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Chemical Mediated Bacterial Ghost Generation of *Pasteurella multocida*

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Abstract

Pasteurella multocida is a prominent animal pathogen that causes serious complications for a variety of animal species. To overcome diseases caused in recent years, the demand for safe vaccinations, biological carriers, and efficient drug delivery methods has skyrocketed. One of the platforms that can address this demand is Bacterial Ghosts (BGs). Bacterial ghosts are empty cellular envelopes that retain surface and antigenic characteristics but lack the interior components. In this study, different chemicals like Sodium Dodecyl Sulphate (SDS), Sodium Hydroxide (NaOH), Sodium Carbonate (Na_2CO_3), Hydrogen Peroxide (H_2O_2), and ethanol were used to standardise a protocol for the production of ghost particles of *Pasteurella multocida.* The optimised concentrations of SDS, NaOH, Na_2CO_3 and H_2O_2 for the production of ghosts were 0.234, 0.046, 0.58 mg/mL and 31.25 µl/mL, respectively. The bacterial cells were completely inactivated with 60% ethanol. Among all tested combinations, the treatment with SDS in conjunction with H_2O_2 and ethanol exhibited the most pronounced effect in facilitating the release of intracellular content. These findings demonstrate that SDS in combination with H_2O_2 and ethanol can be effectively employed to generate structurally intact yet non-viable BGs of *P. multocida*, making them suitable candidates for future use in vaccine development and as antigen-delivery platforms.

Major Findings: The optimised chemical combination of SDS, H_2O_2, and ethanol effectively produced structurally intact *Pasteurella multocida* bacterial ghosts by promoting efficient nucleic acid release.

Keywords: Bacterial Ghosts, Chemical Method, *Pasteurella multocida*

1. Introduction

Pasteurella species are among the most common symbiotic and spoilage microbes found in both domestic and wild animals worldwide[1]. They are identified routinely in the oral, nasal, and respiratory cavities of animals[2–4]. *Pasteurella* species, particularly *P. multocida*, are frequently associated with the severity and acute infections both in animals and humans. These infections can cause significant morbidity, including pasteurellosis, pneumonia, atrophic rhinitis, dermonecrosis, cellulitis, abscesses, meningitis, and Haemorrhagic Septicaemia (HS). They are also a leading cause of mortality[5]. The dearth of effective and safe vaccinations makes it difficult to prevent pasteurellosis by vaccination. The use of *Pasteurella* strains with reduced virulence as live vaccines protects numerous serotypes. However, chemical and physical stress generated by typical inactivation techniques may damage the protective epitopes in inactivated vaccine formulations[6–8]. Live vaccines appear to be advantageous as they offer all antigen epitopes of a cell in their natural conformation. This includes antigens

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that are specific for *in vivo* growth expression. However, current live vaccines are not rationally attenuated. As a result, they have the potential to revert to virulence and cause illness[9]. Considering these factors, a non-living vaccine that contains fewer adjuvants and combines the safety of an inactivated vaccine with the broad spectrum of antigen presentation of a live vaccination is needed[10]. In this context, novel strategies are repeatedly being explored in order to develop potent vaccines that are not only safe, but also mean minimal immunisations and are remarkably efficient. BG are a possible platform that may be used to develop both effective candidate vaccines and efficient adjuvant and vaccine delivery systems.

The whole cell surface features, including outer membrane proteins, adhesins, Lipopolysaccharides (LPS), and the peptidoglycan layer, are retained in BG[11]. Furthermore, foreign antigens were loaded into the cytoplasmic lumen or expressed on the surface and in the periplasmic region of BG[12]. These unique characteristics make BG an appealing technique for vaccine development and antigen delivery in humans and animals. Due to its low toxicity as compared to free LPS, the presence of LPS in the BG does not harm its application as an adjuvant or candidate vaccine[13]. Because of their particle form and the presence of numerous well-known Toll-Like Receptor (TLR) agonists, BG are identified by antigen-presenting cells and induce powerful immune responses[14]. This boosts the production of cytokine genes, which activate adaptive immune cells via signal transduction, boosting the adaptive immune response[10].

