CP302 Capstone Project Report

Submitted in Partial Fulfillment for the
B.Tech Capstone Project (CP-302)

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Title – Low-cost phage- based biosensors for primary healthcare applications

Aim

To detect bacterial strains using low-cost **phage- based biosensors** for primary healthcare applications

Background

Biosensors are measurement devices that can sense several biomolecules, and are widely used for the detection of relevant clinical pathogens such as bacteria and viruses, showing outstanding results. Because of the latent existing risk of facing another pandemic like the one we are living through due to COVID-19, researchers are constantly looking forward to developing new technologies for diagnosis and treatment of infections caused by different bacteria and viruses.

Usually, biosensors detect biomolecules such as nucleic acids, proteins, and cells that are associated with diseases. This is possible because of their three major components: The biologically sensitive element, the detector element, and the reader device. Enzymes, microorganisms, organelles, antibodies, and nucleic acids are used to detect the biomolecules. In addition, researchers must identify the requirements to obtain a functional device according to the intended use. Hence, multidisciplinary studies are fundamental to select the proper material, transducing device, and biological element involved before assembling the biosensor .

At a clinical level, biosensors are applied for detecting disease-associated biomolecules. These devices can monitor the biochemical markers of a disease in body fluids, such as saliva, blood, or urine.

Types of biosensors

- ### Phage based biosensors

In phage-based biosensors, bacteriophage is attached to the sensor surface, and consequently, it can detect the pathogen in the sample. The main advantages of this method are sensitive, accuracy and reliably. Bacteriophage-based biosensors have been used for direct diagnosis of pathogens in fresh foods such as milk, and water food matrices

1. ### Phage-Based Optical Biosensors

Optical-based bioassay systems are used for rapid diagnosis of pathogens in different experimental conditions, with high sensitivity and compatibility. Optical biosensors are used as more suitable diagnostic systems for the detection of pathogens. Detection in optical biosensors is based on the variations induced in the light properties, such as refractive index, wavelength and polarization.

Currently, BIACORE 3000 biosensor and SPREETA biosensor as commercial optical biosensors are used for the detection of foodborne pathogens. BIACORE 3000 biosensor is used for detecting *L. monocytogenes*, with sensitivity of 1×10^5 cells/mL

in milk. *Salmonella enteritidis* and *E. coli* O157:H7, and *S. typhimurium* and *S. enteritidis*, can be successfully detected by BIACORE 3000. and SPREETA biosensors, respectively.

Optical techniques are separated into two major subclasses, fluorescence and label-free, based on their working platform. The most used technique for these biosensors is the measure of the changed fluorescence, in absorbance or luminescence, of the biosensor surface after analyte recognition. Furthermore, one of the advantages of the optical biosensor design of label-free biosensors is the detection of a specific and susceptible bacterial pathogens. The most employed techniques for bacterial detection are fluorescence/phosphorescence spectrometry, surface Plasmon resonance (SPR), and bio/chemiluminescence.

2. Surface Plasmon Resonance Sensors (SPR)

SPR (Surface Plasmon Resonance) biosensors are devices that use SPR to detect and measure biomolecular interactions in real-time. SPR is a phenomenon that occurs when polarized light is directed onto a thin metal film, such as gold or silver, and is coupled to surface plasmons, which are collective oscillations of the free electrons on the metal surface.

In a SPR biosensor, a ligand, such as a protein or a DNA molecule, is immobilized onto the metal film, and an analyte, such as a small molecule or another protein, is flowed over the surface. When the analyte binds to the ligand, it causes a change in the refractive index of the metal film, which changes the SPR signal. By monitoring the changes in the SPR signal over time, the kinetics and affinity of the biomolecular interaction can be determined. SPR biosensors are highly sensitive and can detect binding events in real-time without the need for labelling or other modifications to the molecules being studied.

They are widely used in drug discovery, protein-protein interaction studies, and other areas of molecular biology and biotechnology. They can be used to study a wide range of interactions, from small molecule-protein interactions to protein-protein interactions, and can provide information on binding kinetics, affinity, and specificity.

