Preparation & Characterization of *E. coli* DH5α Bacterial Ghosts and their Evaluation as a Drug Delivery Vehicle

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A thesis submitted in partial fulfillment of the requirement
for the degree of MS in Industrial Biotechnology

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Sadia Masood
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…To my Angelic Mother, **Sajida M. Ghumman**…

May I never have to live a single day of my life without you

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List of Abbreviations

SEM	Scanning Electron Microscope
E. coli	*Escherichia coli*
BG	Bacterial Ghost
EtBr	Ethidium bromide
kb	kilobase
LB	Luria Broth
LPS	Lipopolysaccharides
DDS	Drug Delivery System
N-Agar	Nutrient Agar
N-Broth	Nutrient Broth
O. D	Optical Density
DOX	Doxorubicin

Abstract

Bacterial ghosts (BGs) are nonliving devoid bacterial cell envelopes of gram negative bacteria with conserved cellular morphology. The study focuses on development of a new delivery platform for cancer treatment. Genetic and chemical methods can be used for the preparation of bacterial ghosts. In the following study, BGs of *E. coli* DH5α prepared by the exposure of cells to tween-80 for a prolonged period followed by an immediate decline of pH. Scanning Electron Microscope (SEM) results showed the excellent formation of bacterial ghosts with clear holes of 111nm size in their outer membranes. Furthermore, Release of DNA and protein content was confirmed by agarose gel electrophoresis and Bradford assay, respectively. Bacterial ghosts were loaded with anti-cancerous drug Doxorubicin Loading efficiency is determined using direct method which is 43µg/mg. Release profile is being studied using dialysis tubing for 11 days and loaded BGs showed slow release over a long period of time. As a result, BGs could be considered as a potent drug delivery and targeting vehicle for the reliable distribution of anticancerous drugs for cancer treatment. Subsequently, Doxorubicin dosage could be significantly reduced when BGs deliver the DOX as compare to the free Doxorubicin. These outcomes will help the patient in a several ways, including lowering the dosage, reducing the administration frequency and cytotoxicity. Experimental validation will confirm the potential of bacterial ghost platform.

Key words: Bacterial Ghost, Delivery Vehicle, Doxorubicin, Cancer

INTRODUCTION

1 Introduction

1.1 Cancer

Cancer is the rapid expansion of aberrant cells beyond their normal bounds, allowing them to invade other regions of the body and spread to other organs, a process known as metastasis. According to the global cancer statistics for the year 2020, 19.3 million new cases have been reported for 36 cancers in 185 countries and 9.9 million deaths have been reported in both genders. So, Cancer is the world's second greatest cause of death, accounting for one out of every six fatalities.

1.2 Cancer Treatment and their Limitations

The most common treatments of cancer are Surgery, Chemotherapy and Radiations. Other options include immunotherapy, laser, hormonal therapy and targeted therapy etc. But the most used treatment is chemotherapy in which drugs are being used to kill cancer cells. Chemotherapy has its own limitations including damage to the healthy cells, Non-specific targeting, High dose requirements, Low therapeutic indices and low bioavailability.

To overcome these limitations Drug Delivery Vehicles are being used for the delivery of chemotherapeutic drugs to the tumor cells. Benefits of these delivery vehicles include No damage to healthy cells, site specific targeting, low dose requirements, and minimize administration frequency and side effects of drugs. The chemotherapeutic drug that we used in our study was Doxorubicin which is one of the most used drugs against a wide range of cancers. Doxorubicin was delivered by using a new delivery system known as bacterial ghost.

1.3 What are Bacterial Ghosts?

Bacterial Ghosts (BGs) are cell envelopes derived from Gram-negative bacteria devoid of cytoplasmic content but their surface structures remain intact. The Bacterial Ghost (BG) platform technology is an advanced system for the delivery of vaccine, drug or active substances. BGs also have many practical applications in white biotechnology. Bacterial ghost delivery platform is used for the delivery of chemotherapeutic drugs to treat cancer cells. They are also being used for the delivery of DNA and subunit vaccines because particle structure and surface properties of BGs target the carrier itself primary antigen-presenting cells (APCs) *(Langemann et al., 2010).* These void envelopes contain many immunostimulatory agonists of innate immunity. They are also effective activators of a large number of cells, which are a part of innate and adaptive immunity.BG are not only effective candidate vaccines, but they also work well as adjuvants. *(Hajam et al., 2017).*

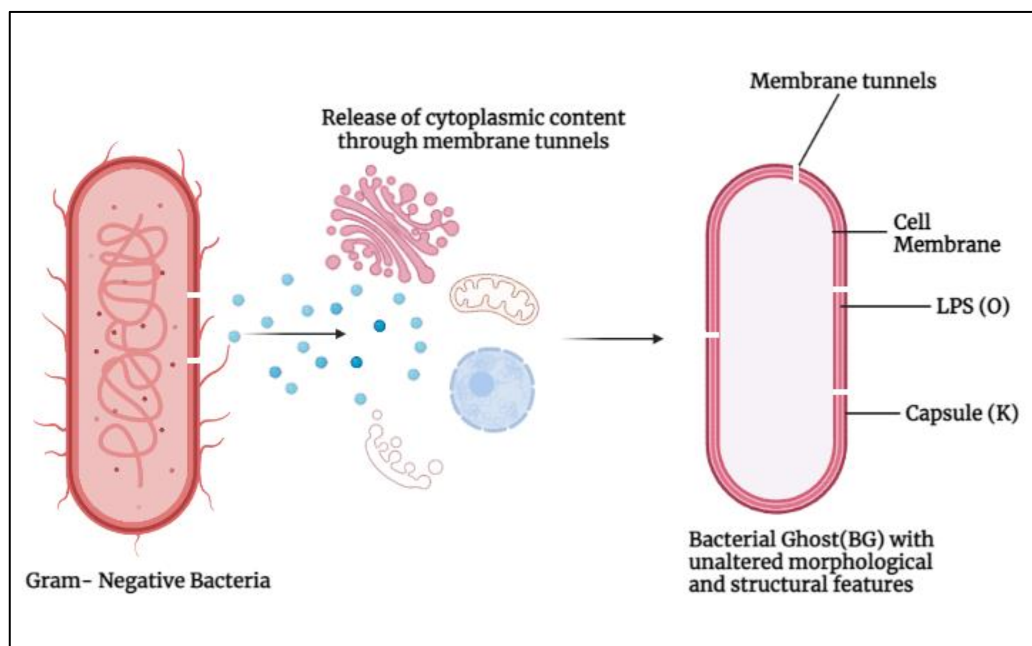

Figure 1: Bacterial Ghosts

1.4 Preparation Methods for bacterial ghost

There are two methods documented for the production of bacterial ghosts (BGs). One method is known as genetic process and the second procedure is chemically induced method *(Rabea et al., 2018).*

1.4.1 Genetic method for bacterial ghost preparation

Genetic method includes the use of lysis gene *E* to produce bacterial ghost. This is a cloned lysis gene *E* (a bacteriophage *ΦX174* gene). It lysed *E. coli* after bacteriophage infection *(C. A. Hutchison & Sinsheimer, 1966).* Nuclease is being used to degrade any present DNA to make sure that there is no pathogenic islands or antibiotic resistance genes are present in the BGs. *(W. et al., 2003).*

1.4.2 Chemical method for bacterial ghost preparation

Preparation of Bacterial Ghosts using chemical method includes incubation of bacterial cells with different chemicals for a small period at their minimum growth or minimum inhibitory concentrations (MGC or MIC). In the following method, active chemical compounds are used in fewer concentrations than the MIC. These active chemical compounds include NaOH, SDS, and H_2O_2. Experimental plan of Plackett-Burman is being used to determine the optimize settings *(A. A. Amara et al., 2013)*.

1.4.3 Novel protocol for bacterial ghost preparation

A study conducted earlier in 2018 where a novel method is mentioned for bacterial ghosts' preparation. In this new method, bacterial ghost is prepared by exposing the bacteria to 7% tween-80 for a certain time period which is being followed by the immediate reduction of pH *(Rabea et al., 2018).*

It is noteworthy to mention that preparation of bacterial ghost by using tween-80 is the simplest, reliable and cost-effective protocol.

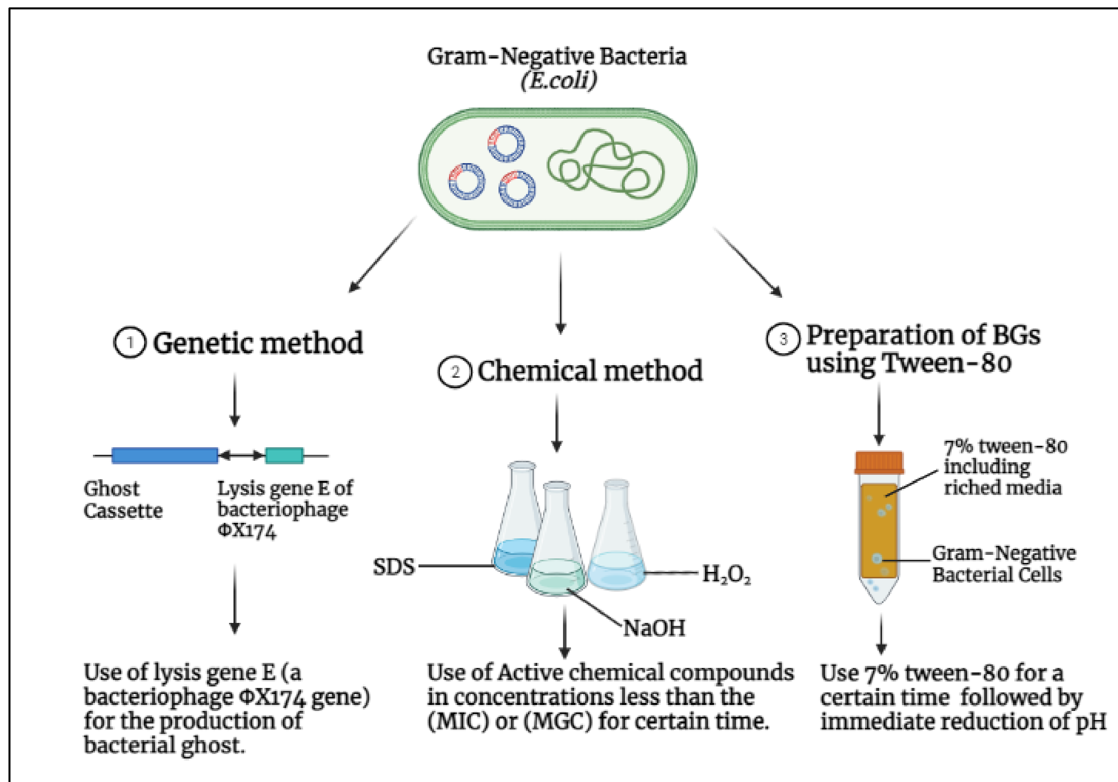

Figure 2: Different Methods of bacterial ghost preparation

1.5 Applications of Bacterial Ghost

Bacterial ghosts (BGs) have a broad range of applications in white biotechnology. Their low production cost makes them a nontoxic and suitable delivery vehicle for targeted drug delivery and active agents. Bacterial ghosts are also used as carriers for the immobilized enzymes *(Langemann et al., 2010)*.