Gram-negative bacteria are the only organisms that can activate the E-lysis gene, which is employed to make bacterial ghosts[15]. However, employing genetic approaches to manufacture bacterial ghosts is limited by the high cost and intricacy. Chemical agents may also be employed to make Gram-positive BGs[16,17], Gram-negative BGs[18–21], yeasts' ghosts and viral ghosts[22]. Chemical agents are chosen for their ability to affect cell outer surfaces by their permeabilisation action on the outer membranes or cell walls[23]. However, the chemical method for generating bacterial ghosts in *P. multocida* has not been reported previously, and this study aims to explore and establish such an approach. In the present study, the chemicals such as SDS, NaOH, Na_2CO_3, H_2O_2, and ethanol were used. They were studied for their inhibitory concentration and ability to affect the outer surface of *P. multocida*. Based on their inhibitory concentrations, the protocol was optimised and compared among the used chemicals for their efficacy in the generation of ghost particles of *P. multocida*.

2. Materials and Methods

2.1 Growth and Confirmation

P. multocida serogroup B:2 (strain P52) was obtained from the Division of Biological Standardisation, ICAR-Indian Veterinary Research Institute (IVRI), Izatnagar, Uttar Pradesh, India. The culture was then revived using Brain Heart Infusion (BHI) broth. From the BHI broth, a loopful of culture was streaked on a BHI agar plate and incubated at 37°C for 24 hrs. A single colony was picked from a BHI agar plate, inoculated in BHI broth, and spread on a glass slide for Gram's staining. The bacterial morphology was checked under a compound microscope.

The screened pure isolates were subjected to molecular identification by amplification of the 16S rRNA gene. Randomly, six colonies were selected and grown in 5.0 mL of BHI broth. The genomic DNA was isolated from these six colonies by the phenol: chloroform method. The isolated DNA was amplified with specific kmt1 gene primers (forward primer KMT1T7 5'-ATCCGCTATTTACCCAGTGG 3' and reverse primer KMT1SP6 5'GCTGTAAACGAACTCGCCAC 3')[24]. The polymerase chain reaction was performed using the following conditions: initial denaturation at 95°C for 5 minutes, denaturation at 95°C for 30 sec., primer annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds, for 35 cycles and final extension at 72°C for 5 minutes. The amplified product was checked on 2% agarose gel. After confirmation, further study was initiated.

2.2 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Concentration for Growth (MCG)

P. multocida culture grown to an optical density (OD_{600}nm) of 0.602 was used for MIC determination. The MIC was determined by the two-fold dilution method as described previously [16]. The initial concentration used for sodium-dodecyl sulphate (SDS), sodium hydroxide (NaOH), sodium carbonate (Na_2CO_3) and hydrogen peroxide (H_2O_2) was 10, 4, 100 mg/mL and 500 µL/mL, respectively. The two-fold dilutions of each chemical were

streaked on a BHI agar plate and incubated at 37°C for 24 hrs. The growth was observed after 24 hrs to check the MIC.

2.3 Generation of *Pasteurella multocida* Ghost (PMG)

Bacterial ghosts were prepared with a protocol that has been suggested earlier[18] with slight modifications in the speed of centrifugation and the concentrations of desired chemicals to achieve optimal emptying specific to *P. multocida*. In brief, overnight-grown pure *P. multocida* culture at $1.2x10^9$ cells was pelleted at 5000g for 20 minutes. To the pellet, 0.234 mg/mL of SDS was added and incubated for 60 minutes. After the incubation, the cells were centrifuged, and the supernatant was collected separately to determine the release of nucleic acid. The pellet was washed twice with normal saline, followed by the addition of 31.25 µL/mL of H_2O_2. After 30 minutes of incubation, the cells were washed again with normal saline and incubated with 60% ethanol for 30 minutes. Upon final washing, the cell pellet was suspended in normal saline. A similar protocol was followed for the production of bacterial ghosts using 0.58 mg/mL Na_2CO_3 and 0.046 mg/mL of NaOH in lieu of 0.234 mg/mL of SDS in the initial step. The chemically produced bacterial ghosts were stored at 4°C for further use and characterisation. The overall workflow has been depicted as an illustration in Figure 1.

2.4 DNA Extraction and Quantification

DNA was extracted from supernatant, Polymicrogyria (PMG) cells, and control, untreated *P. multocida* cells using the phenol: chloroform method[25]. The spectrophotometer measurement was used to determine the DNA concentrations, and its quality was checked on a 0.8% agarose gel.