- ## Bioluminescence Sensors

Bioluminescence through the oxidation of organic compounds (Luciferin), due to the enzyme Luciferase, produces visible light in the living organisms. Commonly in marine environments, some bacteria, including Vibrio strains, are widely and abundantly used as luminescent organisms. The ATP Bioluminescence tests are a sensitive, fast and simple ways for bacterial detection. In this method, the bacterial cell is lysed and releases intracellular ATP which is measured by luciferase bioluminescence reaction. The problem of this method is the low specificity in both Adenylate kinase (AK) and ATP diagnosis.

- **Fluorescent Bioassay**

In this method, fluorescent blended bacteriophages are involved in detecting and binding to the host bacteria. Phage-bacteria is discovered using flow cytometry or epifluorescent filter technique. This method is also used to identify bacterial toxins.

- **Electrochemical Biosensors**

1. Amperometric Biosensors

Amperometric biosensors are a type of electrochemical biosensor that detects the concentration of a target analyte in a sample based on the measurement of electrical current produced by a redox reaction involving the analyte. The reaction takes place at the surface of an electrode that is coated with a bio-recognition element, such as an enzyme or antibody, that specifically interacts with the analyte.

The measurement of the current is based on Faraday's law, which states that the amount of charge that flows through a circuit is directly proportional to the number of electrons involved in a reaction. In amperometric biosensors, an electrical potential is applied to the electrode, and the current that flows between the working electrode and a reference electrode is measured. As the analyte interacts with the bio-recognition element, it undergoes an oxidation or reduction reaction, which produces a change in the current that is proportional to the concentration of the analyte.

Amperometric biosensors are widely used in clinical and environmental applications to detect a wide range of analytes, including glucose, lactate, cholesterol, and various environmental pollutants. They are highly sensitive and selective, and can be designed to operate over a wide range of concentrations. However, they may be subject to interference from other electroactive species present in the sample, and may require frequent calibration to maintain accuracy over time.

2. Electrochemical Impedance Spectroscopy (EIS) Biosensors

Electrochemical Impedance Spectroscopy (EIS) is a technique used in biosensors to measure the impedance of a system as a function of frequency. EIS biosensors utilize electrochemical reactions to detect and quantify analytes, with the aim of providing accurate and sensitive measurements in a range of biological and environmental applications.

In EIS biosensors, a small-amplitude alternating current is applied to the system, and the resulting potential response is measured. By analyzing the impedance of the system as a function of frequency, information about the chemical and physical properties of the system can be obtained, including the resistance, capacitance, and inductance of the system.

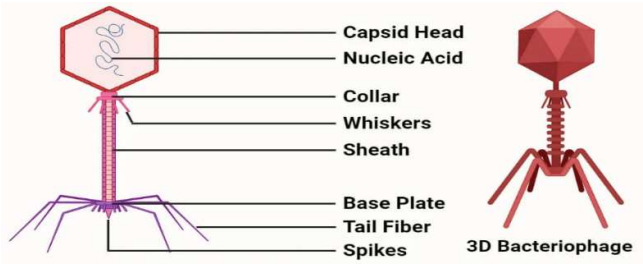
EIS biosensors typically employ a bio-recognition element, such as an antibody or enzyme, that is immobilized on the surface of an electrode. When the target analyte interacts with the bio-recognition element, it causes a change in the impedance of the system, which can be measured by EIS.

EIS biosensors are highly sensitive and selective, and can be used to detect a wide range of analytes, including biomolecules, bacteria, and viruses. They can be used for a variety of applications, including clinical diagnostics, environmental monitoring, and food safety. However, EIS biosensors can be complex to design and require careful optimization of the experimental conditions to achieve optimal sensitivity and accuracy.

Biosensor used in this project- **phage based biosensor**

Bacteriophages

Bacteriophages, also known as phages, are viruses that infect and replicate only in bacterial cells. They are ubiquitous in the environment and are recognized as the most abundant biological agent on earth. They are extremely diverse in size, morphology, and genomic organization. However, all consist of a nucleic acid genome encased in a shell of phage-encoded capsid proteins, which protect the genetic material and mediate its delivery into the next host cell. Electron microscopy has allowed the detailed visualization of hundreds of phage types, some of which appear to have "heads," "legs", and "tails". Despite this appearance, phages are non-motile and depend upon Brownian motion to reach their targets.