BGs can also be employed as an enzymatic activity carrier for a unique idea of probiotics that can manufacture active chemicals from environmental substrates when delivered with a preference for the gut system. In conclusion, Bacterial ghosts symbolize a favorable technology platform as drug delivery vehicle for therapeutic

approaches in cancer treatment, for new vaccines such as combinations or DNA vaccines, and as novel probiotics *(P. Lubitz et al., 2009).*

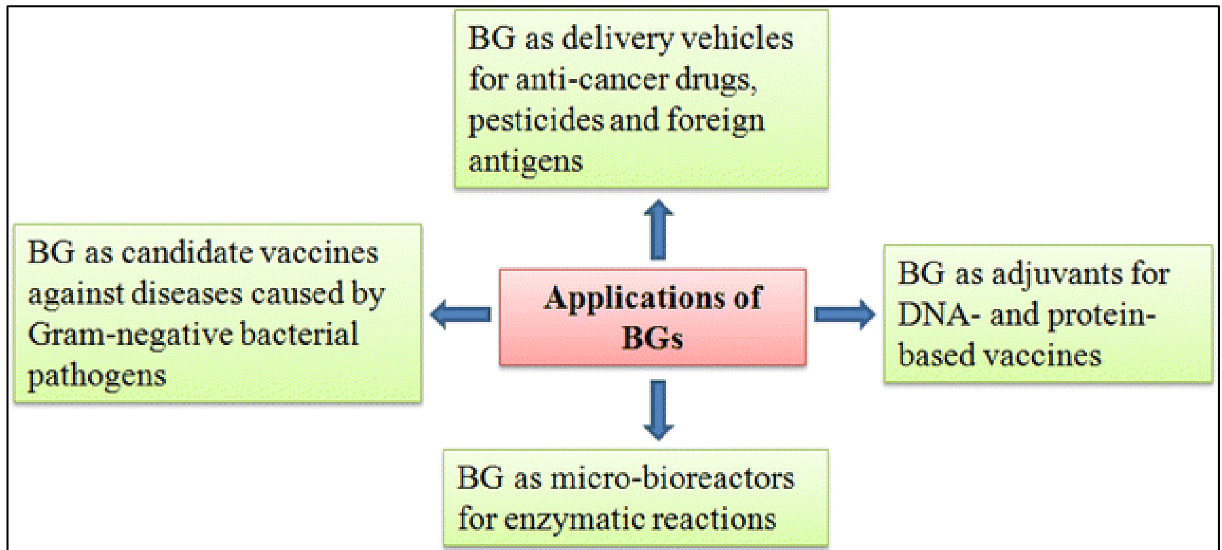

Figure 3: Applications of Bacterial ghost (Hajam et al., 2017)

1.6 Why bacterial ghost technology platform?

Based on the literature review, a bacterial ghost technology platform possesses the following properties:

- Intrinsic adjuvant properties
- Generation of humoral and cellular immune responses
- No cold-chain storage required because of their lyophilized status
- Safe as they have no involvement of live organisms or host DNA
- Uncomplicatedness of Bacterial Ghost production
- Ability to load multiple target antigens
- Exhibit improved potency concerning target antigens as compare to conventional methods

- Adaptable with respect to DNA or protein antigen choices
- High bioavailability as a delivery vehicle
- Targeted drug delivery vehicle for cancer treatment
- Could be manufactured on an industrial scale
- Long Shelf life
- Biocompatible
- Minimal Side effects
- Cost-effective

1.7 Bacterial Ghost as a Drug Delivery Vehicle

Bacterial Ghosts (BGs) are being used for the delivery of anti-cancerous drugs to the selective tumor tissues. The most advantageous thing about bacterial ghosts as delivery vehicle is their ability to overcome the limitations of currently used delivery methods as these anti-cancerous drugs are unable to localize the tumor cells and specifically act at the site of action. These limitations are making their use limited. Bacterial Ghosts are a promising approach for the targeted delivery of anti-cancerous drugs to cancerous cells.

The principal aim of this study was to prepare the bacterial ghosts using chemical method and to use these bacterial ghosts as a delivery platform for an anti-cancerous drug Doxorubicin. The existing study is the first study on the use of *E. coli* DH5α BGs as a DDS for Doxorubicin globally.

LITERATURE REVIEW

2 Literature Review

Background

2.1 Malignancy

Till date, cancer is the second reason for mortality worldwide (Ferlay et al., 2019). In science, disease is characterized as the threatening development because of uncontrolled cell division. It is currently utilized as an overall term for more than 100 sicknesses described by the uncontrolled, unusual development of cells. At cutting edge organizes, the phones spread (metastasis) locally or through the circulatory system and lymphatic framework to different pieces of the body. The main recorded portrayal of this condition was corresponding to bosom carcinoma. The expression "disease" came from Ancient Greek καρκίνος (karkínos), signifying "crab", since like a crab malignant growth cells appear to "**grab on and won't let go**".

Cancers and tumors are similar in a way that both of these conditions are characterized by abnormal cell division that ends up in the formation of a mass of cells with no useful function. Tumors (also called neoplasms) are the more inclusive term for all abnormal cell growths. Thus, it can be said that cancer is a type of tumor. A cancerous tumor is one that has the potential to grow continuously and then spread to other tissues. Other hallmarks of cancers are as follows: new blood vessels form on the affected tissue, avoiding programmed cell death, and an unlimited number of cell divisions.

2.2 Cancer therapeutic options

Besides the fact, that chemotherapy is the most well-known therapy for disease, the

2.3 Innovative therapeutic strategies

It is important to develop innovative therapies for cancer therapy. This includes either passive targeting (which depends on the size of leaked fenestrations from arteries and veins) or active targeting (based on the association with target cells (Alanazi et al., 2020). Various therapies have been applied to deliver chemotherapeutic medications to malignant cells and different cancers utilize hydrogels, polymeric substances, vesicular particles like liposomes and noisomes, magnetic particles, lipoproteins, clay, minerals, ion exchange resins and metals. Despite the fact that these conventional treatments are promising, useful, and give effective outcomes, chemotherapy remains unsuccessful and clinical results are always lower than anticipated outcomes. Furthermore, lack of site specificity is the main justification for ineffectiveness of these conventional treatments as they also cause damage to healthy cells. Hence, there is an increasing demand for new potential therapies to overcome shortage of already established treatments. To achieve successful delivery of active drug molecules to target cells, drug targeting is a significant consideration. Cell-targeting therapies are recently being focused to deliver anti-cancerous drugs selectively and efficiently.

2.4 Bacterial ghosts (BGs)

Bacterial ghosts are considered as one of the advanced targeting vehicle technology on the basis of cell-based targeting approaches *(Paukner et al., 2004).* BGs presented one of the most advanced drug delivery vehicles (DDVs) that can be used to distribute chemotherapeutic agents to tumor cells. Bacterial ghosts have more specificity of targeting tumor cells, production simplicity, loading capability and packaging of different compounds in various carrier compartments. These benefits make BG

product ideal as drug delivery vehicle and their clarification of superiority as compare to other delivery platforms. Bio-recognitive features of BGs have been established allowing them to stick to various surfaces of bodily tissues depending on the species.

2.1 History of BGs

Bacterial ghosts were prepared through the cloning of lysis gene *E* with controlled expression in 1966. The vital character of gene *E* was discovered after the infection of *E. coli* with bacteriophage φX174 *(C. A. 3rd Hutchison & Sinsheimer, 1966).* Sixteen years later, After the development of genetic engineering, it was demonstrated that the individual expression right after the cloning is enough to cause successive *E. coli* lysis *(Wang et al., 2011,Young & Young, 1982).*

2.2 BG preparation from bacteriophage ΦX174

Gene E was the first bacteria-killing gene that could be inactive on plasmids. When phage expression of E is developed in bacteria which comes under the range of non-host, Gram-negative bacterial cells are converted to BGs, whereas Gram-positive bacterial cells are eliminated without lysis *(Langemann et al., 2010)*. Unlike other lytic proteins of other phages, Gene E codes for a 91-aa polypeptide *(Barrell et al., 1976)* that has no inherent enzymatic function *(C. A. 3rd Hutchison & Sinsheimer, 1966, Markert & Zillig, 1965)*. E is a membrane lytic protein which can create a transmembrane tunnel when oligomerized *(Bläsi et al., 1989,WITTE & LUBITZ, 1989)*.

Basic configuration of protein E shown a hydrophobic area at its N-terminal end, indicating co-translational incorporation in the inner membrane of *E.coli*

(Bläsi et al., 2019) The fact that host cells in the stationary phase do not react to E-lysis induction yet lyse when given new medium, as well as other discoveries like the inhibition of E-lysis by non-physiological pH levels, suggest that E-mediated lysis is dependent on allowing them to stick to various surfaces of bodily tissues depending on the species.

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The fact that host cells in the stationary phase do not react to E-lysis induction yet lyse when given new medium, as well as other discoveries like the inhibition of E-lysis by non-physiological pH levels, suggest that E-mediated lysis is dependent on the host cells' growth phase and its autolytic system *(G Halfmann et al., 1984, W. Lubitz & Pugsley, 1985)*.

An E-specific holes bridging the inner and outer membrane was discovered by examining the hydropathicity sections of protein E. This tunnel is most likely positioned at membrane bond sites inside the host *(Bayer, 1968)*. The BG is formed by E-mediated lysis, which releases all cytoplasm in the environment while keeping periplasmic components attached to the void cell membrane *(WITTE & LUBITZ, 1989)*. The initiation of E-lysis is preceded by the breakdown of the bacterial membrane potential *(A Witte et al., 1987)*. The E-specific lysis tunnel was visible when high-magnification SEM and TEM were used to examine the E-lyzed *E. coli*. E-lysis of *E. coli* was also escorted by a union of the inner and outer membranes, which sealed the periplasmic space (PPS), according to electron microscopic images *(A Witte et al., 1990)*.

Several studies of E-mediated lysis of *E. coli* cells demonstrated that the holes produced by the E protein usually present in the center or at the poles of bacterial cells which are actually divisional areas of bacteria *(A Witte et al., 1992)*. Protein E-mediated lysis is reliant on the physical situations of the host bacterium *(BLASI et al., 1985, W. Lubitz et al., 1984)* and research of lysis in other *E. coli* division mutants suggests that cell division machinery are required for lysis *(A Witte et al., 1992, A Witte et al., 1998)*.

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The observed lysis tunnel has a diameter ranging from 40 to 200 nm with no regular pattern. The quick discharge of cell content is fueled by the differential osmotic pressure between the cytoplasmic content and the surrounding environment. The unique configuration of the peptidoglycan inside the envelope complex is retained and firm *(Angela Witte et al., 1998)*. The 10% increase in peptidoglycan turnover found in consistent with genetic proof

that protein E hinders MryA translocase A *(Bernhardt et al., 2000)*.

Based on these outcomes and comprehensive experimentations by a protein (E-FXa-StrpA) *(Schön et al., 1995)* defined the procedure of E-mediated hole creating with a model of three phases: (1) Protein E is integrated into the IM with its C-terminus towards the cytoplasmic content; (2) Protein E undergoes a conformational transition that translocate the domain of c-terminal to the PPS, followed by oligomerization and targeting of the division initiation complex via lateral complex; (3) By exposing the C-terminus of protein E to the cell surface, fusion of IM and OM at membrane adhesion sites is stimulated.

The lysis tunnel is not only bounded by protein E oligomers, according to this model, but its development also necessitates protein E-mediated union of outer and inner membranes *(Schön et al., 1995)*. The idea of E-lysis can be confirmed with further Gram-negative bacterial cells after the unique properties of protein E-mediated inactivation in *E. coli* were discovered *(Jalava et al., 2002,W. Lubitz et al., 1999)* However, Gram-negative bacteria are not affected *(Gabriele Halfmann et al., 1993)*. A range of Gram-negative strains (such as *E. coli* strains, *Salmonella enteritidis, Salmonella typhimurium* and etc) are effectively being produced. This means that the BG system may be adapted to work with any Gram-negative bacterium *(W. Lubitz et al., 1999)*.

2.4.1 Need of BG inactivation

A new quality standard has been in action for the past two years, requiring that the

prepared BGs should have no active cells prior to freeze drying. Though BG production efficacy exceeds 3 to 5 times of magnitude within the E-lysis time period, any residual living cells should be dead afterwards. The existence of protein E do not guarantee that every bacterial cell will be killed by E-lysis. Furthermore, the presence of protein E in complex makes every bacterial cells extra susceptible to inactivation by freeze drying, and no activated cell counts could previously be found in freeze dried Bacterial ghosts.

Applications in which bacterial ghosts without nucleic acid are generated, killing can be achieved by combining E-lysis with the production of an extra "kill gene" inside host *(W. et al., 2003)*. SNUC enzyme is employed for this and it decreases the concentrations of DNA below the real-time PCR identification range because it breaks the DNA of host into contigs no more than hundred base pairs, this enzyme is accountable for vacuuming up residual DNA in BGs, which can cause the killing of entire culture *(W. et al., 2003)*. Mg2+ and Ca2+ were added, as a pH change to 8.0, activates the favorable upshot of SNUC expression, decreasing the cell inactivation and remaining DNA in the bacterial ghosts product.

After harvesting, adding the BPL is successful in totally killing the active cells, either in combination with or instead of SNUC. Nucleic acids, particularly guanine, are known to react with BPL. BPL is commonly used to sterilise vaccines, human tissue transplants, and plasma, as well as to inactivate viruses *(Perrin & Morgeaux, 1995)*. The presence of BPL produces changes in nucleic acids (transition mutations, cross-linking, and nicks). BPL is entirely hydrolyzed in the hydroxypropionix acid, which is non-toxic, in the presence of water at room temperature *(Perrin & Morgeaux, 1995)*.

The quantity of BPL needed for complete bacterial ghosts inactivation in BG

production is determined by three factors: the amount of DNA in liquid phase, temperature, and time. The bulk of DNA in the BG solution is present in the liquid phase as a result of cytoplasmic material evacuation, making it feasible to use BPL in the BG concentrate after preparation but before infiltration. Roughly 97% of the inventive fermentation fluid has been removed from the product, and thus 97% of the free DNA.

2.4.2 Challenges in BG inactivation

Bacterial inactivation is a term used to describe bactericidal treatments that exterminate bacteria through disrupting by synthesis of protein or DNA, causing growth to stop. While there are various approaches for bacterial cells inactivation, many research studies emphasis on a specific bacterium, and there are no methodologies to inactivate bacteria in general. Bacterial inactivation before application is common in trials that are vulnerable to host-microbe interaction. Vaccinations, cell sensitivity to bacterial surface-membrane features, and inactivated bacterial cells as medication or antigen vehicles are all examples *(Langemann et al., 2010)*.

- **First Challenge**

The diversity of the bacteria used for BG production poses a challenge for a consistent bacterial inactivation approach. The structural differences between Gram-positive bacterial walls and Gram-negative bacterial walls, for example, might prevent assertive inactivation techniques from working. One great example is creation of BGs by using plasmid containing an insert of E gene found by bacteriophage X174, which lysis bacteria by producing a tunnel transversely in the cell membrane through which DNA and contents of cytoplasm can escape *(Langemann et al., 2010)*.