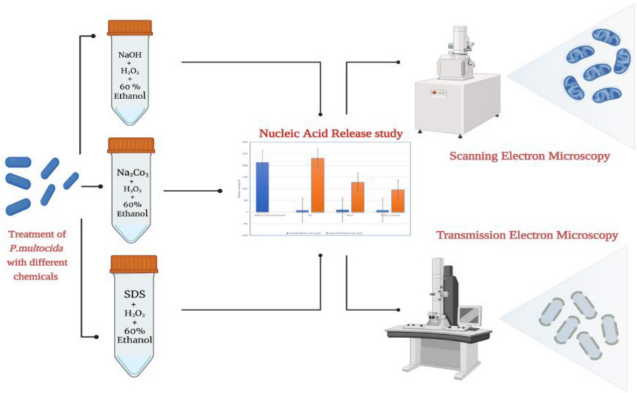


Figure 1. Illustration depicting the flow of work for the generation of bacterial ghosts.

2.5 Cell Viability and Cell Integrity Assay

A loopful of culture from a chemically produced bacterial ghost pellet and an untreated cell pellet was streaked on a BHI agar plate to check the cell viability, if any. Gram's staining was performed to check the integrity of the cell. Based on the bacterial structure, the quality of the cells has been classified as either acceptable or distorted[18].

2.6 Microscopy

The bacterial ghost samples were processed for Scanning Electron Microscopy (SEM). The sample was mounted on a stub holder with carbon tape and air dried for inspection under vacuum in the microscope model Gemini - SEM – 300 at M/s Carl Zeiss India (Bengaluru) Pvt. Ltd.

For transmission electron microscopy (Hitachi, HT 7700), the samples were placed on para film, and a copper grid was placed on them for 10 minutes. The grid was transferred using forceps onto an absorbent paper and then placed on uranyl acetate. The samples were stained with uranyl acetate for 5 minutes. Then the grids were collected, washed with distilled water and allowed to dry on filter paper before imaging.

3. Results

3.1 Molecular Identification of *P. multocida* Using the *kmt1* Gene

A specific Polymerase Chain Reaction (PCR) that targeted an established *kmt1* gene fragment was carried out in order to detect *P. multocida*. The observed band size was 460bp, confirming that the colonies belonged to *P. multocida.* The pure culture of *P. multocida was* successfully isolated and confirmed by molecular characterisation as indicated in Figure 2.

3.2 Determination of Minimum Inhibitory Concentrations

To produce chemically induced bacterial ghosts, the Minimum Inhibitory Concentration (MIC) of SDS, H_2O_2, NaOH and Na_2CO_3 for *P. multocida* was determined.

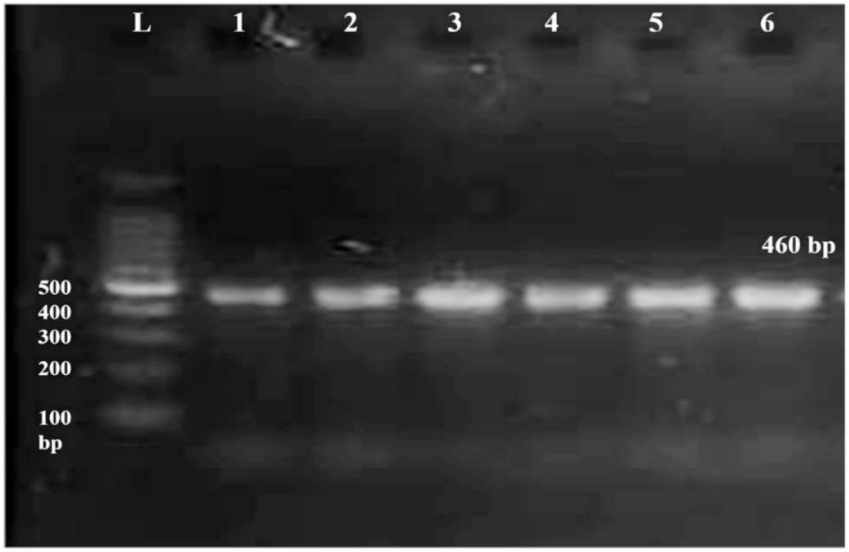


Figure 2. The PCR amplification of *P. multocida* on agarose gel (2%). L, 100bp DNA ladder (Thermoscientific, Germany), L1-L6, expected PCR product of *P. multocida*.

