Preparation of Phage Enzyme Infused Nanolipid Carrier Against Respiratory Infections

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**Abstract:** The creation of phage enzyme-infused nanolipid carriers (NLCs) as a novel treatment approach against respiratory infections—Klebsiella pneumoniae in particular—is presented in this paper. Using high-pressure homogenization procedures, the phage enzymes, which are recognized for their particular bactericidal activity, were successfully encapsulated within NLCs, guaranteeing stability and bioactivity. Comprehensive FTIR evaluation verified the integrity of the functional groups, and XRD examination showed that the NLCs were semi-crystalline, indicating structural robustness. An in vitro antibacterial experiment was used to assess the formed NLCs' antibacterial activity. The results showed a distinct dose-dependent reduction of bacterial growth, with notable zones of inhibition seen at different doses. The outcomes highlight how this cutting-edge delivery method can improve the targeted delivery and effectiveness of phage enzymes, providing a viable substitute for traditional antibiotic therapies. This novel strategy offers a regulated release mechanism in addition to increasing the stability and bioavailability of phage enzymes, making it an appealing option for further research and clinical use in the management of respiratory infections.

**Keywords**: Phage enzyme, nanolipid carrier, respiratory infections, Klebsiella pneumoniae, antimicrobial therapy, enzyme encapsulation, targeted delivery, FTIR, XRD, antibacterial assay.

# INTRODUCTION

Bacteriophages, also known as phages, are viruses that enter bacterial cells and, in the case of lytic phages, cause bacterial lysis by interfering with bacterial metabolism. Long-running discussions about the history of bacteriophage discovery have included a dispute over priority claims.[(Dokuz et al., 2024)](https://paperpile.com/c/nGJIPX/VxER) [(Harsha & Subramanian, 2022)](https://paperpile.com/c/nGJIPX/wsExT)[(Deepika et al., 2022)](https://paperpile.com/c/nGJIPX/27r2a)[(Solanki et al., 2022)](https://paperpile.com/c/nGJIPX/opgSU). The second-generation, smarter drug carrier technology, known as nanostructured lipid carrier (NLC), has a solid matrix that may be used at ambient temperature. Regulatory bodies have approved the use of this carrier system, surfactants and physiological, biodegradable, and biocompatible lipid molecules, in several drug delivery systems. The rapid proliferation of items on the market attests to the efficacy of this delivery technique.[(Yılmaz Usta et al., 2024)](https://paperpile.com/c/nGJIPX/8m3n) Approximately thirty different NLC preparations have been made commercially accessible since the first product's launch. Since NLC outperforms other colloidal carriers—such as liposomes, polymeric nanoparticles, nanoemulsions, SLN, etc.—it has been thoroughly investigated in the field of pharmaceutical technology. The entire collection of special benefits, which includes increased drug loading capacity and prevention of drug expulsion, results in more[(Iqbal et al., 2012)](https://paperpile.com/c/nGJIPX/6s5v)[(Chidambaram et al., 2022)](https://paperpile.com/c/nGJIPX/2a6VA).[(Ajay, Sasikala, et al., 2022)](https://paperpile.com/c/nGJIPX/5tD0U). Worldwide, the leading three causes of death are respiratory disorders (chronic obstructive pulmonary disease and lower respiratory infections), cardiovascular disorders (ischemic heart disease and stroke), and neonatal disorders (birth asphyxia, birth trauma, neonatal sepsis and infections, and preterm birth complications).[(Pramanik et al., 2021)](https://paperpile.com/c/nGJIPX/3VOk) [(Neha et al., 2021)](https://paperpile.com/c/nGJIPX/GkqaO)[(Maliael et al., 2021)](https://paperpile.com/c/nGJIPX/wYkDy)[(Lakshmi, 2021)](https://paperpile.com/c/nGJIPX/hD6Dt) The respiratory system is always coming into contact with microorganisms (fungi, viruses, and bacteria). Respiratory tract infections result when harmful germs are inhaled and are not eliminated from the lungs. Upper and lower respiratory tract infections are the two categories into which these illnesses fall. Bacterial infections are the primary cause of lower respiratory tract infections, which have greater harmful effects and the greatest fatality rates in developing nations. Consequently, the health care system will bear an excruciating financial burden (Z. Huang et al., Citation2021) [(Ajay, Rakshagan, et al., 2022)](https://paperpile.com/c/nGJIPX/4aOum) [(Ajay, Suma, et al., 2022)](https://paperpile.com/c/nGJIPX/O4IkH) [(Katyal et al., 2021)](https://paperpile.com/c/nGJIPX/8II3W). Pathogens that are most common in respiratory tract infections include S. pneumoniae, H. influenzae, Moraxella catarrhalis, S. aureus, P. aeruginosa, M. tuberculosis, and S. pyogenes.[(Chen et al., 2022)](https://paperpile.com/c/nGJIPX/MjpH) Lower respiratory infections were the fourth most common cause of mortality globally in 2020, according to WHO data.