Additionally, BPL and staphylococcal nuclease A destroy DNA. In conclusion, to assure inactivation, bacterial cells which were not activated were freeze dried. Gram-negative bacteria are inactivated by using this method, whereas Gram-positive bacteria are not. For the creation of holes, a double membrane, just like in Gram-negative bacteria, is required *(Langemann et al., 2010) (Gabriele Halfmann et al., 1993)*.

- **Second Challenge**

Finding ways for high-throughput applications is a second challenge. Use of combination chemicals in a succession, *Listeria monocytogenes* (also known as *L. monocytogenes* ghosts or LMGs) were generated *(Wu et al., 2016)*. The minimal inhibitory concentrations for these substances had to be determined ahead of time in order to create inactivated bacteria with maintained structure. Individual bacteria may have different minimum inhibitory concentrations due to which some inactivation approaches are incompatible with high-throughput procedures.

- **Third Challenge**

The methods for quantifying bacterial cells pose a third trial while standardizing inactivation protocol. To achieve efficient inactivation, many approaches necessitate the supply of equivalent numbers of microorganisms. Colony forming units (CFU) plating & counting, 4,6-diamidino-2-phenylindole (DAPI) staining, fluorescent-activated cell sorting (FACS), and optical density measurement (OD) are all methods for quantifying bacteria with varying degrees of precision and adaptability for use in high-throughput *(Eun-Young et al., 2010)(Davis, 2014)*. DAPI and FACS both necessitate more bacterial handling, which takes time. Measuring OD is a faster and easier approach than plating and counting CFUs, although it is less precise. As a result, OD can be determined next to plating in order to begin

result, OD can be determined next to plating in order to begin with investigations right away while incubating bacteria on the plates.

2.1 BG Preparation Using Chemicals

Another study reported the feasibility of developing a BGs preparation procedure that does not involve lysis gene E. The procedure relies on the reducing effects of SDS, CaCO3, NaOH, and H2O2, which are used to create BGs instead of the E lysis gene. To know the right amounts of chemicals to be used for the optimum BGs production, the randomization and optimization strategy of Plackett-Burman was used. *(A. A. Amara et al., 2013)*.

Therefore, a method of producing BGs by utilizing chemicals instead of lysis gene E was discovered. The procedure depends on developing bacterial cells in a setting that takes into consideration proper formation of cell wall, then, after that, presenting the cells to synthetic substances that change the cell wall in a manner that takes into account the creation of BGs. The concentration of chemicals according to the strain in order to produce effective BGs for that particular strain. Concentration can be determined by calculating MIC and MGC of the H_2O_2, NaOH, and SDS for a more accurate estimation. MIC provides information about the minimum quantity of chemicals that may be required for bacterial lysis and killing. MGC will provide the bacterial cells with the lowest concentration possible, permitting them to survive. Vegetative cells are reportedly more vulnerable to the available chemical substances. Utilization of more than one variable to change the microbes' cell wall, different variable randomization and optimization were the most ideal choice for enhancing the BGs production process. Plackett-Burman method helps us to randomize the variables allowing us to find best conditions for the cells where they are deprived of most of their cellular content.

(Plackett & Burman, 1946)(A. A. al fattah Amara, 2011). With the help of a spectrophotometer, such protein and DNA release may be measured. It could also serve as a gauge of each experiment's effectiveness. A spectrophotometer at either 260 or 280 nm, may be used to determine the release of DNA and proteins during each phase of the preparation. Significant degrees of DNA and protein discharge recommend that the cells have lost their DNA and protein content and have changed to BGs. On the other hand, complete cell lysis should be prevented. The cells were separated and washed to eliminate any fatty substances, debris, DNA/protein, and substance buildup. An example from each examination was taken and cultured on nutrient agar plate for quite a period to check whether any live cells existed.

The experiment started with the cells being treated with NaOH, SDS, and CaCO3; after that, the cells were washed with saline, incubated with H_2O_2, and again washed with saline. After treatment with NaOH, SDS, and CaCO3, how much DNA and protein let out of the cells was estimated (initial step). In the second step, amount of DNA and protein delivered after H_2O_2 treatment was assessed. Cell quality was assessed using a light microscope and expressed as a percentage. Quality of the prepared BGs is evaluated by examination under scanning electron microscope (SEM). Miniscule pores at the surface of E.coli cells are responsible for the eviction of cells. The cells were in optimal condition and kept up with their three-dimensional structure. Furthermore, AGE was utilized to evaluate a definitive amount of bacterial DNA for each examination. Results of AGE clearly revealed that the DNA has been substantially destroyed. In comparison to the control, any type of DNA, either genomic or plasmid have been totally destroyed. Plackett-Burman design was successful in mapping the points where the protein and DNA of the cell could be precisely evacuated. The SEM showed that the cells were in great structure and has held their intact 3D shape.

The cellular lysis lysozyme gene was also used in this process, which degraded any surviving cells. This could be useful in applications like the preparation of fragmented BGs. All of the genetic elements were degraded, according to the AGE. In fact, this technique is a compilation of lessons learned from a variety of earlier protocols, including the competent cells formation, which doesn't compromise on the viability of cells during transformation. SDS breakdown the cell walls of bacteria; nevertheless, in the right dose, it might be utilised to achieve our goal. Instead of employing nucleases, H_2O_2 possesses oxidising action and can damage genetic material *(A. A. Amara et al., 2013)*. The action of NaOH on the cell wall is likewise well-known.

2.1 BG preparation using Tween-80

Bacterial ghosts (BGs) can be made in two ways: genetically and chemically. Using the lysis gene E as a genetic approach is one option. Incubation of cells at minimum inhibitory concentration of different substances for a brief period of time is a chemical procedure to produce BGs.

The study reported the production of BGs using a novel method in which cells are exposed to Tween 80 for a short period of time. This is followed by substantial decreasing pH of the bacterial culture. SEM analysis revealed the presence of perforated cells with at least one intramembranous pore to release intracellular content while keeping the outer cell wall intact. Subculturing confirmed the absence of essential cells. Another evidence of successful ghost preparation is the release of proteins and DNA. Furthermore, the cells integrity was demonstrated using light microscopy and Gram-stained cells. To summarize, this new methodology for BG preparation is straightforward, cost-effective, and realistic *(Rabea et al., 2018).*

2.2 Applications of Bacterial ghosts (BGs)

- ***BGs solo***

BG-based vaccination against Gram-negative bacteria has been evaluated in many animal studies *(Jalava et al., 2002)(Walcher et al., 2004)*. Various BGs have been utilized in animals models for the preparation of A. pleuropneumoniae vaccine (*App*) to be used in swine. *App* BGs protected the animal from developing the aerogenic infection with the potentially devastating pathogen. Bacteria was unable to colonize lungs and tonsils, indicating that BG vaccination is better than bacterin therapy (Andreas Hensel et al., 1996). More notably, there have been no reports of clinical adverse effects.

Mucosal inoculation of BGs is far better than parental inoculation. Administration of App BGs via Mucosal route *(A Hensel et al., 1995)* or aerosols *(Andreas Hensel et al., 1996)(Huter et al., 2000)* depicted not only the immunity but also cross-protection in pigs against other serotypes *(Huter et al., 2000)*. On the other hand intramuscular immunization *(Andreas Hensel et al., 2000)* totally ensured immunized pigs against infection after challenge, however, sterile immunity was not observed because the test bacteria was re-isolated from the tonsils of vaccinated pigs.

In rabbit and mouse models, BG from P. multocida and M. haemolytica was employed. Cross-protective antibodies were produced, and they were efficacious against different Pasteurella strains as well as the one used for immunisation (Marchart, Dropmann, et al., 2003). Cattle inoculation with M. haemolytica BG provided protective immunity comparable to commonly produced vaccines *(Marchart, Rehagen, et al., 2003)*.

Preclinical investigations on V. cholera have been completed. Rabbits were fully protected using the ilea loop challenge concept. Partially cross-protection was detected against the classic O1 strain and the newly discovered O139 strain *(Eko et al., 2003)*. Mucosal injection of BG candidate vaccines has proven to be a suitable method for generating both type of immune response in most models. *(Riedmann et al., 2007) (Walcher et al., 2004)*.

- ***BGs as Adjuvants***

During the lysis phase, the BG morphology is not denaturated. As a result, all of the major immune-stimulating components are preserved. Lipopolysaccharides (LPS), peptidoglycan, monophosphoryl lipid A (MPL) and flagella are most common example of pathogen-associated molecular patterns (PAMPs). PAMPs induce the innate immune response because they are identified by toll-like receptors (TLR). As a result, most bacterial strains used for the BGs production set off innate immune responses as a first response. Inherently, they also have adjuvant qualities, making them exceedingly adaptable in terms of inducing both responses, humoral and cellular, in laboratory animals *(Riedmann et al., 2007)*.

- ***BGs as carriers of foreign protein antigens***

Foreign antigens can be incorporated into or associated with the microorganisms' envelope before lysis utilizing recombinant DNA techniques, and in this way they may become part of the BGs. AGs are presented on the cell surface as membrane

anchors fused with N-, C-, or N/C-terminal. They are also present on IM fused with N-, C-, or N/C-terminal and target the cells via outer membrane protein like ompA or

via fusion *(Hobom et al., 1995)*. 30 fusion with the membrane anchoring proteins had no impact on AG assembly and folding, nor did it decrease the functionality of enzyme, inferring that AGs are in the right conformation. BGs can be directly fused with AGs, but another technique for loading BGs has also been devised. In this strategy, the BGs are attached with streptavidin, which is a membrane- anchored. Such streptavidin loaded BGs can be fused with a biotinylated drugs after lyophilization *(Huter et al., 1999)*.

Another method to incorporate foreign antigens into BGs is the export directed to PPS via fusion protein or signal sequences. During lysis, the PPS is fixed, holding by far most of periplasmic parts inside the envelope complex *(WITTE & LUBITZ, 1989)(A Witte et al., 1990)*. The PPS's membrane oligosaccharides provide protection during lyophilization in order to prevent inactivation. *(Mayr et al., 2005)*.
When target antigen DNA sequences are fused to the Bacillus stearothermophilus S-layer genes sbsA or sbsB and produced heterologously in bacteria (gram-negative), they make sheet-like self-assembling structures inside the cytoplasmic space *(Kuen et al., 1996)*. S-layers, during lysis, are not released with the cytoplasm because they are made up of several 100,000 subunits. S-layer gene may incorporate foreign sequences of large proteins which are foreign to host microorganism. Protein subunits can likewise be exported out to the PPS preceding S-layer arrangement by connecting MalE to SbsA *(Riedmann et al., 2003)*.

- ***BGs as carriers of DNA vaccines***

Virus and bacterial based vaccines with ability to transfect the host cells run the

danger of reverting to their pathogenic states. Transfection efficiency have been reduced in many systems which are non-viral such as DNA complexes, attenuated bacteria, and nucleoporation *(Gentschev et al., 2001) (Schumacher et al., 2004)*. With another very productive gene delivery technology, the BG offers an option in contrast to current viral and bacterial methodologies in vaccine development. The safety profile of BGs is one of the most significant characteristic of the DNA-carrier framework. In vitro studies have shown that following mutual co-incubation, No cytotoxic or genotoxic effects of BGs have been observed for human cells. This finding was made regardless of the strain used for BG production (*Koller and Lubitz, personal communication).* DNA vaccines are recently being used in veterinary medication *(Weiner, 2006)*.

Before DNA vaccines may be declared safe to be used for human, more study and development is required. It requires high dose of plasmid but low immunogenicity, which is usually linked to the lack of an efficient techniques for delivery, is one reason for the slow pace of DNA vaccine research and licensing approval *(Liu et al., 2006) (Wiendl et al., 2005)*. BGs are being used in several research to deliver DNA vaccines, and an easy process for loading of plasmid DNA onto BGs have been devised. BGs, in lyophilized form, are suspended in solution containing DNA, then washed many times to detach unbound plasmid DNA from the BGs. The concentration of DNA solution used has a direct relationship with the quantity loaded DNA on to the BGs. This loading method is very efficient, with the ability to load 6,000 plasmid copies on each BG *(Paukner et al., 2005)*.

The fact that BGs are non-living is one of their key advantages. They preserve surface morphology, antigenicity and structure which are living counterparts of a cell. The loading capacity of bacterial ghost is likewise exceptional *(A Witte et al., 1990)*. The

inside space of the envelope of an empty BG can be jammed with a variety of peptides, medicines, or DNA, allowing us to create novel polyvalent vaccines *(Paukner et al., 2005)*(Usanne et al., 2005). Tumour cells and antigen presenting cells (APCs) both successfully internalise and phagocytize BGs equipped with DNA encoded in plasmid. The heterologous genes are delivered to non-dividing as well as dividing cells using BGs. Reported genes are present on plasmid and delivered by BGs was expressed by up to 82 percent of cells in the study. On the other hand, no cytotoxicity was seen in target cells *(Ebensen et al., 2004) (Paukner et al., 2005)(Usanne et al., 2005) (Kudela et al., 2008)*. Balb/c mice were immunised intradermally and intramuscularly with plasmid pCMV, containing beta-galactosidase, loaded onto BGs and elicited more effective innate and adaptive immune response against antigens than naked DNA. After intravenous inoculation of mice with dendritic cells (DCs), which undergo tranfection with pCMV BGs, a beta-galactosidase-explicit safe reaction was found *(Ebensen et al., 2004)*. In mice inoculated with BGs containing DNA, increased expression of MHC (class I) molecules as well as costimulatory molecules was observed. *(Ebensen et al., 2004)*. Cross-presentation of antigens expressed on DCs and delivered via BGs has the ability to induce both CD4+ and CD8+ immune response significantly enhancing the immune response. Lipopolysaccharides of bacteria promotes maturation of DC, changes DC acidification, and enhance cross presentation of antigens *(Sergio et al., 2003)(Trombetta & Mellman, 2004)*.