Table 1. MIC values of specific chemical compounds against *P. multocida*

Chemical Used	Dilutions									
	1	2	3	4	5	6	7	8	9	10
SDS Concentration (mg/mL)	10	5	2.5	1.25	0.625	0.31*	0.15	0.07	0.03	0.01
Growth Observation	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(+)	(+)	(+)
H_2O_2 Concentration (µl/mL)	500	250	125	62.5	31.25*	15.62	7.81	3.90	1.95	0.97
Growth Observation	(-)	(-)	(-)	(-)	(-)	(+)	(+)	(+)	(+)	(+)
NaOH Concentration (mg/mL)	4	2	1	0.5	0.25	0.12	0.06*	0.03	0.01	0.007
Growth Observation	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(+)	(+)
Na_2CO_3 Concentration (mg/mL)	100	50	25	12.5	6.25	3.12	1.56	0.78*	0.39	0.19
Growth Observation	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(+)

Foot Note: +, Presence of growth; -, Absence of growth; *, MIC

The BHI agar plates were streaked with the cells that were treated with different concentrations of SDS, H_2O_2, NaOH and Na_2CO_3. No colonies formed on BHI agar plates by the cells that were treated with concentrations more than 0.156 mg/mL SDS, 15.62µl/mL H_2O_2, 0.0312 mg/mL NaOH and 0.39 mg/mL Na_2CO_3. Therefore, the minimum inhibitory concentration of SDS, H_2O_2, NaOH and Na_2CO_3 was 0.31 mg/mL, 31.25 µL/mL, 0.06 mg/mL and 0.78 mg/mL, respectively, as indicated in Table 1.

3.3 Generation of *Pasteurella multocida* Ghost (PMG)

The final concentration for each chemical-mediated ghost preparation was optimised after obtaining the MIC. A concentration of 0.234 mg/mL, 0.046 mg/mL and 0.58 mg/mL of SDS, NaOH and Na_2CO_3, respectively, was used for bacterial ghost generation that was less than the MIC for each respective chemical treatment. After treatment, all the treated bacterial cells were streaked on BHI agar plates with control (untreated *P. multocida*) and no growth was observed in the plates streaked with ghosts, confirming its successful inactivation (Figure 3)

The integrity of bacterial ghost cells or shape was checked under a light microscope by Gram's staining. The Gram-negative coccobacilli cells were observed with intact shape or integrity. There was no complete cell lysis or debris observed. Therefore, the predetermined concentration of each chemical used for BG did not lyse

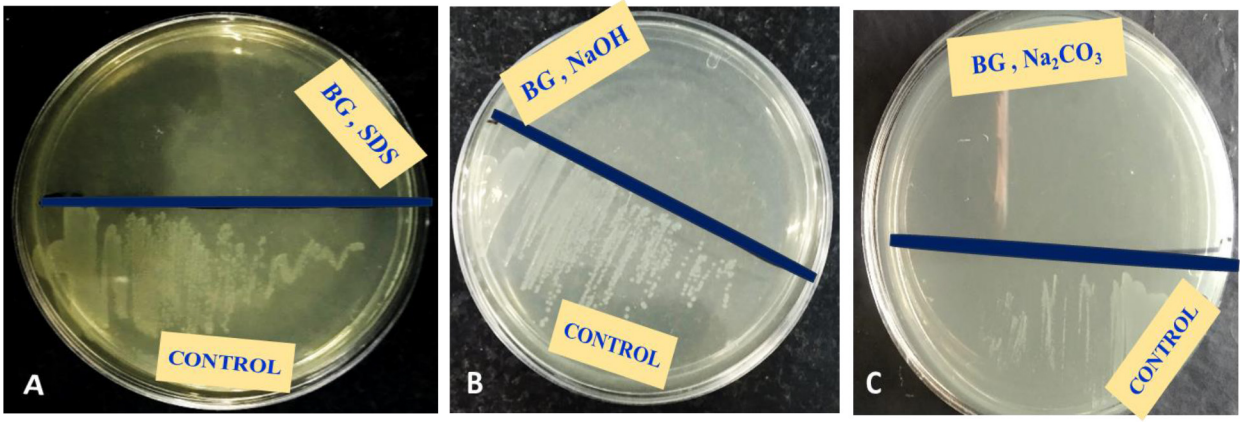


Figure 3. No growth observed in the streaking area of the bacterial ghost, while the control (untreated *P. multocida*) area exhibited growth.
A. Cell viability of PMG after SDS treatment and without treatment
B. Cell viability of PMG after NaOH treatment and without treatment
C. Cell viability of PMG after sodium carbonate treatment and without treatment