There were 2.6 million deaths in 2019, which is 460,000 lower than in 2000. Research examining how well medications work in response to infectious diseases is vital given the harm they pose[(Marzaman et al., 2023)](https://paperpile.com/c/nGJIPX/AdKqk)[(Jabin et al., 2021)](https://paperpile.com/c/nGJIPX/89uJV)[(Balaji Ganesh S & Sugumar, 2021)](https://paperpile.com/c/nGJIPX/iPSpP) [(Govindaraj & Dinesh, 2021)](https://paperpile.com/c/nGJIPX/H44og) .Phage therapy trials and compassionate use instances are among the latest clinical experiences that have been reviewed. These demonstrate encouraging results for patients with both acute and chronic respiratory infections. Genetically engineered phages present a promising treatment option for diseases that are resistant to conventional therapies because they can be engineered to more efficiently target particular bacterial pathogens. This has been especially important in the treatment of severe respiratory infections brought on by organisms like Acinetobacter baumannii and Pseudomonas aeruginosa, which are frequently linked to chronic lung diseases and pneumonia that requires the use of a ventilator.​It is being investigated to use nanocarriers, such as nanolipid carriers, as phage enzyme delivery systems. By improving their stability and bioavailability, these carriers can make sure that phage lysins are present at the infection site in sufficient amounts. This technique is promising for use in both intravenous and inhaled settings, offering a focused strategy of treating respiratory infections with no side effects[(Ruan et al., 2024)](https://paperpile.com/c/nGJIPX/HhAH4) [(Tiwari & Jain, 2023)](https://paperpile.com/c/nGJIPX/4sRE5)[(Graf et al., 2023)](https://paperpile.com/c/nGJIPX/3oRVk) .

Phage therapy trials and compassionate use instances are among the latest clinical experiences that have been reviewed. These demonstrate encouraging results for patients with both acute and chronic respiratory infections. Among the noteworthy research are studies on the use of phage combinations for infections caused by methicillin-resistant Staphylococcus aureus (MRSA) and inhaled phage therapy for Acinetobacter baumannii in critically ill COVID-19 patients[(Vaezi et al., 2024)](https://paperpile.com/c/nGJIPX/NF0U3).An essential barrier that separates the body's internal physiology from the external environment is the lung. Because of this, this interface is always under a lot of strain to protect against foreign particles and pathogenic bacteria. Acute and chronic respiratory disorders are among the most common and pervasive illnesses that affect people.

Many lung and respiratory tract disorders have shown benefit from direct medication delivery through the airway. Lipid nanoparticles, or LNPs, are a potent non-viral drug delivery vehicle that has been made possible by the development of nanotechnology. In this study, we assess the advancements made thus far in the delivery of inhaled medicines via LNPs as well as the obstacles still standing.[(Website, n.d.)](https://paperpile.com/c/nGJIPX/ou6Z)Millions of people worldwide are afflicted with lung cancer, idiopathic pulmonary fibrosis (IPF), cystic fibrosis (CF), asthma, and chronic obstructive pulmonary disease (COPD), among other deadly and persistent illnesses. The efficacy of conventional pharmacotherapeutic treatment techniques, such as peptide-based medicines, corticosteroids, chemotherapeutics, and bronchodilators, in curing or impeding diseases, is questionable. Even with the development of nanotechnology, tailored medicinal delivery remains challenging; nonetheless, the physicochemical features of nanoparticles can facilitate this process. Nanoparticles have gained prominence in the field of drug delivery studies because to their increased pharmacokinetics of active ingredients, which can be attributed to their surface, size, density, and physical-chemical features[(Pramanik et al., 2021)](https://paperpile.com/c/nGJIPX/3VOk)[(Sabarathinam & Madhulaxmi, 2021)](https://paperpile.com/c/nGJIPX/I9iT5)[(Sushanthi et al., 2021)](https://paperpile.com/c/nGJIPX/nFsi2)[(Harsha et al., 2022)](https://paperpile.com/c/nGJIPX/Rclu8)