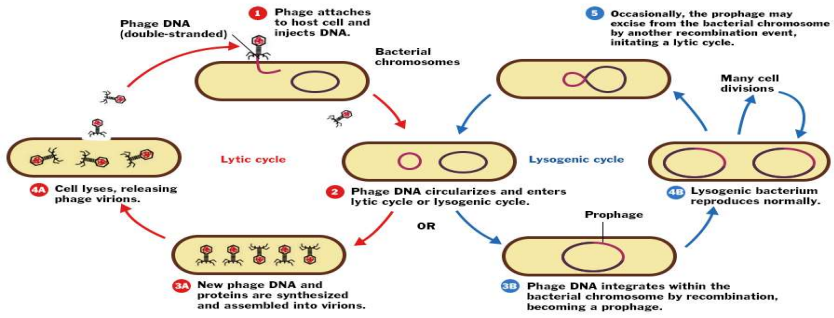


Like all viruses, bacteriophages are very species-specific with regard to their hosts and usually only infect a single bacterial species or even specific strains within a species. Once a bacteriophage attaches to a susceptible host, it pursues one of two replication strategies: lytic or lysogenic.

During a lytic replication cycle, a phage attaches to a susceptible host bacterium, introduces its genome into the host cell cytoplasm, and utilizes the ribosomes of the host to manufacture its proteins. The host cell resources are rapidly converted to viral genomes and capsid proteins, which assemble into multiple copies of the original phage. As the host cell dies, it is either actively or passively lysed, releasing the new bacteriophage to infect another host cell.

In the lysogenic replication cycle, the phage also attaches to a susceptible host bacterium and introduces its genome into the host cell cytoplasm. However, the phage genome is instead integrated into the bacterial cell chromosome or maintained as an

episomal element where, in both cases, it is replicated and passed on to daughter bacterial cells without killing them.



Since in this project I am interested to kill the susceptible and AMR strains using phages we will use phages which follows lytic cycle.

Advantages of bacteriophages

- High specificity: Phages have a high specificity for their host bacteria, allowing for the detection of specific strains of bacteria with high accuracy. This specificity can be used to selectively capture and detect bacteria in complex samples such as food or environmental samples.

- Rapid detection: Phage-based biosensors can detect bacteria in as little as a few hours, which is significantly faster than traditional culture-based methods that can take several days. This rapid detection can help to prevent outbreaks of foodborne illness or other infections.