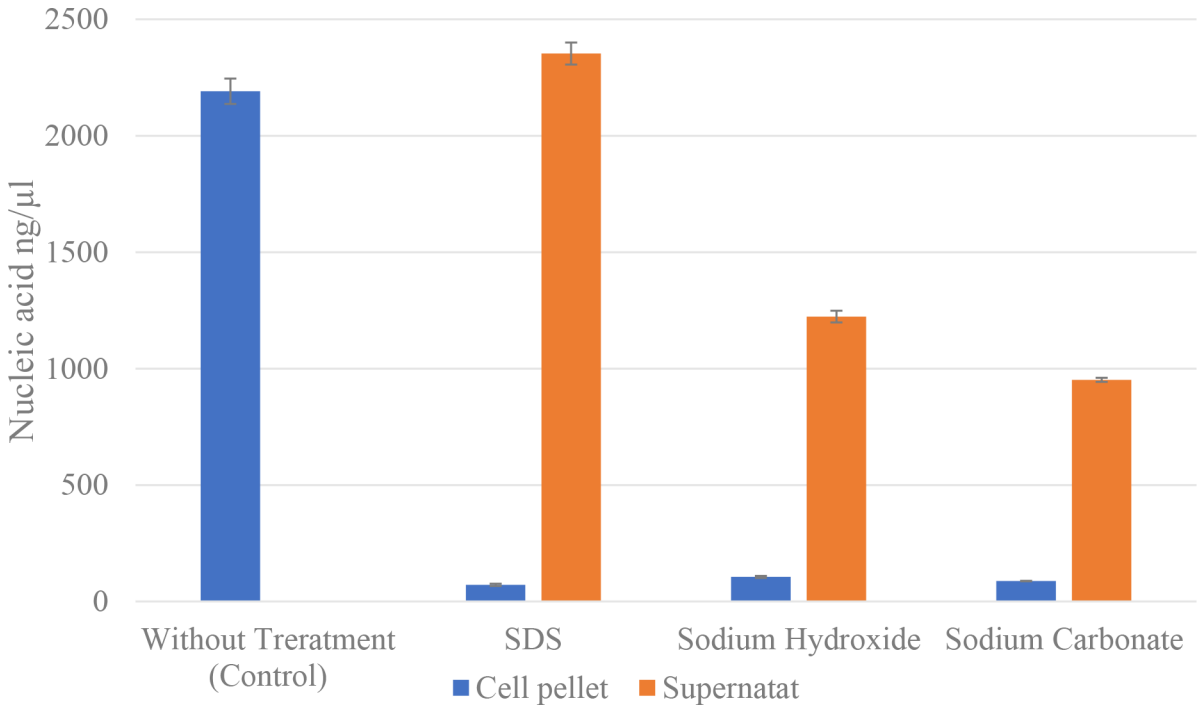


Figure 4. Total Nucleic acid content in control (without treatment), SDS, NaOH, sodium carbonate-treated bacterial ghost cell pellet and supernatant.

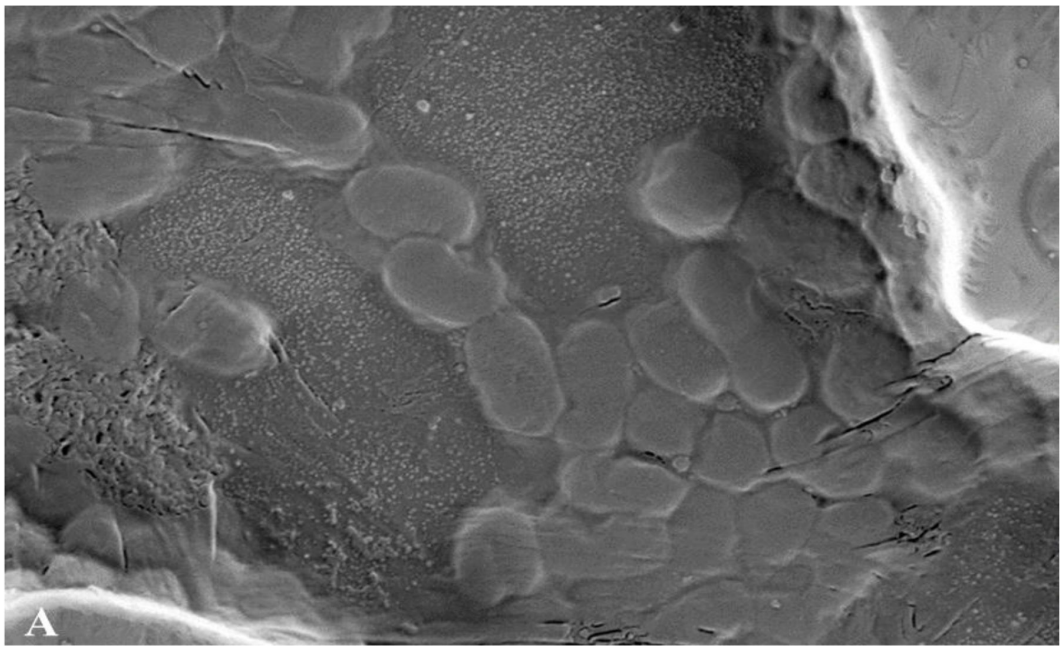
the cells completely and was found to be optimum to generate the ghosts.