# MATERIALS AND METHODS

## ISOLATION OF BACTERIOPHAGE

Samples from different cattle farms were gathered in order to isolate the bacteriophage, and they were then processed using SM Buffer. Following a 15–20 minute centrifugation at 10,000 g, the samples were collected, and the supernatant was filtered through a 0.22 µm filter. After that, the filtrate was added to Luria Bertani (LB) media that contained the host cell and 1 mM CaCl2, and the mixture was incubated for two to three hours at 37°C. Following the incubation period, the media was centrifuged for 15 minutes at 8,000 g, and the supernatant was once more filtered using a 0.22 µm filter. The filtrate that was ultimately obtained was used for further analysis.

## SPOT TEST ANALYSIS

A spot test was performed to determine whether bacteriophage was present in the obtained filtrate. After making Luria Bertani (LB) agar and adding 1 mM CaCl2, the host bacteria was grown in a lawn culture and applied to the agar's surface. The phage lysate was then spotted onto the inoculated media in an amount of 5 µl. Next, the plates were incubated at 37°C for the entire night. Following incubation, the presence of bacteriophage was verified by looking for clear areas or plaques where the lysate was applied.

## PURIFICATION OF PHAGE PARTICLES

The agar overlay method was employed to purify the phage lysate. Agar was prepared in two concentrations and placed in Luria Bertani (LB) media. The bottom layer was left to solidify and contained 2% agar. After solidifying, the bottom agar was covered with 3 millilitres of 0.75% agar that contained the host cell and diluted phage lysate. The mixture was then incubated at 37°C for an entire night. After the incubation period, each plaque was removed with a sterile tip and placed in SM Buffer. For more research, this refined phage solution was duplicated.

## EXTRACTION OF PHAGE ENZYME

Thermal extraction was used to extract the bacteriophage enzyme, and centrifugation at 7000 g for 20 minutes at 4 C produced the phage lysate. The supernatant was collected and filtered through a 0.22um syringe filter following centrifugation. The obtained filtrate was dialysed against sterile distilled water for a duration of 48 hours. After obtaining the phage lysate, it was incubated for 30 minutes at 90C. Following this, a sample containing 1 mM Mg+ ions was added, and it was kept at 37C for an hour. Following that, the SDS-PAGE was loaded with the sample. followed by staining and destaining to look for bands that could be compared to the protein marker.

## PREPARATION OF NLP

With a few minor adjustments to the microemulsion technique, nanostructured lipid carriers (NLC) were created. Mixing 0.35% solid beeswax and 0.25% liquid coconut oil in 50 mM Tris buffer (pH 6.3) and quickly stirring the mixture at 500 rpm on a magnetic stirrer at 80°C for two hours produced Solution A. Concurrently, 1% SDS and polyvinyl alcohol were added to 50 mM Tris buffer (pH 6.3) and allowed to stir at room temperature for 1.5 hours in order to prepare Solution B (emulsifier). After adding seagrass extract gradually to Solution B, it was stirred for a further half-hour. After 30 minutes of stirring, Solution B was gradually added to Solution A. After that, the mixture was freeze-dried and stored at -80°C for the next day. As a result, the NLC was characterized further.

## FTIR

The material's functional groups were investigated using Fourier-transform infrared (FTIR) spectroscopy, which has a spectral range of 4000 to 1000 cm⁻¹. With a resolution of roughly 4 cm⁻³, a total of 32 scans were carried out to guarantee accurate and thorough characterisation of the sample's composition.