BGs hold their inward and outside membrane components, such as LPS and successfully invigorating AG-cross-presentation *(Sergio et al., 2003) (W. Lubitz, 2001)*. In general, the creation of BGs and the loading of plasmid DNA into them are two independent activities. This lengthy technique was streamlined into a single step

technique, a cost effective procedure with the introduction of our novel self immobilising plasmids (pSIP). The DNA in the form of plasmid, containing an operator sequence is attached to a particular binding protein of DNA found on the bacteria's internal membrane during this process *(Mayrhofer et al., 2005)*. For gene therapies and vaccination strategies containing DNA sequences, antibiotic resistance genes and bacterial essential genes are regarded as an important safety measure. To overcome this issue, researchers developed novel pSIP BG-DNA vaccines. These are based on minicircle (mc) DNA that is free of physiologically dangerous residues. The ParA resolvase mechanism is used to create mcDNA that is destined to the receptor of inner membrane in this upgraded version of pSIP. In the lysis mediated by gene E, the equivalent sis pair miniplasmid formed in the procedure is ejected to the culture media *(Jechlinger et al., 2004)*. Based on the activity of endonucleases from the I-SceI gene programmed by the original plasmid, a modified system for minicircle formation digesting the miniplasmid is being documented *(Chen et al., 2005)*.

The stimulation of cell-mediated and antibody mediated immune responses is the primary benefit of DNA vaccines. Antigen epitopes show both MHC class I and II molecules after processing through both endogenous and external routes *(Wolff & Budker, 2005)(Yu & Finn, 2006)*. Gene therapy that is well-designed and implemented should be able to successfully transport required AG DNA to APCs. The expression, natural dispensation, and appearance of AG-derived epitope follows. T lymphocytes that have been developed against AGs that have been supplied, naturally digested, and presented by APCs may be more effective in recognising the epitopes offered by cells expressing similar antigens. A gene which is expressed should elicit powerful immune reactions or cause targeted cells to behave differently.

Peptidoglycan and LPS are examples of BGs with intact envelope structures. These

materials not only "wake up" professional phagocytic APCs, but also give tumor cells stimulatory signals. Melanoma cells, for example, have been demonstrated to have the ability to act as non-professional APCs, phagocytose both apoptotic and living cells *(Lugini et al., 2006)*, and respond to BG challenge *(Kudela et al., 2008)*. Despite BGs' strong DNA loading capacity, melanoma cells can effectively deliver and express genes with relatively low DNA quantities. After incubating BGs with cancerous cells, more transfection proficiencies were found.

- ***BGs as carrier of biologically active substances***

The bacterial ghosts platform offers a unique and advanced system for medication and other biologically active material delivery. Because BGs lack cytoplasmic content due to which their loading capacity increases about 250 fm/BG. This space can be packed with medications of your own concern like a liquid, immersed into the lipid partitions, or selectively linked to receptors in the BGs.

In vitro administration of the mild DOX to human cancer cells was achieved using BGs generated by *M. haemolytica*. The release of endogenous drugs has been confirmed. In Caco-2 cells, there were increased cytotoxic and antiproliferative actions. When compared to the drug alone, bacterial ghosts loaded with DOX were 2–3 times more good and effective *(Paukner et al., 2005)*.

Another delivery strategy used the water-soluble chemical calcein, with bacterial membrane vesicles inserted into the old lysis pores *(Paukner et al., 2003)*.

With the lipophilic fungicide tebuconazole, Bacterial ghosts of *P. cypripedii* were employed as pesticide delivery devices. Due to the BGs' adherence to the plant, this formulation provided a stronger resilience to rainfalls, according to the research.

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Against agricultural plant diseases, this BG treatment has both protective and curative effects *(Hatfaludi et al., 2004)*.

2.1 Anti-Cancerous Drug Doxorubicin Drug Delivery

Anticancer medications are frequently administered systemically, resulting in significant lethal drawbacks. For reduction of these side effects, improved drug delivery strategies based on particular cell targeting vehicles are required. DOX could be loaded into bacterial ghosts and that the DOX was released in a long period. Furthermore, the BGs loaded with DOX was transported to cancerous cells and strong cytotoxicity was achieved thanks to the effective targeting characteristics of *M. haemolytica* ghosts. Because of transport by bacterial ghosts, the DOX dose might be greatly reduced with the same cytotoxicity as free DOX. Overall, DOX-loaded bacterial ghosts provide effective DDV that could be helpful in the treatment of cancer.

MATERIALS & METHODS

3 Materials and Methods

3.1 Materials

This part of materials consists of all the instruments, equipment, chemicals, bacterial strain, buffers and media used during the research work.

3.1.1 Equipment

Table 1: Equipment used during this study

Equipment	Manufacturer
SEM	Jeol JSM-6490A
Nanodrop	Colibri
Shaking Incubator	Jsr
Gel Electrophoresis Tank	Cleaver Scientific Ltd
Ultraviolet Viewing cabinet	Extra Gene
Tabletop Balance	ShiMADZu
Hot Plate	Velp-Scientifica
Centrifuge Machine	Hermle
pH Meter	WTW inoLab
Microwave Oven	Haier
Laminar Flow cabinet	Esco
Microcentrifuge	Sigma
Vortex Mixer	Heidolph
Spectrophotometer	Optima
Incubator	Memmert
UV-Vis Spectrophotometry	Agilent, USA

DNA Extraction Kit	New England Biolabs
Bench top freeze dryer	Christ alpha 1-2/LD plus

3.1.2 Reagents/Chemicals

Table 2: Reagents/Chemicals used during this study

Reagents/Chemicals	**Manufacturer**
Agar	Bioworld USA
Agarose	Bioworld USA
Nutrient Broth	Lab M UK
Tryptone	Bioworld USA
Tween-80	Sigma-Aldrich
Yeast Extract	Lab M UK
Lactic Acid	Sigma-Aldrich
Ethanol Absolute	Sigma Aldrich
Ethidium bromide	Sigma Aldrich
PBS	Inovatiqa
Glutaraldehyde	Sigma-Aldrich
NaCl	Sigma-Aldrich
Sodium acetate	Sigma-Aldrich
Doxorubicin	Pfizer
SDS	Sigma-Aldrich
Sulfuric Acid	Bioworld USA
Dialysis tubing	Sigma-Aldrich

3.1.3 Molecular biology grade Markers used

Table 3: Table 3.3 List of Molecular biology Markers used

Marker	Manufacturer
GeneRuler 1kb DNA Ladder	Thermo scientific
GeneRuler loading dye	Thermo scientific

3.1.4 Bacterial strains used

Table 4: Bacterial strain

Bacteria	Description	Source/Reference
DH5α	Used for preparation of Bacterial ghost	Plant virology Lab, ASAB, NUST

3.1.5 Microbiological Media

Distilled water was used to prepare all the required solutions, buffers and media. The media was prepared and autoclaved in order to maintain the sterility. Autoclaving was carried out at 121℃ for 15 minutes. pH was adjusted at 7.0 unless otherwise mentioned.

Table 5: LB Broth

Sr. No	Components	Quantity (g/100ml)
1	Tryptone	1.0
2	Yeast Extract	0.5
3	NaCl	1.0

Table 6: Nutrient Agar (N Agar)

Sr. No	Components	Quantity (g/100ml)
1	Peptone	0.5
2	Beef Extract	0.3
3	Agar	1.5

Table 7: Luria Agar (L Agar)

Sr. No	Components	Quantity (g/100ml)
1	Tryptone	1.0
2	NaCl	1.0
3	Agar	1.5
4	Yeast Extract	0.5

3.1.6 Solutions used in bacterial ghost preparation

Table 8: 7% tween-80 solutions

Sr. No	Components	Quantity $100ml^{-1}$ (v/v)
1	Luria broth	93.0
2	Tween-80	7.00

3.1.7 Washing Buffer

Table 9: Half Normal saline

Sr. No	Components	Quantity (g/100ml)
1	NaCl	0.45
2	Distilled water	100.0

3.1.8 Solution used in SEM slide preparation

Table 10: PBS buffer

Sr. No	Components	Quantity (g/100ml)
1	PBS Tablet	1.0
2	Distilled water	100.0

Table 11: 2.5% Glutaraldehyde solution

Sr. No	Components	Quantity $100ml^{-1}$ (v/v)
1	Glutaraldehyde	16.00
2	PBS	84.00

Table 12: 70% Ethanol solution

Sr. No	Components	Quantity $100ml^{-1}$ (v/v)
1	Ethanol	70.00
2	Distilled water	30.00

3.1.9 Buffer used for Gel Electrophoresis TAE (1x)

Table 13: TAE (1x) Buffer

Sr. No	Components	Quantity
1	Tris-HCl	40 mM
2	Acetic acid	40 mM
3	EDTA	0.4 mM (pH 8.0)

3.1.10 Solution used for resuspention/Loading of bacterial ghosts

Table 14: Tris-HCL (pH 8.0)

Sr. No	Components	Quantity (g/100ml)
1	Tris	12.11
2	Distilled water	100.0
3	HCL	pH maintenance

Table 15: Doxorubicin Solution

Sr. No	Components	Quantity (mg/ml)
1	DOX	10.0
2	Tris-Hcl	1.00

Table 16: Loading Solution

Sr. No	Components	Quantity (mg/µl)
1	Lyophilized BGs	10.0
2	DOX solution	100.0

3.1.11 Solutions used for quantification of loaded drug

Table 17: 10% SDS Solution

Sr. No	Components	Quantity (g/100ml)
1	SDS	10.0
2	Distilled water	100.0

Table 18: 10mM H2SO4

Sr. No	Components	Quantity
1	H_2SO_4	1M
2	Distilled water	99

3.1.12 Solutions used for release profiling of loaded BGs

Table 19:PBS solution (6.4 pH)

Sr. No	Components	Quantity (1 pc/100ml)
1	PBS Tablet	1
2	Distilled water	100.
3	Lactic Acid	Few drops

Table 20:PBS solution (7.4 pH)

Sr. No	Components	Quantity (1 pc/100ml)
1	PBS Tablet	1
2	Distilled water	100.

3.2 Methodology

3.2.1 Target Strain

The bacterial strain which was used for the preparation of bacterial ghosts was *E. coli DH5a.* It was obtained from plant virology lab, ASAB, NUST. DH5α cells from cryovials were taken and streaked on L-agar plates and incubated them at 37°C for overnight to get isolated colonies. Next day, isolated colonies from the petri plates were taken and were added to the conical flasks containing Luria broth (LB). Conical Flasks were incubated overnight in a shaking incubator at 37°C by keeping speed of incubator at 200 rpm in order to get growth.

3.2.2 Stock Preparation of E. coli DH5a and storage

Add 800µl of the overnight culture to the 200µl of 100% sterile glycerol in the already autoclaved Eppendorf tubes. Vortex was used in order to thoroughly mix the glycerol and the culture having cells. Eppendorf tubes were labeled properly with bacterial strain name, date and then they were stored at - 80°C and -20°C for downstream processing.

3.2.3 Preparation of 7% tween-80 solution

100ml of 7% tween-80 (v/v) solution was prepared by adding 7ml of tween-80 into the 93ml of LB media. This LB media containing tween-80 will be used for the ghost preparation.

3.2.4 Bacterial ghost production

Bacterial ghost were prepared using a unique procedure mentioned by (Rabea et al., 2018) with minor changes. 200µl of primary inoculum was added to the 5ml of the 7% tween-80 solution under the sterile Conditions. Flasks were placed in the shaking incubator for 24 hours at 37°C.

3.2.5 pH maintenance

After 24 hours of incubation, cultures were transferred from flask to the 50ml falcon tubes. Rod of pH meter was dipped into the distilled water to check the pH of water, which was 7 indicating the correct working condition of pH meter. After that, I checked the pH of a sample, which was round about 10. This pH was highly basic, and then we add 50 µl of lactic acid to make it acidic drop wise. I checked the pH of all the samples and maintain their pH at 3.6. After maintaining 3.6 pH of samples, incubate them at 37°C for one hour.

3.2.6 Separation of pellet and supernatant

Took the samples from incubator after one hour and centrifuged them at 4000xg for 10 min. Then I saved the supernatant in the falcon tubes for further testing and evaluation and washed the pallet with half-normal saline. Repeat the washing step two times.

3.2.7 Lyophilization and storage

After washing bacterial ghost were freeze dried using bench-top Christ freeze dryer and then stored at 4°C until further use.