Total genomic DNA extracted from bacterial ghost cells, control (untreated *P. multocida*) cells and supernatant after chemical treatment was used to check the release of nucleic acid content in the supernatant and ghost particles. The nucleic acid (DNA and RNA) quantity and quality were estimated spectrophotometrically at OD_{260nm}. It was observed that the maximum amount of nucleic acid content was released in the supernatant after chemical treatment, and a very minute quantity of nucleic acid was present in the treated cells as compared to that of the control. BG generated from chemical treatment of the SDS has released most of the nucleic acid into the

supernatant as compared to other chemical treatments (Figure 4).

3.4 Microscopy

Bacterial ghosts formed by the treatment with SDS were processed for microscopy. In the SEM, the cells showed perforations on the surface morphology from which the nucleic acid was expected to release into the cell surrounding (Figure 5). The Transmission Electron Microscope (TEM) revealed empty shells of bacteria without any genetic material internally, suggesting successful formation of a ghost (Figure 6).



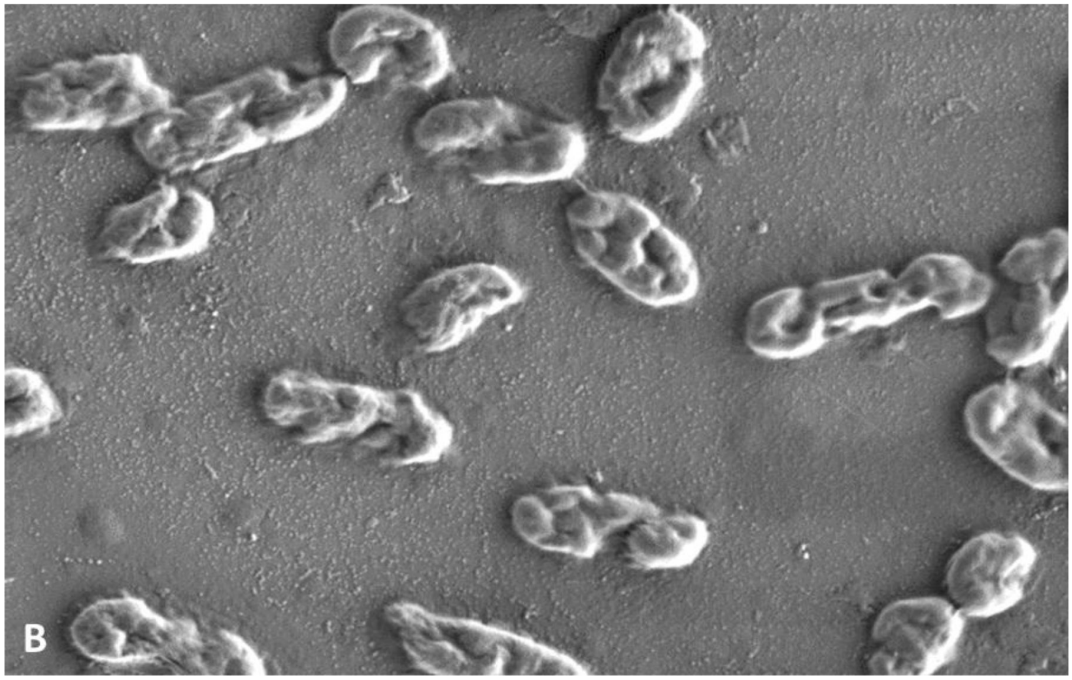


Figure 5. SEM images- **A:** Untreated *Pasturella multocida* bacterial cells, **B:** SDS-treated *Pasturella multocida* ghosts with perforations on the surface.