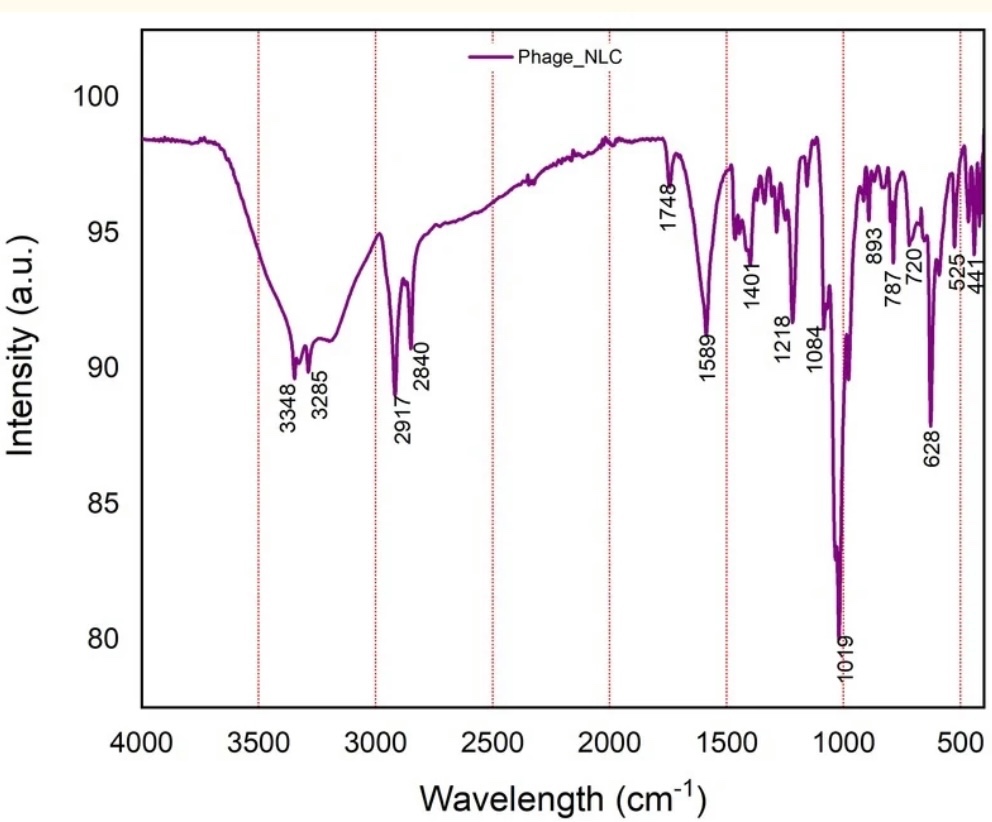
## XRD

The crystal's lattice dimensions, exact atomic locations within the lattice, phase identification, degree of crystallinity, and other structural characteristics were all clarified by using X-ray diffraction (XRD). In materials science, chemistry, and biology, this method is invaluable for analysing a broad range of materials, including minerals, polymers, and pharmaceuticals. The formation of the stereo complex was investigated in this work using a Bruker D8 Advance X-ray diffractometer that operated at 40 kV and 40 mA and produced Cu Kα radiation (λ = 0.15418 nm). Prior to analysis, the lyophilised hydrogel material was evenly loaded into the XRD apparatus and allowed to dry completely.