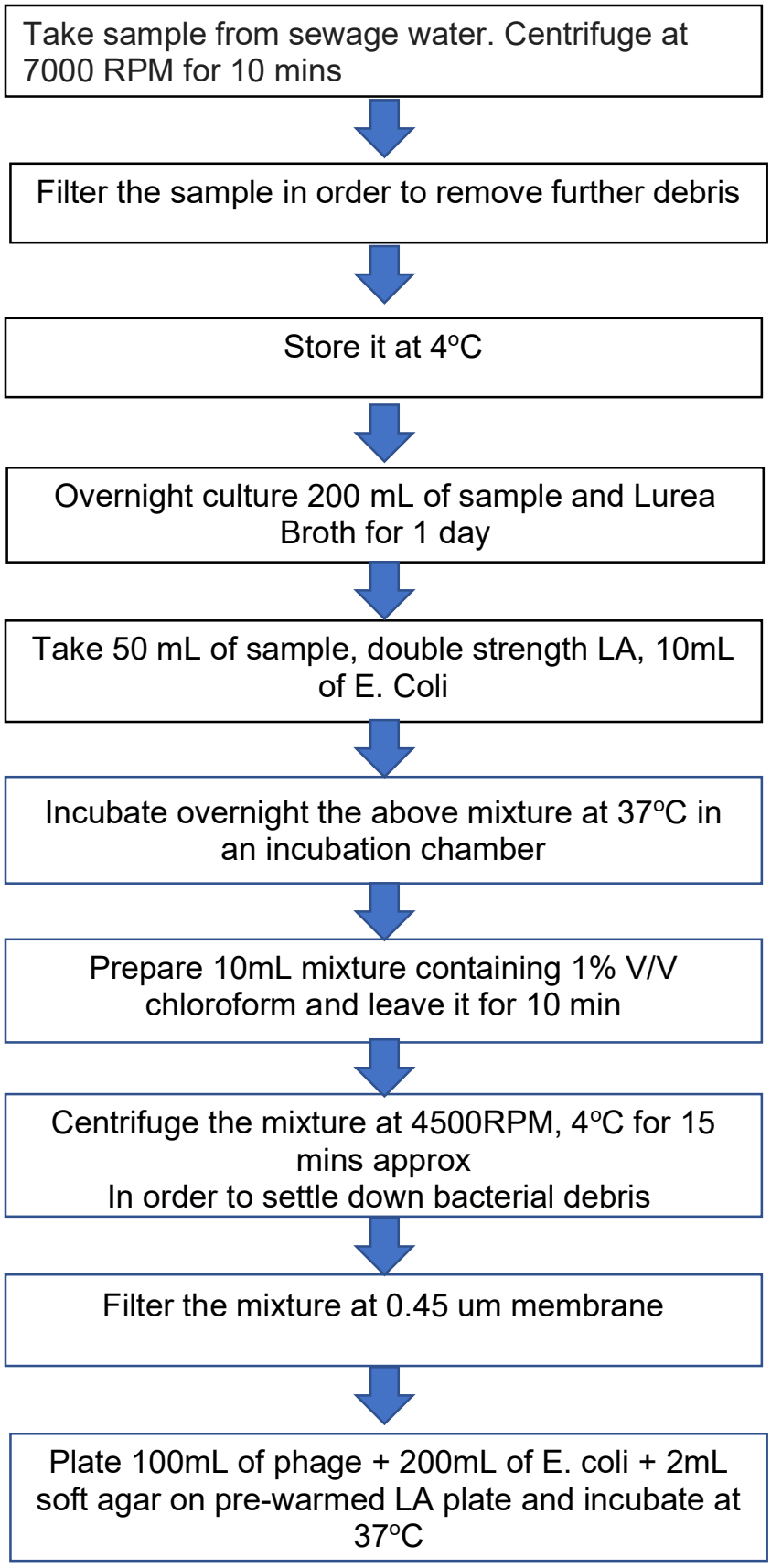
- Low cost: Phages are inexpensive and easy to produce, which can reduce the cost of biosensor development and production. No need for complex sample preparation: Phage-based biosensors can detect bacteria directly from complex samples such as blood, food, or environmental samples, without the need for extensive sample preparation. This can simplify and speed up the detection process.

- Real-time monitoring: Phage-based biosensors can be designed to provide continuous real-time monitoring of bacterial populations, which can be useful for monitoring bacterial growth in bioreactors or other industrial applications.

Methodology

1. Phage isolation

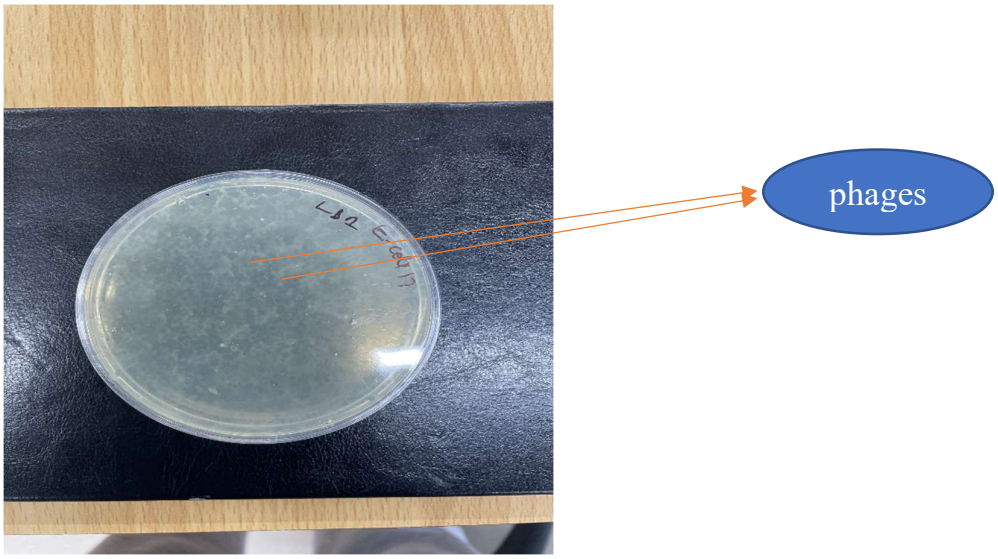
The following steps are implemented in order to isolate the phages from the samples.