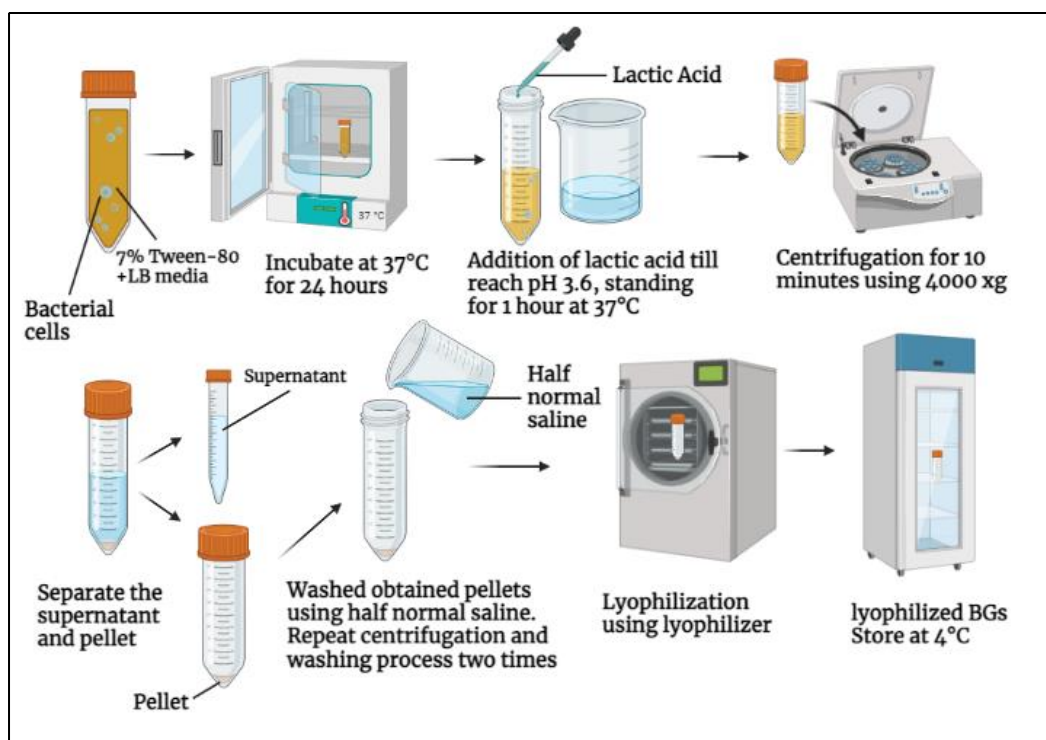


Figure 4: Detailed diagram of bacterial ghosts preparation

3.3 Characterization of bacterial ghost

3.3.1 Optical Density

Optical density (O. D) was measured to know the growth rate of normal and treated *E. coli* DH5α. O.D was measured before the addition of Tween-80 using Spectrophotometer. Then O.D measurements were taken after 24 hours of tween-80 addition that too using a spectrophotometer.

3.3.2 Sub-culturing

Possibility of any viable cell is confirmed by spreading the 25 microliter treated inoculum over the surface of NA plates. Then plates were incubated for overnight at 37˚C.

3.3.3 NanoDrop

At 260 nm, the NanoDropTM 2000/2000c spectrophotometer was used to measure the amount of DNA released in the supernatant.

3.3.4 Bradford Assay

Bradford method for protein quantification was applied for the quantification of released proteins from ghost cells by using a NanoDrop™ 2000/2000c spectrophotometer.

BSA delivered by the industrialist was used for standard curve generation. All samples were analyzed at 595 nm. The proteins in the media were considered and quantified the proteins in un-inoculated media.

3.3.5 Extraction of DNA from Supernatant

DNA was extracted from the supernatant in small volume. Supernatant, which was separated during ghost preparation. DNA extraction was done using AxyPrep™ multisource genomic DNA miniprep kit (Tewksbury, MA, USA). The whole process was processed according to the supplier's specifications.

DNA was extracted from the supernatant in small volume. Supernatant, which was separated during ghost preparation.

3.3.6 Agarose gel electrophoresis

For preparation of 1% gel, 0.5 grams of agarose was dissolved in 50ml of 1X TAE buffer (Table 3.10) by boiling. After complete dissolution, the gel was

cooled down for few minutes and then ethidium bromide was added for stain. The gel was poured into a taped gel-casting tray which already had comb.

After polymerization, the agarose gel was shifted into the gel tank and the gel tank was filled with 1X TAE buffer. Sample was mixed with 6x DNA loading dye. 1kb ladder was used for reference. Electrophoresis was carried at 80V for 45-50 min.

3.3.7 SEM slide preparation

The bacterial cells pellets were investigated by SEM (JEOL-JSM-5500 LV): The bacterial cell pellets were fixed by 2.5% glutaraldehyde and ethanol dilutions were used for the dehydration. The samples were dried in the incubator and then samples were coated by gold sputter coater (SPI-Module). Lastly, all the samples were studied by SEM with x9500 amplification power, 20 kV, and using high vacuum mode at the school of chemical and Mechanical Engineering (SCME), NUST, Islamabad.

3.3.7.1 *Fixation step:*

Wash obtained pallets with PBS (phosphate buffer saline) several times and then centrifuge. After centrifugation, incubate pallet in 2.5% glutaraldehyde for 30 minutes at room temperature. Then incubate the pallets at 37°C for overnight.

3.3.7.2 *Dehydration step:*

Dehydrate with increasing percentage of absolute ethanol (40%, 50%, 60%, 70%, and 100%). Incubate in 40% ethanol for 10 minutes and then remove it. Do the above step for 50%-100% ethanol for 10 minutes each. Do not mix the sample with ethanol. Just add ethanol and leave it for 10 minutes on the table. If it is mixed, centrifuge it at high speed. Remove ethanol and dry the pallet in the incubator for 1 hour and then put it in the laminar airflow for complete dehydration for some time.

3.4 Loading of bacterial ghost

3.4.1 Resuspension of Bacterial ghost & Doxorubicin

After the preparation of bacterial ghosts, Freeze-dried bacterial ghosts were loaded with anti-cancerous drug Doxorubicin. The drug was hydrated in Tris-Hcl (pH 9.0) at a concentration of 5mg/ml. The lyophilized BG were resuspended in drug solution. Bacterial Ghosts uptake the drug by diffusion through the membrane tunnels.

3.4.2 Loading of bacterial ghosts

A total of 10mg of lyophilized Bacterial ghosts were incubated with 200µ DOX solution (5 mg/ml), in a total weight of 1 mg while keeping all the factors constant; pH = 9, DOX quantity =1 mg and period of incubation =10 min, incubation temperature 27°C. The Drug loaded BGs were separated by centrifugation at 5000rpm for 5min. The obtained pellets were washed by the Tris-HCl buffer three times and then freeze dried them.

3.4.3 Extraction of the loaded DOX

For the evaluation of loading capacity, the bacterial ghosts were disturbed for the elaboration of loaded DOX as mentioned before (*Alsuwyeh et al., 2018*). In short, 10 mg freeze dried bacterial ghosts were resuspended into 1ml distilled water and then mix the same volume of 10% SDS solution and incubated at 65°C for 10 min. 1ml of sulfuric acid (10 mM) were added and further incubated at the same conditions. We performed centrifugation for the separation of supernatant which was used in DOX analysis.

3.4.4 Encapsulation Efficacy of Doxorubicin

The amount of drug loaded in the bacterial ghosts was quantified by UV-Vis Spectrophotometry. The collected supernatant was analyzed for this purpose. The

absorption at 470nm was used to quantify the exact amount of DOX loaded in the BGs. A standard calibration curve was generated by using the standard solutions of pure drug in 10% SDS, 10mM H_2SO_4 and Distilled water. All the measurements were completed in triplicate. The Entrapment Efficiency (EE %) was calculated using following equation:

$$EE= \frac{Ws}{Wtotal} \times 100$$

Where, W_s is the weight of loaded Drug in bacterial ghost and W_{total} is the initial weight of drug used.

3.5 *In vitro* drug release profiling

Determination of drug release pattern from loaded BGs was carried out through dialysis assay. Prior to use dialysis tube was soaked in distilled water at room temperature in order to properly hydrate it and to remove preservatives. In each dialysis experiment, 2 ml of drug encapsulating BG suspensions was placed in dialysis tubing (MWC ranging from 35,000) and sealed at both ends using a cotton thread. This dialysis tube was then placed into a beaker with 100ml of release medium which is PBS in both cases with (A) pH 7.4 corresponds to the pH of human blood (B) pH 6.4 corresponds to the pH of tumor tissues. This dialysis tube having sample was hanged in a beaker so as it was completely dipped under the surface of release medium. The beaker was kept on a magnetic stirrer and continuous agitation was provided at room temperature.

With every one hour's interval, an aliquot of 2 ml was taken out for the spectrophotometric analysis. Aliquots were withdrawn regularly at predetermined intervals and release medium was kept on changing with same volumes of fresh PBS

at regular intervals. DOX presence was quantified by UV/Vis spectrophotometer *(Shabbits et al., 2002)*.

3.5.1 Standard calibration curve for drug release assay

A standard curve for Doxorubicin from 0.01 to 0.1 mg/ml drug was generated. For making this standard calibration curve, stock solution of 1 mg/ml of Doxorubicin was prepared in PBS, and then a serial dilution was done in order to obtain the required range known sample concentrations (0.01-0.1 mg/ml). All measurements were taken using independent samples in triplicates at the λmax = 470 nm. After quantifying each sample separately, cumulative release was calculated using subsequent formula;

Cumulative Drug Release Percentage

$$\frac{Concentration\ of\ drug\ released\ in\ medium}{Concentration\ of\ initially\ added\ drug} \times 100\ (\%)$$

3.6 Statistical Analysis

Statistical analysis was completed with the help of GraphPad Prism 5, Microsoft Excel and Origin software.

RESULTS

4 Results

4.1 Bacterial Ghosts Preparation & Characterization:

Preparation using Tween-80:

Different methods are being used to prepare the bacterial ghost. In all the chemical methods that are being used to synthesize bacterial ghost Tween-80 is the best. Tween-80 with combination of lactic Acid gives best results. *(Y. Han, Han, Wang, Meng, Zhang, Ding, & Yu, 2014)*

4.1.1 Growth rate Untreated vs. Treated

E. coli cultures with OD levels greater than 0.4 demonstrated poor lysis, whereas culture with OD levels of 0.2–0.3 produced extremely effective BG.

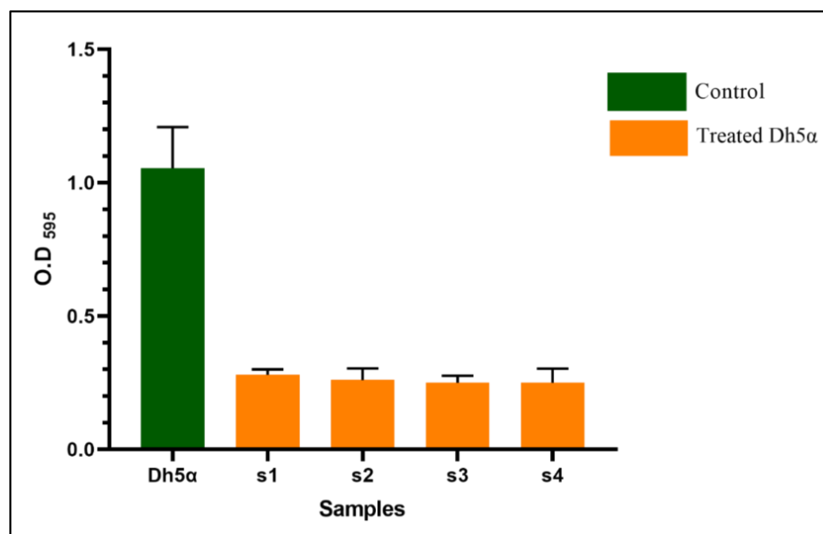


Figure 5: Optical Density measurements before and after treatment with Tween-80

4.1.2 Viability of E. coli cells

Untreated bacterial cells showed very good growth on N-agar plates after an incubation period of 24 hours while bacterial cells which were treated

with Tween-80 showed no growth on N-agar plates even after the incubation period of 72 hours. This shows that the treated bacterial cells were inactivated.