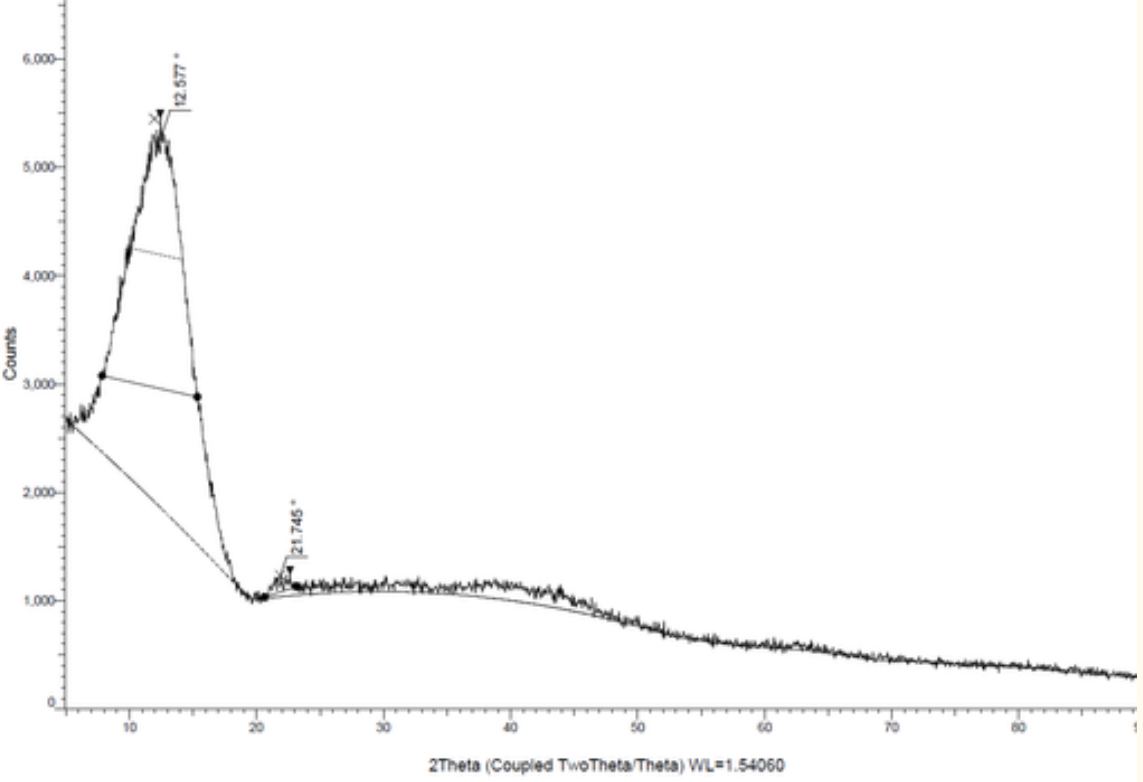
## ANTIBACTERIAL ACTIVITY

The antibacterial activity of the samples was assessed using the agar well diffusion method. After making nutrient broth and adding the bacterial strains Streptococcus mutans, Staphylococcus aureus, and Enterococcus faecalis, it was incubated for two to three hours at 37°C. The turbidity was adjusted to meet the 0.5 McFarland standard after incubation. A bacterial lawn culture was conducted on sterile petri plates containing Mueller Hinton agar that had been aseptically prepared. Using a sterile gel puncture, four wells were created, each measuring 10 mm in diameter and 4 mm in depth. As a positive control, an antibiotic disc was added, and dimethyl sulfoxide (DMSO) was added as a negative control. The inhibition zones on the plates had a diameter of after 24 hours of incubation at 37°C.

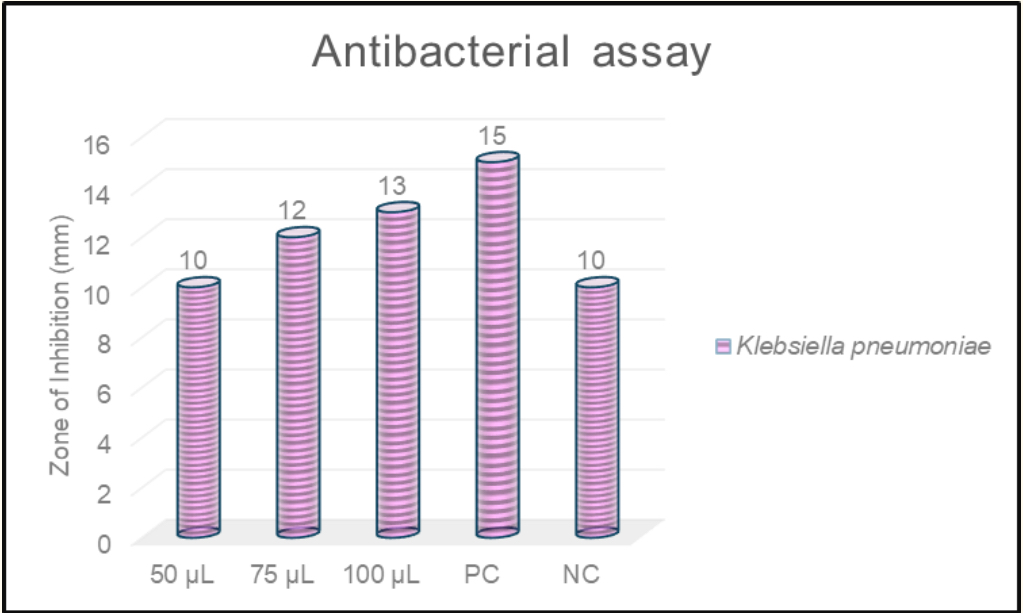
# RESULTS & DISCUSSION



**Figure 1:** FTIR spectroscopy analysis showing the incorporation of NLC-encapsulated phage enzymes.



**Figure 2:** XRD analysis showing the incorporation of NLC-encapsulated phage enzymes.



**Figure 3:** Antibacterial activity of the NLC-encapsulated phage enzymes against Klebsiella pneumoniae in various concentrations.