Take sample from sewage water. Centrifuge at 7000 RPM for 10 mins
Filter the sample in order to remove further debris
Store it at 4°C
Overnight culture 200 mL of sample and Lurea Broth for 1 day
Take 50 mL of sample, double strength LA, 10mL of E. Coli
Incubate overnight the above mixture at 37°C in an incubation chamber
Prepare 10mL mixture containing 1% V/V chloroform and leave it for 10 min
Centrifuge the mixture at 4500RPM, 4°C for 15 mins approx
In order to settle down bacterial debris
Filter the mixture at 0.45 um membrane
Plate 100mL of phage + 200mL of E. coli + 2mL soft agar on pre-warmed LA plate and incubate at 37°C

After conducting the above steps on our collected samples we were able to get positive results of phase isolation from three samples whereas the other samples showed negative result indicating that the target phages were not present in the sample water. In our project we are only looking for phages for E. Coli bacterial strains. The table below shows the result of each sample after conducting phage isolation procedure

No of sample	Source	Phage isolation result
1	Satluj hostel	
2	Pond 1(outside campus)	
3	Pond 2(outside campus)	
4	Utility block	
5	Pond 3(outside campus)	
6	Bus stand	
7	Drain near bus stand	
8	Pond 4(outside campus)	
9	Stagnant water near UB	

10	Satluj river (outside campus)	✓
11	Hospital road (outside campus)	✓
12	Market (outside campus)	✗



2. Phage stability

The Stability of Bacteriophages can be enhanced by using Physical, Chemical, and Nano-Based Approaches

A. Polymer-Based Stabilization

A polymer is a substance or material of large molecular mass composed of repeating subunits. Because of a large number of subunits, removing one of them does not change the properties of the entire molecule. Polymers can be divided into natural (proteins, nucleic acids, and polysaccharides) and synthetic (polyethylene, polytetrafluoroethylene, and polypropylene)

The effect of sugars as stabilizers against protein unfolding is known. Because of this effect, sucrose has been used as a cryo-stabilizer for freeze-dried vaccines.

Yet, it has been shown that sucrose-induced stabilization of the viral capsid proteins alone does not necessarily lead to virus capsid stabilization. More importantly, the protein stabilization effect is broadly observed with many sugars, but only sucrose and trehalose seem to be effective in stabilizing viral capsids. Sucrose is known to enhance protein–protein binding at molar concentrations by modifying protein hydration properties, and thus, it is effective in the stabilization of vaccines. Other carbohydrates, such as agar or alginate, can also be used for the stabilization of phage particles.

Polyacrylamide can be used to stabilize phage particles for strictly research purposes, such as protein nuclear magnetic resonance spectroscopy.

PEGylation is the attachment of polyethylene glycol (PEG) groups to the target molecule, commonly used in food and drug formulation to increase molecules' stability. PEG is biocompatible and reduces the immunogenicity of the molecule, but it is non-degradable. PEG is known to affect protein–protein interactions by changing their hydration, similarly to sucrose. Since phage capsids are made exclusively of proteins, PEGylation was found to be suitable as a phage stabilizer. This approach uses PEG polymer conjugate with phage proteins

Additionally, an inorganic polymer nanocomposite (gold nanoparticles embedded in polyoxoborates) can be used to cover plastic container surfaces. Such a nanocomposite not only prevents the uncontrolled absorption of phages, but also has antibacterial and antifungal properties. This, indirectly, allows for more extended storage of liquid phage stocks.

B. Encapsulation

Polymers are also used as protective matrices in which phages are embedded. Encapsulation allows for better stability and modulates the long-term release of active phage particles. Various techniques are used for encapsulation, for example, emulsion, polymerization, spray-drying, and extrusion dripping.