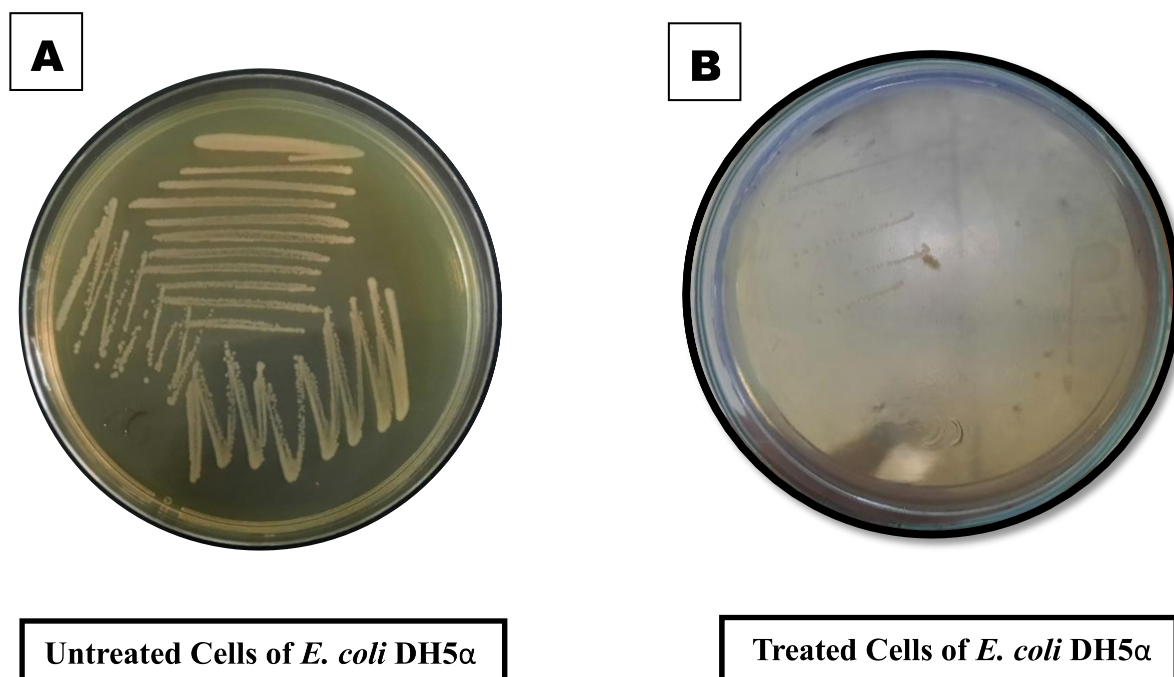


Figure 6: Sub-culturing of E. coli cells before and after treatment with Tween-80

4.1.3 Quantification of Released DNA

The amount of released DNA was significantly changed in different conditions. The maximum DNA was released at 1 hour standing period after pH maintenance with 5ml volume of LB containing 7% tween-80 into 200µl of inoculum. In case of experiment A, following conditions were applied.

- 200µl of inoculum into 5ml of LB Containing 7% of tween-80
- pH of Media 3.6
- Incubation temperature 37°C for different time period

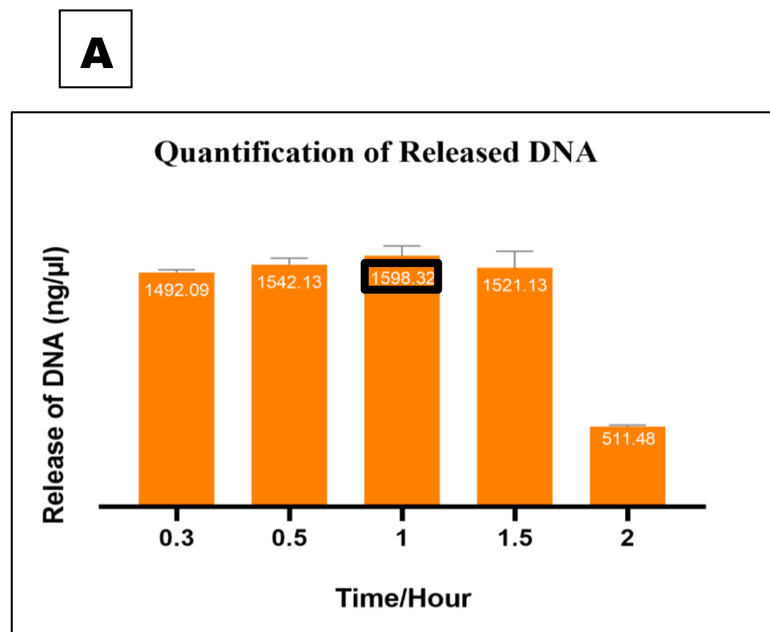


Figure 7: Quantification of released DNA at different time period

In case of experiment B, following conditions were applied.

- 200µl of inoculum into different Concentrations of LB 7% of tween-80
- pH of Media 3.6
- Incubation temperature 37°C for 1 hour

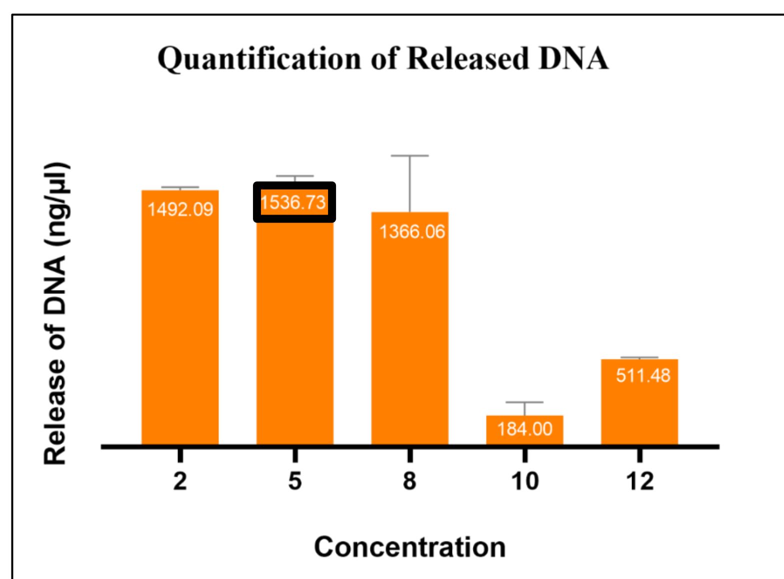


Figure 8: Quantification of released DNA at different concentration

So, the optimized conditions for preparing *E. coli* DH5α bacterial ghosts was the addition of 200µl of inoculum into 5ml volume of LB containing 7% tween-80 after standing period of 1 hour at pH 3.6.

4.1.4 Confirmation of Genetic Material Degradation

The Agarose gel electrophoresis simply proves that the genomic and plasmid DNA have been completely destroyed, as evidenced by the lack of band. The prepared BGs are free of most genetic elements, according to AGE data. As BGs are entirely inactivated cells, this underlines that they are non-living.

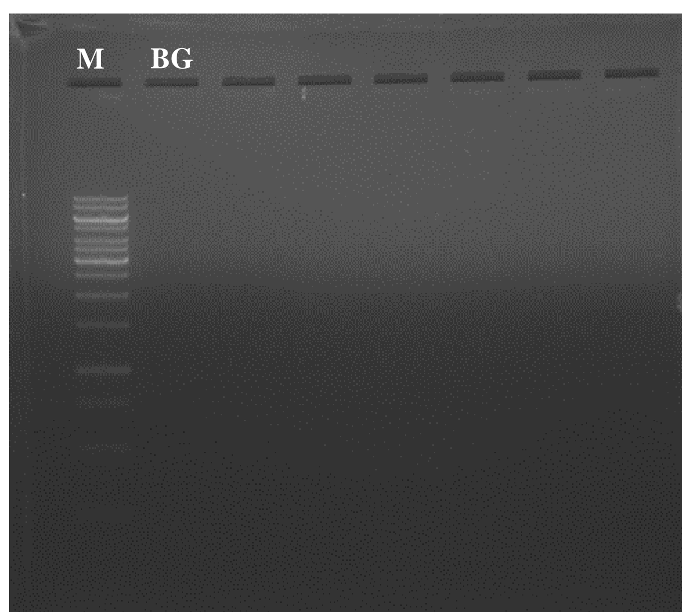


Figure 9: A) AGE for the Marker (M) and Bacterial Ghost (BG)

4.1.5 Quantification of Released Protein

Bradford Assay was performed for the quantification of released protein. Standard curve of BSA (Bovine Serum Albumin) was generated to quantify the maximum protein released from bacterial ghost which was 7.10µg/ml.

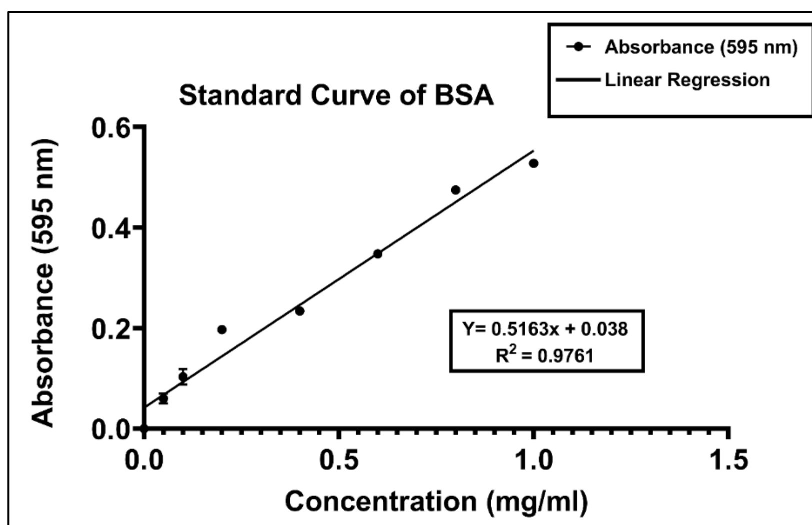


Figure 10: Standard curve of Bradford assay for quantification of released protein

$$Y = ax + b$$

$$X = y - b / a$$

Table 21: Maximum released proteins from the prepared bacterial ghosts

Sample	Absorbance	Concentration	Dilution factor	Actual concentration
1	0.391	0.32	20.00	6.50
2	0.423	0.36	20.00	7.10
3	0.391	0.32	20.00	6.50

4.1.6 Characterization of bacterial ghost:

Preparation of bacterial ghosts is confirmed using different methods.

4.1.6.1 SEM (Scanning electron microscope):

The centrifuged pallets of bacterial cells were investigated for bacterial ghosts using Scanning electron microscope. SEM Analysis showed the successful production of bacterial ghost with an average size of 111.60nm.

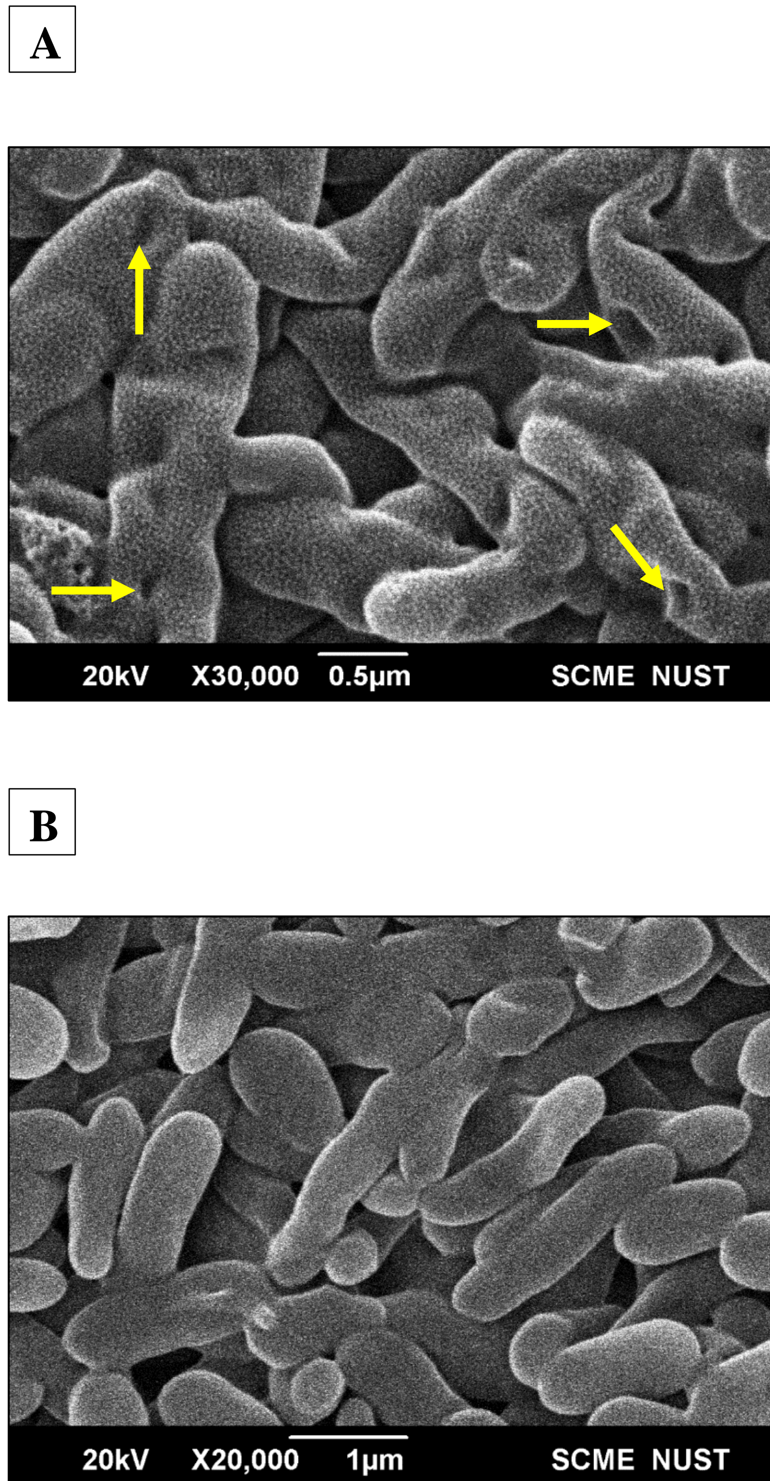
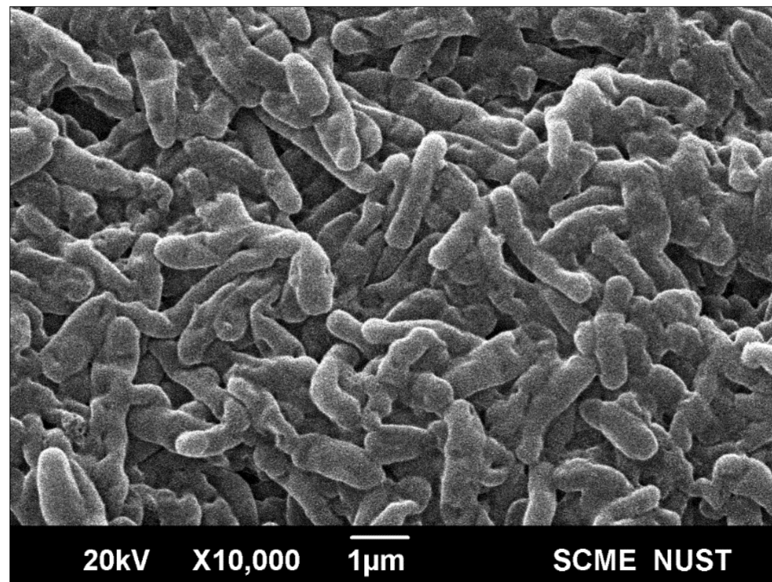