## ISOLATION, PURIFICATION AND PROPAGATION OF BACTERIOPHAGE

To guarantee the acquisition of high-purity phage preparations, a number of careful steps are involved in the isolation, purification, and propagation of bacteriophages. First, soil or sewage samples are taken from the environment and enriched by culturing them in a nutrient-rich medium with a suitable bacterial host.After that, the mixture is filtered through a 0.22 µm filter to get rid of any remaining debris and bacteria. To separate individual phage plaques, the filtrate—which contains bacteriophages—is put through a plaque assay. A single phage is represented by each plaque, which is selected and then multiplied by infecting new bacterial cultures. To guarantee phage purity, repeat this step multiple times. The phages are concentrated and purified from the lysate using methods like polyethylene glycol (PEG) precipitation or caesium chloride (CsCl) gradient centrifugation. Plaque-forming unit (PFU) assays are used to quantify the resulting high-titer phage preparation, which can then be stored under suitable conditions for use in therapeutic or research purposes

## EXTRACTION OF PHAGE ENZYME

The protein profile of the phage enzyme was ascertained by SDS-PAGE analysis after it was extracted thermally. Based on their relative mobility in relation to a standard protein marker, each of the four major protein bands identified by the analysis could be found. Among these, the depolymerase enzyme at a relative molecular mass of [specific molecular mass] was clearly observed. The successful extraction and characterisation of the enzyme were confirmed by the comparison with the protein marker, which made for a more accurate identification. The bands observed suggest the existence of various protein constituents, among which the depolymerase enzyme is a noteworthy component, offering valuable information regarding the enzyme's molecular weight and purity.

## SPOT TEST

To find out whether there were any bacteriophages in the purified lysate, a spot test was conducted. The host bacteria was grown in a lawn culture and added to LB agar plates along with 1 mM CaCl2. After that, different volumes of the phage lysate (5 µl, 10 µl, 50 µl, and 75 µl) were spotted onto the agar, and the plates were incubated overnight at 37°C. The results, as displayed in the pictures supplied, demonstrate distinct lysis zones where the phage lysate was applied. The presence and activity of bacteriophages in the lysate were confirmed by the observation of different plaques at every volume tested. The assay's specificity was confirmed when the negative control (-ve) exhibited no lysis and the positive control (+ve) also displayed clean zones. These results validate the bacteriophage's effective purification and anti-host bacterial action.

## PREPARATION OF NLC

The microemulsion method was altered in order to create the NLCs. Solution A was first created by rapidly stirring 0.35% solid beeswax and 0.25% liquid coconut oil in 50 mM Tris buffer (pH 6.3) at 500 rpm with a magnetic stirrer at 80°C for two hours. In parallel, 50 mM Tris buffer (pH 6.3) was mixed with 1% SDS and polyvinyl alcohol to create Solution B, an emulsifier solution, which was then left to stand at room temperature for 1.5 hours. Then, after slowly adding seagrass extract to Solution B, it was stirred for an extra thirty minutes. Solution B was gradually added to Solution A after it had been thoroughly mixed, and the combination was then agitated for a further thirty minutes. After that, the mixture was freeze-dried and kept for further analysis at -80°C. NLCs with potential uses in targeted drug delivery systems and other biomedical applications were produced by this method.

