intravenal drugs. Perinatal risk factors can happen in neonates who acquire *Enterobacter* septicemia (Jha et al., (2016)).

References

[1] Wesevich. A et al., (2020). Newly Named *Klebsiella aerogenes* (formerly *Enterobacter aerogenes*) Is Associated with Poor Clinical Outcomes Relative to Other *Enterobacter* Species in Patients with Bloodstream Infection. J Clin Microbiol;58(9):e00582-20. doi:10.1128/JCM.00582-20

[2] Qin.s et al., (2022). Pseudomonas aeruginosa: pathogenesis, virulence factors, antibiotic resistance, interaction with host, technology advances and emerging therapeutics. Nature;Signal Transduction and Targeted Therapy volume 7, Article number: 199 (2022)

[3] Eswari beeram et al., (2024). Isolation of Gram -ve Bacteria from the Root Extracts of Kalanchoe pinnata, Euphorbia tithymaloides and Murray koenigii. ENVIRONMENTAL SCIENCE ARCHIVES; Volume III Issue 2,pp- 116-123.

[4] Kadri O etal., (2010). Performance of Chromogenic Candida Agar and CHROMagar Candida in recovery and presumptive identification of monofungal and polyfungal vaginal isolates. Medical Mycology, Volume 48, Issue 1,, pp 29– 34, https://doi.org/10.3109/13693780802713224.

[5] Gokul P et al., (2021). Nanomaterials: Synthesis and Applications in Theranostics.Nanomaterials (Basel) ;11(12):3228. doi: 10.3390/nano11123228.

[6] Nadeem B et al., (2021).Nanomaterials: a review of synthesis methods, properties, recent progress, and challenges.Mater. Adv., Vol, 2, pp.1821–1871.

[7] Chakkour M et al., (2024). Overview of Proteus mirabilis pathogenicity and virulence. Insights into the role of metals.Front. Microbiol., Sec. Infectious Agents and Disease, Volume 15 | https://doi.org/10.3389/fmicb.2024.1383618.

[8] Wang et al., (2022). Antibiotic Sensitivity of Proteus mirabilis Urinary Tract Infection in Patients with Urinary Calculi.International Journal of Clinical PracticeVolume 2022, Article ID 7273627, 6 pages https://doi.org/10.1155/2022/7273627.

[9] Gu et al., (2022). A case report of Klebsiella aerogenes-caused lumbar spine infection identified by metagenome next-generation sequencing.BMC Infectious diseases 22:616.https://doi.org/10.1186/s12879-022-07583-0.

[10] Jha et al., (2016). Transmission of Enterobacter aerogenes septicemia in healthcare workers.Springerplus; 23;5(1):1397. doi: 10.1186/s40064-016-3011-x.