The applications of encapsulation also include phage cocktails. For example, a cocktail of three phages against *Salmonella* was encapsulated within alginate microparticles associated with calcium carbonate to prolong their gut residence time. the types of polymers used for phage encapsulation are mainly agarose, alginate, chitosan, pectin, whey protein, gelled milk protein, hyaluronic acid methacrylate, hydroxypropyl methylcellulose poly(N-isopropyl acrylamide), poly(DL-lactide: glycolide), polyesteramide, polyvinyl pyrrolidone, polyethylene oxide/polyvinyl alcohol, cellulose diacetate, and polymethyl methacrylate. Alginate is often used to encapsulate phages for applications that require exposure to acidic media.

Liposomes are biocompatible nanoparticles composed of a lipid bilayer surrounding therapeutic cargo. This approach is promising in achieving directed delivery while surviving the extreme conditions of the stomach and intestine when administered orally. Liposome-encapsulated phages were used to target *Salmonella*. Nanoparticles of cationic liposomes can also be designed to encapsulate anti-*Salmonella* phages, protecting them against the acidic conditions of the intestine. This technique is popularly adopted to

C. Lyophilization

Lyophilization (dehydration process), first used for food storage, has become a very commonly used method for phage stabilization and long-term storage. At first, lyophilization involved freezing the phage stock, lowering the pressure, and removing the water (so-called freeze-drying). Now, there is a branch of similar methods, including: (i) spray-drying, where the concentrated liquid is atomized and exposed to the hot air—causing evaporation of the water—and then, dried and separated (ii) hot-air-drying, where the sample is pre-treated with ethyl oleate and potassium carbonate solutions, and then, exposed to a temperature of about 50–60 °C and (iii) drum-drying, where hot-air-drying is enhanced by placing the sample in a rotating drum to increase the heat transfer.

For phage storage, freeze-drying and spray-drying methods are the most important. Freeze-drying is, relatively, the cheapest method for preparing phage powders. One of the first attempts was the freeze-drying of mycobacteriophages; when stored in the dark, phage lyophilizates were suitable for over two years. The activity of phages after freeze-drying can be regulated by the drying time and the number of times they have previously been refrigerated. The authors proved that a long duration of drying (over 150 min) provides three-times-higher survivability of phages during freeze-drying than a short duration (90 and 120 min). Additionally, every subsequent freezing causes the titers to decrease by about 3 log. The efficiency of freeze-drying also depends on the size of the grains of phage powder during the procedure—smaller bead formulation provides a smaller reduction in phage titer than in the case of macro-beads.

Freeze-drying and spray-drying were combined into a novel atmospheric spray-freeze-drying (ASFD). Both spray-dried and ASFD phage powders are believed to be a promising medicine for bacterial infections, e.g., caused by *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Burkholderia cenocepacia*, *Salmonella enterica*, *Campylobacter jejuni*, or *Mycobacterium tuberculosis*. However, at this moment, such projects are in the stage of optimization.

Another long-term method of storing and stabilizing bacteriophages is freezing mature virions within bacterial cells. In this approach, bacteriophages at a proper multiplicity of infection (MOI) are mixed with their host bacterium and incubated for a short time. Next, infected cells are frozen and stored at −80 °C. After reviving and washing, phages are released and can actively infect bacterial cells.

D. Nano-Assisted Stabilization

The implementation of nanotechnology in medicine has the potential to solve the stability issue.Phages can be associated with nanoparticles to remain detectable in the bloodstream for about 24 h longer than in the control. The majority of bacteriophages fulfill the classical definition of nanomaterials (i.e., having one geometrical dimension in a range from 1 nm to 100 nm). Therefore, they integrate well with abiotic nanomaterials, ensuring higher efficiency.

Nanoscience plays an essential role in the immobilization of phages; bacteriophages can be chemically or genetically modified to bind strongly to nanomaterials. Gold nanoparticles are most commonly used to stabilize T4-like bacteriophages to detect *E. coli* cells. Additionally, the DNA of *B. anthracis* can be targeted using phage probes modified and stabilized with gold nanoparticles. For more inexpensive applications, silica nanoparticles are popularly employed for their ability to bind to phages.

In some experiments on vaccine formulations, nanolayers of aluminum oxide were used to stabilize the λ bacteriophage, which ensured the controlled release of antigens in vivo. Negatively charged gold nanoparticles can improve the storage time of adenovirus.