Figure 11: SEM images of the prepared A) DH5α ghosts Vs. B) Control. The arrows point to certain surface holes formed when E. coli cultures were incubated with tween-80

A



B

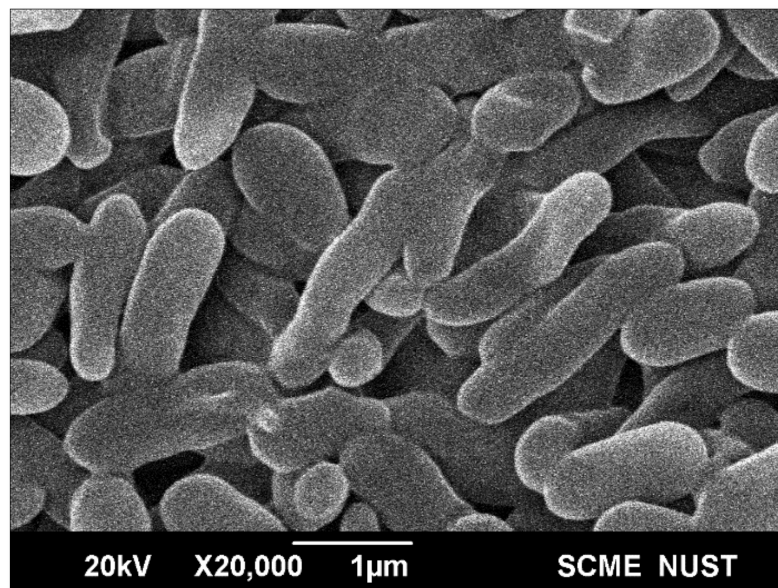


Figure 12: Confirmation of Bacterial Ghost: (A) Holes of different nanometer can be seen on the surface of bacterial cell (B) Untreated cells showing unaffected surfaces

4.2 Drug encapsulation Efficiency:

The absorbance was measured with a UV-vis spectrophotometer at 470 nm in various concentrations of Doxorubicin. For the standard curve for Doxorubicin, a graph was generated between the absorbance of the UV spectrophotometer and the concentrations. The concentration of the free drug in the supernatant was determined through this curve using the following equation.

$$y=mx+b$$

Therefore.

$$\frac{y-b}{m}=x$$

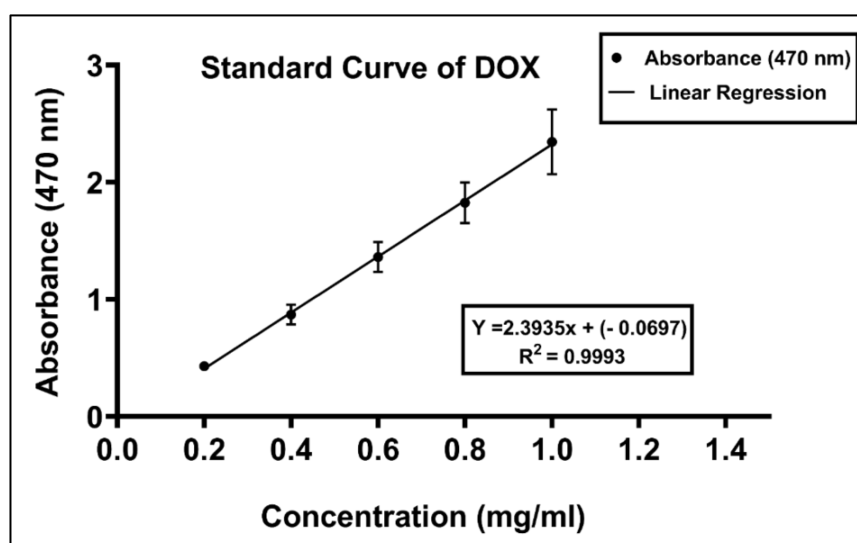


Figure 13: Standard curve for Doxorubicin in concentration 0.2- 1 mg/ml, for drug encapsulation efficiency analysis. Each sample was run in triplicate (n=3, STD ±0.23) and the graph was plotted against mean values

Therefore, Doxorubicin encapsulation efficiency in the bacterial ghosts was easily calculated. At first, the total drug for was 1g/ml. This has resulted in 43.71% of Doxorubicin being encapsulated using the EE% formula. Table 3 shows detailed results.

Table 22:Drug (DOX) encapsulation efficiency percentage of Bacterial ghosts, Absorbance at 470 nm of Doxorubicin known concentrations

DOX (mg/ml)	Replicate 1	Replicate 2	Replicate 3	MEAN	SD
0.2	0.413	0.470	0.405	0.429333	0.035445
0.4	0.843	0.804	0.964	0.870333	0.083429
0.6	1.226	1.374	1.483	1.361	0.128992
0.8	1.673	2.015	1.789	1.825667	0.173923
1	2.286	2.102	2.646	2.344667	0.276704

Table 23: Drug (DOX) encapsulation efficiency percentage of Bacterial ghosts, Absorbance at 470 nm for released Bacterial ghosts in the medium

OD-1	OD-2	OD-3	Average	SD	SK in Medium
0.97	0.96	0.98	0.977	0.00888351	0.43

Y= mx + b

Y= 2.57x + 0.26

Y= Line intercept (Absorbance)

X= any point on the line (Drug Concentration)

$$x = \frac{y - 0.267}{2.57}$$

X= 0.43 mg/ml

$$\text{ENCAPSULATION EFFICIENCY } (\%) \frac{\text{DRUG RELEASED IN THE MEDIUM}}{\text{CONCENTRATION OF DOXORUBICIN ADDED INITIALLY}} \times 100$$

$$= \frac{0.43}{1} \times 100$$

ENCAPSULATION EFFICIENCY (%) = 43%

4.3 *In vitro* drug release assay

Six point's standard calibration graphs were generated covering a wide range of known drug concentrations i.e. 0.02–0.1 g/ml. Three independent measurements were obtained using each concentration. Significantly linear relationship was observed between the sample absorbance and the corresponding drug concentration. Results are presented in the form of linear curve (y= 2.4014x + 0.0384, $R^2 = 0.9619$)

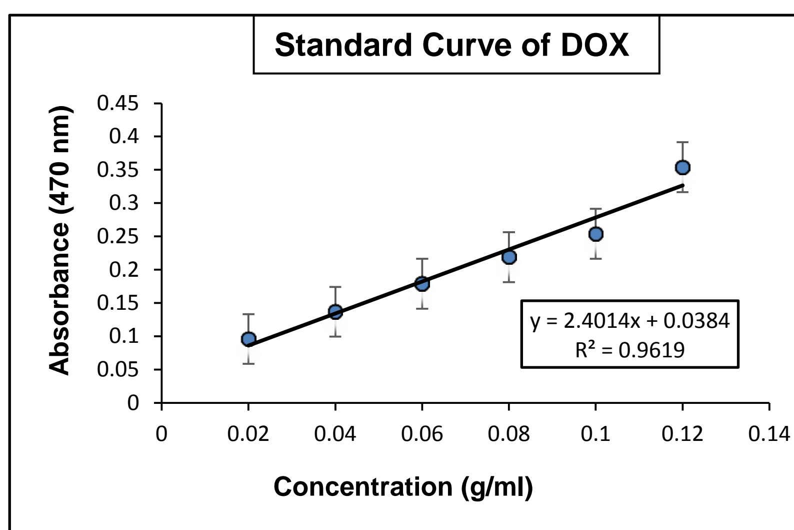


Figure 14: Standard calibration curve of DOX at 470nm for drug release assay

The drug release from loaded BGs was studied in featured circumstances such as maintaining temperature and pH. This evaluation was carried out by dialysis method. Aliquots from release medium were regularly withdrawn and analyzed spectrophotometrically against standard curve in order to calculate drug quantity in release medium at that specific time point. Results are shown as cumulative drug release percentage over 11 days' study period.

4.3.1 Drug release pattern from bacterial Ghosts at pH 6.4

Diffusion of the drug from bacterial ghosts through the dialysis membrane in release medium at pH 6.4 which corresponds to the pH of cancerous cells was completed in 10 days. This could also assure that dialysis membrane was not the only barrier to the drug release but bacterial membrane can also impose significant hindrance. Generally, the release profile of loaded BGs suspension was two phasic which slower release of drug. The release profile shows that 100% of the drug was diffused out in the medium after 240 hours.

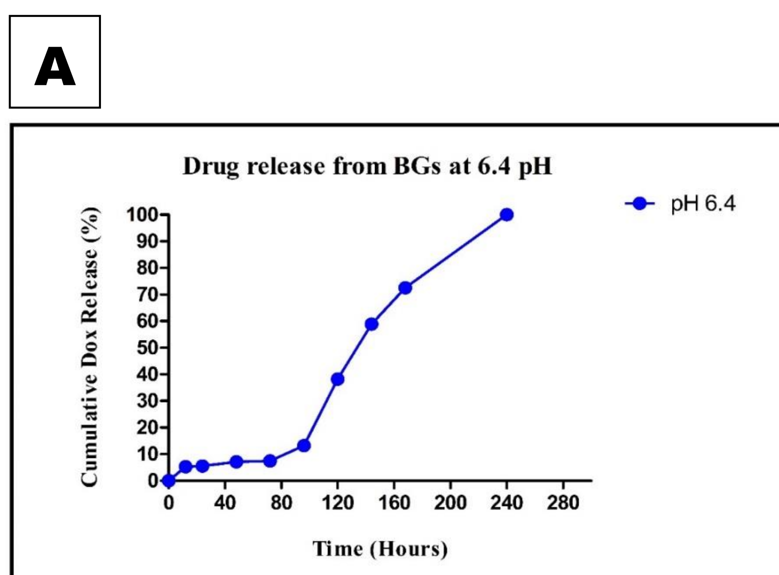


Figure 15: In Vitro Drug release from BGs at PBS (pH 6.4)

4.3.2 Drug release Bacterial Ghosts at pH 7.4

The release rate of drug was lower in the release medium at pH 7.4. This pH corresponds to the pH of human blood. Although the difference between both release profiles was not much significant yet it showed some minor differences. In case of loaded BGs in release medium at pH 7.4, Maximum drug release was recorded at 264 hours.

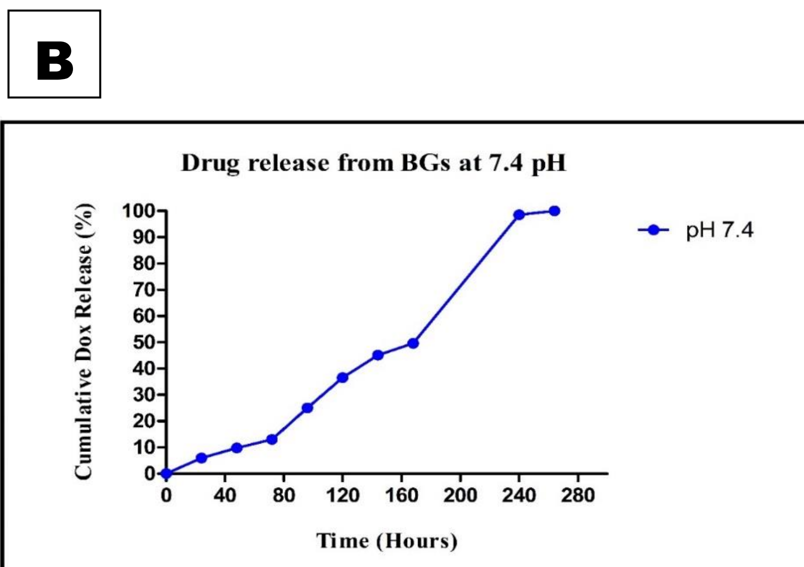


Figure 16: In Vitro Drug release from BGs at PBS (pH 7.4)

4.3.3 Comparison of both drug release profiles

However, the discrepancies between the two release profiles were not significant, they did reveal some modest differences. In case of loaded BGs in the release medium at pH 6.4, 100% drug was released in 10 days earlier than the BGs present in the release medium at pH 7.4. In case of BGs in the release medium at pH 7.4, 100% drug was released in 11 days.