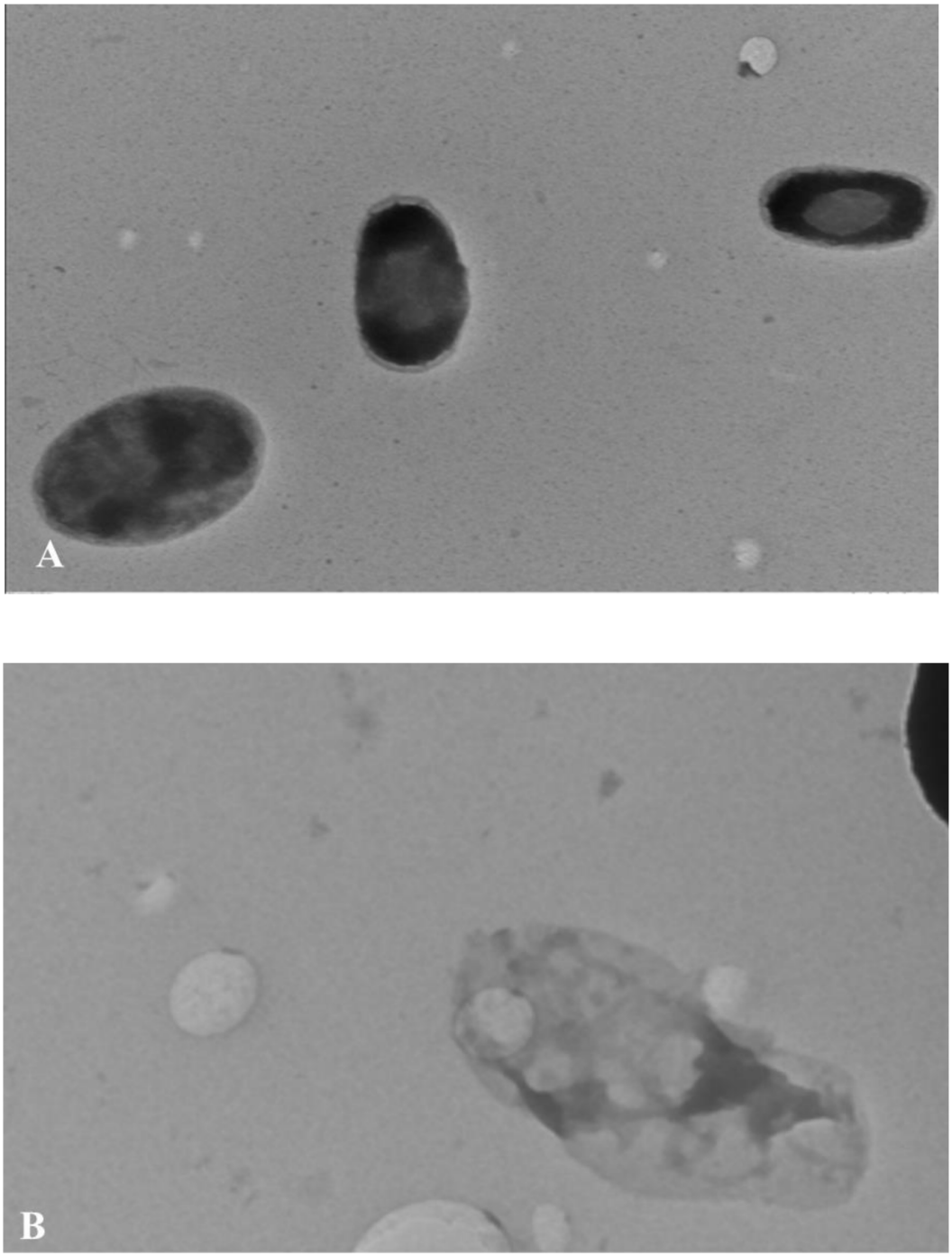


Figure 6. TEM images – **A:** Untreated *Pasturella multocida* bacterial cells with dense nucleic acid contents internally, **B**: SDS-treated *Pasturella multocida* ghosts without any genetic material internally but with the outer shell.

4. Discussion

BG were produced earlier under control using the bacteriophage *phi X174* lysis protein E, which is present in Gram-negative bacteria. However, the application of genetic approaches to prepare bacterial ghosts is constrained by their high cost and complexity. Lately, BG has been produced successfully by treating the bacteria with common chemicals at their optimal concentrations for a particular period. What makes these agents effective in common is their ability to permeabilise either the cell walls or the outer membrane[23].