Carbon-based nanomaterials, in particular, have emerged as potential next-generation miniaturized biosensors to obtain susceptible and selective detection. This was displayed when virions were chemisorbed on a glassy carbon electrode decorated with gold nanoparticles.

The synergistic effect of phages and nanoparticles has been widely used for targeting biofilms and eliminating pathogenic infections. Polyvalent phages were attached to magnetic colloidal nanoparticle clusters (CNCs) to facilitate biofilm penetration. Phage virions may be used as stabilizing agents for synthesizing gold nanoparticles, which have antibacterial and antibiofilm properties. Metallic nanoparticles and bacteriophages impart a synergistic effect against pathogenic bacteria

Substrate used for phage stabilization in the project- **glycerol**

3. Immobilization

Cell immobilization is a general term describing the **physical confinement of viable microbial cells to a certain defined region of space** (carrier) in order to limit free migration and exhibit hydrodynamic characteristics different from those of the surrounding environment.

Immobilized cell systems are far more tolerant to changing environmental conditions and less vulnerable to toxic substances present in the bulk phase. General techniques used for immobilization include: **adsorption on surfaces, covalent bonding to carriers, electrostatic binding and nanocoating**

Method implemented for immobilization

Physisorption

- Physical adsorption, or physisorption, refers to the adhesion of particles onto a surface brought about by van der Waal's forces, dipole-dipole moments, electrostatic forces and steric and hydrophobic interactions. Van der Waals forces, although weak, occur in all molecular species.
- It represents a quick and relatively simple ways to immobilize a species onto a given surface and, because it occurs through non-chemical interactions, it typically does not result in any chemical alteration of the absorbate.

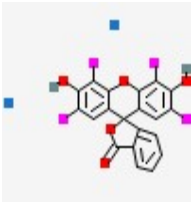
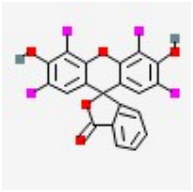
- However, because of these physical stresses as well as extremes of acidity, temperature and ionic strength can act to reduce attachment or reverse it post immobilization

Substrate used

Nitro cellulose membrane

Dye used

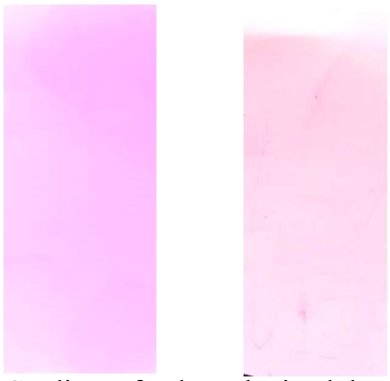
Tetraiodofluorescein- It is an organoiodine compound, specifically a derivative of fluorone. It is a pink dye which is primarily used for food coloring. Its maximum absorbance is at 530 nm in an aqueous solution, and it is subject to photodegradation. It is used as a red coloring in some foods (cherries, fish), as a disclosure of DENTAL PLAQUE, and as a stain of some cell types. It has structural similarity to THYROXINE.

Molecular formula	$C_{20}H_8I_4Na_2O_5$
Structure (3D)	
Structure (2D)	
Parent compound	 CID 3259 (Tetraiodofluorescein)

Procedure

1. The phage dipped in glycerol is physically attached to the surface of nitro cellulose membrane strip
2. The strip is dipped in the sample containing the target bacteria for 2-3 hours for the phages to action on it
3. We apply Tetraiodofluorescein dye to the surface of the membrane and wait for 5-10 mins
4. We can observe clear dark pink spots on the nitrocellulose membrane which indicates that the phages were able to kill the host bacteria present in the sample. Darker spots represent greater number of killed bacterial cells

5. We can observe light pink spots on the nitrocellulose membrane which indicates that the phages were not able to kill the host bacteria present in the sample



Gradient of colors obtained due to the number of dead cells

Discussion

- We have isolated the phages from various samples
- We have immobilised the phages and used colorimetric assay to detect bacterial samples

Future plans

- we need to work on the stability of bacteriophages using different methods and check their effectiveness
- we need to work on the di-optimization of the assay

References

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