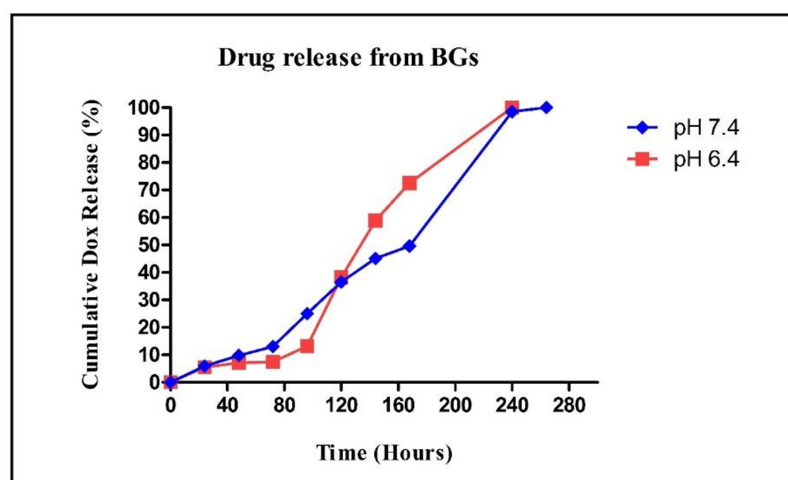


Figure 17: Comparison of drug release profiles

The release pattern of the drug shows that the doxorubicin was released very slowly from the ghosts which increases the half-life of drug and makes them desirable as DDV.

DISCUSSION

5 Discussion

Because of the beneficial properties, bacterial ghosts take focus in white biotechnology. Several studies have produced several approaches for the production of bacterial ghosts with improving ability. Gram negative bacteria are the only ones that can control the activation of the E-lysis gene to prepare bacterial ghosts. *(Kudela et al., 2010)*. To achieve 100% non-reproductive lysed cells, several stages should be followed *(Kwon et al., 2006).* The cost and complexity of genetic procedures for the creation of bacterial ghosts are limiting considerations. On the other hand, chemical compounds can be utilized in critical concentrations for long periods of time to produce the gram negative bacterial ghosts *(Nagarajan et al., 2015,Wu et al., 2016)*, gram negative bacterial ghosts *(A. A. Amara et al., 2013,Vinod et al., 2014,Park et al., 2016)* , yeasts' ghosts *(Amara, 2015)* and even viral ghosts *(Abd El-Baky and Amara, 2014).*

The same principles that are used to make gram-negative bacterial ghosts may be employed to make gram-positive bacterial ghosts *(Nagarajan et al., 2015)*. Bacterial ghosts of good quality are being produced by utilizing the suitable chemicals, optimized concentrations, temperature and shaking speed *(Amro et al., 2014).* Chemical compounds were employed according to their ability to affect cell outer surfaces. The main functional component in these compounds is the permeabilization effects on the surface membranes or cell walls *(Vaara, 1992).* In prior methods of chemically induced ghosts, bacterial cells in stationary phase were subjected to one or more chemical agents, *(Park et al., 2016)* with or without physical stimuli, over a brief period of time *(A. A. Amara et al., 2013,Amro et al., 2014).* Sodium hydroxide

was shown to be very successful in preparation of high-quality bacterial ghosts of gram-negative bacteria such as *Salmonella enteritidis* *(Vinod et al., 2014)* and *Vibrio parahaemolyticus* *(Park et al., 2016).* Additionally, It was also used to prepare gram-positive bacteria such as *Staphylococcus aureus* *(Nagarajan et al., 2015)*. Sodium hydroxide was mixed with other substances in particular amounts and added for brief periods of time to generate the high quality ghosts in a chemically induced BG production technique known as the sponge-like protocol *(A. A. Amara et al., 2013)*. The Surface membranes of gram-negative bacteria is damaged by alkalies *(Hinton JR. & Ingram, 2006)*.

SDS is an amphoteric chemical with a hydrophilic head and a hydrophobic tail that changes the permeability and hydrophobicity of the surface membrane, making bacteria more susceptible to disinfectants and antimicrobial *(Walton et al., 2008)*. Hydrogen peroxide produces hydroxyl free radicals, which oxidise critical exterior biological components. As Gram-positive bacteria lacking the catalase enzyme, hydrogen peroxide has a limited effect on their cellular membranes. Gram-negative bacteria, on the other hand, are more susceptible to hydrogen peroxide. *(Gerald & Denver, 1999).*

Tween-80 is the main player of the procedure used to prepare bacterial ghosts in our research. By using different amounts of tween-80, ideal three dimensional bacterial ghosts are being produced without limiting growth or eliminating the entire population. In this protocol, bacterial cells are being exposed to the tween-80 from lag growth phase to stationary phase for long period of time. Tween-80 is nonionic surfactant that is often used inn food and pharmaceutical industries. Tween-80 is being used for the solubilization of hydrophobic compounds. In the case of *Pseudomonas aeruginosa*, the longer the cells are exposed to tween 80, the more

outer membrane components leak *(Brown & Winsley, 1969).* Tween-80 in fewer concentrations was clearly efficient in potentiating bactericidal action of a variety of antibiotics against *Pseudomonas aeruginosa.* Conversely, the resistance of *Pseudomonas aeruginosa* to these antibiotics without tween-80 shows that tween-80 disrupts the permeability of surface membrane through damaging porin proteins, efflux pumps, and eventually uptake mechanisms *(A. Al- Obaydi et al., 2010).* Organic acids are permeabilizers as well. By penetrating and acidifying the cytoplasmic content, benzoic and lactic acids can disrupt surface membrane permeability, resulted in the inactivation of acid-sensitive enzymes. *(H.-L. et al., 2000).* LPS release and NPN absorption might suggest disruption of the surface membrane of *Salmonella typhimurium*, *Escherichia coli*, and *Pseudomonas aeruginosa*. In comparison to other acids, lactic acid was the most powerful organic acid capable of liberating LPS. The release of a sufficient amount of DNA and proteins is good sign of successful cellular discharge. In this procedure, the maximum released DNA and proteins were 1598 ng/ml and 7.10 µg/ml respectively.

The released proteins in another approach for producing E. coli BGs were 3425 µg/ml, whereas the DNA released levels were 179 µg/ml *(A. A. Amara et al., 2013).* The calculated amount of released DNA was clearly compatible with the gel electrophoresis data. The intensity of DNA bands rose proportionately as the amount of DNA increased. The suggested procedure of bacterial ghosts production is because of bacterial cells are being exposed to the tween-80 for a long period of time which dissolve the hydrophobic regions of outer membrane of bacteria by creating weak regions. These regions help in the creation of punctures produced by abrupt lactic acid exposure.

Moreover, the whole antigenic characteristics of produced ghosts need to be investigated by examining their potential to induce a useful immune response. The effectiveness of this procedure for the production of gram positive BGs should be investigated. The produced ghosts were found to be complete bacterial cells with many membrane tunnels. The pH was the most significant component in increasing the entrapment efficiency of bacterial ghosts. The maximum loaded DOX (43 µg/mg) was attained when pH 9 was employed with following components (Temp = 27 °C, Time = 10 minutes and DOX = 1 mg). When compared to the other parameters (Temp, Time and Drug amount), pH 9 obtained a substantial difference in efficiency of loading with other influencing parameters. Furthermore, there were also no major differences between the other parameters (temperature, time, and DOX amount). The maximum loaded capacity or maximum entrapment efficiency are calculated for DOX and this amount is the total of encapsulated DOX either in the lumen of ghosts or attached to the inner membrane of bacterial ghosts. Apart from BGs, several DOX delivery methods have varying loading capabilities. The minimum loaded quantity was 0.43% through chitosan-polysaccharide polymer-nanoparticles *(Janes et al., 2001)*, and the maximum quantity was 39% through the synthetic conjugated glycol-chitosan *(Nasongkla et al., 2004)*. Furthermore, the entrapment efficacy of BGs for Doxorubicin was around 10%. This was previously disclosed in a research that used a different BG technology (*M. haemolytica* ghosts) *(Paukner et al., 2004)*. With the *E. coli* DH5α BG, 43% entrapment efficiency was attained in this investigation.

According to more rational theories, this might be attributed to two primary factors. Firstly, the loading factors utilised in this investigation. Second, the kind of BGs utilised as a platform or the process for producing BGs were considered. DOX chemical characteristics as loading compound are critical for a better understanding.

DOX is a glycoside antibiotic made up of adriamycinone, a tetracyclic quinoid aglycone coupled to the sugar-amine moiety daunosamine *(Arcamone et al., 1972).* The charged internal and outer membranes of bacterial ghosts may be used to bind the amino-sugar moiety of DOX. The DOX component has a single positive charge *(Novotna et al., 2008).* This permits the DOX molecules to bind with fatty acids of negative charge around LPS that are entrenched in the membranes of *E. coli* DH5α. Moreover, the published strength of plasma protein binding of Doxorubicin is around 74% *(Sieczkowski et al., 2010)* which might potentially imply its strong attachment to proteins of surface membrane like porins and OmpA *(Hiroshi, 2003).* Moreover, it is possible that other DOX features, such as DOX hydrophilicity, water solubility (2600 mg/L) *(Wong et al., 2009)* and preferential LPS permeability, contribute to the ease of loading and binding of DOX to *E. coli* BGs utilizing the aquatic buffered solution *(Nikaido & Vaara, 1985)*. In addition, DOX solubility in water was increased at high temperature and lead the way to the LPS permeability. The increment in concentration gradient is also a contributing parameter that can improve the permeability of DOX through the membranes with tunnels. Weak polyprotonic and acidic nature of DOX (pKa 1 = 7.34 and pKa 2 = 9.46) *(Wong et al., 2009)* helps it retain the positive charge in the basic pH (8 and 9). As a result, the strong positively charged components exist which are more likely to attach to membranes. Doxorubicin have 12 hydrogen ion acceptors and 6 hydrogen ion donors, Additionally single positive charge in physiological buffers, total binding of DOX with BGs is thought to have happened via hydrogen and ionic bonding *(ChemAxon, 2019).*

The maximum amount of released DOX from loaded BGs in release medium at pH 6.4 was 100% at 240 h, while it was 100% at 264 h in release medium at pH 7.4. According to the literature, DOX releases faster at pH 6.4 which corresponds to the

pH of cancerous cells and DOX is more likely to dissolve in slightly acidic medium. Also, DOX releases quicker from free solution than BGs. This might be justified by the fact that free DOX molecules dissolve immediately. Bacterial ghosts, on the other hand, dispersed in two phases (firstly from ghost membrane and then from dialysis membrane bag). Only two *in-vitro* release investigations of DOX through the bacterial ghosts' delivery platform was published *(Paukner et al., 2004)*. The higher but slow DOX release in this study could be due to the different BG employed. This release pattern makes these BGs desirable for the delivery of chemotherapeutic drugs to tumors.

CONCLUSION

6 Conclusion

The study was conducted to prepare and evaluate the bacterial ghosts which are used as a non-viral delivery and targeting vehicle for vaccines and drugs, respectively. The findings of the present research revealed that proposed modified approach successfully prepared the bacterial ghosts. After the successful preparation of BGs, the Loading of anti-cancerous drug Doxorubicin into the bacterial ghosts was done successfully. *In vitro* release profile of Doxorubicin loaded BGs was performed. Additionally, the doxorubicin was released very slowly from the ghosts over a long period of time. As a result, BGs could be considered as a potent drug delivery and targeting vehicle for the reliable distribution of anticancerous drugs for cancer treatment. Subsequently, Doxorubicin dosage could be significantly reduced when BGs deliver the DOX as compare to the free Doxorubicin. These outcomes will help the patient in a several ways, including lowering the dosage, reducing the administration frequency and cytotoxicity. In conclusion, although further experiments are needed to check the cytotoxicity of BGs in vivo, all results of current study point out bacterial ghosts as potentially useful candidates for drug delivery of chemotherapeutic drugs for the treatment of cancer.

FUTURE PROSPECTS

7 Future Prospects

For further studies of cytotoxicity of *E. coli* DH5α, Cytotoxicity assays and antiproliferative analysis can be performed on cancerous cells. Adherence studies can be done to check the targeting efficacy of the Dox loaded BGs. For further confirmation of endotoxicity of bacterial ghosts*, LAL ASSAY* of bacterial ghosts can also be performed. Additionally, more research regarding DOX cellular trafficking using bacterial ghosts is needed to fully understand the benefits of BGs as delivery mechanism. Furthermore, In Future, Animal models with cancer will be used to test this new delivery platform for the treatment of cancer.

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6. References

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