A variety of chemical agents are in use to treat the bacteria to generate ghosts such as Tween 80, sulphuric acid, sodium dodecyl sulfate, sodium hydroxide, hydrogen peroxide and hydrochloric acid[20,26–30]. Gram-negative bacteria experience adverse effects from alkalis on their outer membrane[31]. Gram-negative bacterial ghosts have been successfully produced in earlier studies, which include *Salmonella typhimurium*[29], *Escherichia coli*[18], *Vibrio parahaemolyticus*[20] and *Klebsiella pneumoniae*[19]. The chemical method of bacterial ghost generation has been proven to be successful even in Gram-positive bacteria such as *Listeria monocytogenes*[17]. Determining the right concentrations of the chemical substances proves to be an essential challenge for the production of BG, as higher concentrations would prove toxic to the cells. Whereas lower concentrations would lead to inefficient ghost formation and inactivation of bacterial cells. In addition to forming pores, the chemical approach is quick, easy, and leaves cells with their three-dimensional shape unchanged. It may be due to the small pore size that allowed only a small size of RNA rather than DNA content, but in the cell pellet, the DNA content was also reduced, and the observed quantity was very less as compared to the control.

Pasteurella multocida ghosts have been produced successfully earlier by the expression of phage *PhiX174* lysis gene *E* and found to be immunogenically potent[32]. However, no reports on the production of ghosts of *P. multocida* were found by employing chemical treatment. Therefore, this study explores the possibility of ghost production using different chemicals. After determining the MIC, the bacteria were subjected to different chemicals at different concentrations to analyse the potential of respective chemicals to form pores without any detrimental effects. These substances proved to have no negative effects on the integrity of the cells, as the bacterial cells remained intact and were not fully lysed. A valid sign of efficient cellular evacuation is the discharge of adequate quantities of proteins and DNA. The bacterial cells must initially be subjected to the chemical compounds, followed by multiple centrifugations and washings to remove the residual chemicals and to eliminate DNA and protein content. The delicate centrifugation allows the cells to be finely pressed, which helps them to eliminate the components of their cytoplasm[19].

In the present study, the chemical agents such as SDS, NaOH and Na_2CO_3 were used in appropriate concentrations after determination of their respective MICs. These chemicals did not show any detrimental effect on cell integrity at the concentrations tested. The bacterial cells were intact and not lysed completely. The optimised concentration of the chemicals for the preparation of ghosts was slightly below their respective MIC concentrations; however, the combination of chemicals used and the final ethanol treatment has rendered the ghosts inactive. In the attempts to use higher concentrations of chemicals to achieve the emptying of ghosts, the integrity of the cells has been lost, turning the bacterial cells into an undesirable, pasty consistency.

In the current investigation, compared to other chemicals utilised for ghost creation, the application of SDS in combination with H_2O_2 and ethanol resulted in the almost complete release of nucleic acid content in the supernatant. SDS and H_2O_2 were proven to be successful as a part of a chemical combination for the generation of gram-positive ghosts[17]. Due to its hydrophilic head and hydrophobic tail, the amphoteric compound SDS modifies the hydrophobicity and porosity of outer membranes[33]. Critical external components of the cell are oxidised by the hydroxyl free radicals produced by hydrogen peroxide. The presence of the catalase enzyme makes Gram-negative bacteria more susceptible to the effects of hydrogen peroxide[34]. Previous studies also reported a significant release of DNA into the supernatant[17,29], and remains of insignificant amount of nucleic acid in the bacterial ghosts is an occurrence that has been reported in prior attempts at generation by chemical methods[17]. A negligible amount of DNA detected in the ghost pellet did not hinder the inactivation of the cells. The discharge of nucleic acid content indicated that these chemicals created pores in the bacterial membrane, resulting in the effusion of genetic material into the supernatant. The morphological observation of ghosts revealed multiple pores that were formed over the surface of bacteria in SEM examination. These pores formed on the surface post-chemical treatment would aid in emptying cytoplasmic contents, including the genetic material. The empty shells of bacteria were pictured in the TEM examination

P. multocida ghosts produced by the expression of phage *PhiX174* lysis gene *E* have been tested for immune potentiation in cattle[32], mice and rabbits[35] and proved to be effective. Hence, further studies need to be taken up for testing the immune potentiation capability of chemically produced ghosts for assessing their immune potentiating effect along with certain bacterial or viral vaccines.

5. Conclusion

In the current study, bacterial ghosts of *P. multocida* were successfully generated through a chemical method to preserve cell structure while releasing the cytoplasmic contents. The study explored different chemical treatments, with SDS demonstrating the highest efficiency in terms of the release of intracellular components compared to other chemical treatments. SDS can be considered a viable chemical agent for the production of bacterial ghosts from *P. multocida*. However, further research into their immunogenic properties is essential to fully understand their potential as vaccine candidates or for other applications in biotechnology.

6. Acknowledgements

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8. Author's Contribution

Investigation, K.S.L and M.G.S; Writing - Original draft preparation, K.S.L; Review and editing, V.K.N and V.B; Supervision, M.G.S, V.K.N and V.B.

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