## FTIR

Multiple different peaks can be seen in the supplied FTIR spectrum for Phage\_NLC, suggesting the presence of multiple functional groups in the sample. It is possible to trace the broad peak between 3348 and 3285 cm^-1 to O-H stretching vibrations, which are suggestive of hydroxyl groups, potentially originating from alcohols or phenols. The peaks at 2840 and 2917 cm^-1, which are probably from aliphatic chains, are typical of C-H stretching vibrations. The presence of carbonyl (C=O) stretching, which is usually linked to esters or ketones, is suggested by the prominent peak at 1748 cm^-1. Peaks located at 1589 cm^-1 and 1401 cm^-1 may be indicative of the existence of aromatic rings since they correlate to aromatic C=C stretching vibrations.It is possible to credit the bands at 1218 cm^-1 and 1084 cm^-1 to C-O stretching vibrations, which are frequently present in alcohols, ethers, and esters. Peaks at 893, 787, 1019, and 720 cm^-1, as well as C-H bending in aromatics, indicate the existence of many bending modes. The lesser peaks at 628 cm^-1, 525 cm^-1, and 441 cm^-1 might be the result of the molecule's skeletal vibrations. All of these findings point to the presence of a complex variety of functional groups in Phage\_NLC, including aromatic, carbonyl, and hydroxyl groups. These groups are typical of many organic compounds and may be essential to the structure and functionality of the chemical.

## XRD

A crystalline structure is indicated by the sample's strong peaks at 2θ values of 12.577° and 21.745° in the X-ray diffraction (XRD) pattern. A high degree of crystallinity in that particular plane is indicated by the steep peak at 12.577°, which may be related to the sample's organised molecular or atomic arrangement.There may be more than one crystallographic plane present, as indicated by the extra peak at 21.745°, which further supports the crystalline structure(Rafi et al., 2024). A substantial amount of crystalline material is suggested by the strength of the peaks, whilst a degree of amorphous content is indicated by the wider baseline in the range beyond 25°. Sharp peaks and a wide baseline suggest a semi-crystalline structure in which amorphous and crystalline regions are scattered throughout. The interplanar spacing, which is essential for comprehending the molecular structure and possible functioning of the material, can be computed using Bragg's law and the crystalline peaks. All things considered, the XRD pattern sheds light on the sample's structural qualities, emphasising its semi-crystalline nature, which is relevant for applications needing particular structural qualities.

## ANTIBACTERIAL ASSAY

The findings of the antibacterial assay for Klebsiella pneumoniae show a dose-dependent increase in the zone of inhibition, indicating that the tested chemical is effective in preventing the development of bacteria (Tuluwengjiang et al., 2024). The zone of inhibition measured 10 mm at the lowest dose of 50 µL, indicating a moderate antibacterial activity.This inhibition increased to 12 mm and 13 mm at 75 µL and 100 µL dosages, respectively, showing that the antibacterial effect enhanced with increasing dosage. The positive control (PC) at 15 mm showed the largest zone of inhibition, which was used to calculate the assay's maximal antibacterial efficiency. The negative control (NC), which measured a 10 mm zone of inhibition and provided a baseline comparison, demonstrates the intrinsic antibacterial activity of the test drug even at lower concentrations. These results suggest that the compound has potent antibacterial action against Klebsiella pneumoniae, and that its efficacy is comparable to that of conventional medicines at larger dosages. The outcomes show that this substance has the potential to be studied further and used in antibiotic therapies.

Nanolipid carriers (NLCs) injected with phage enzymes present a viable approach to targeted antibiotic therapy for respiratory infections. Encapsulating phage-derived enzymes, these carriers increase the stability and bioavailability of the enzymes. The procedure comprises the selection of suitable surfactants and lipids to create a stable lipid matrix, guaranteeing even dispersion and maximum encapsulation effectiveness. The deep lung penetration and extended retention in the respiratory system made possible by the nano-sized NLCs improve the effectiveness of treatment against respiratory infections such as Klebsiella pneumoniae. Enzyme inclusion is confirmed by FTIR measurement, and the NLCs' semi-crystalline structure is shown by XRD patterns. Phage enzyme-infused NLCs have the potential to be an efficient therapeutic option, as demonstrated by the antibacterial experiment, which demonstrates a dose-dependent reduction of bacterial growth.

## CONCLUSION

In conclusion, the creation of nanolipid carriers (NLCs) injected with phage enzymes presents a novel strategy for treating respiratory infections, demonstrating important developments in antimicrobial medicine and nanotechnology. The phage enzymes' novel encapsulation into NLCs maintains their enzymatic activity while also improving their stability and tailored distribution to lung tissues that are infected. The structural integrity of the NLCs and the effective integration of the enzymes are verified by characterisation methods including FTIR and XRD. The findings of the antibacterial experiment clearly show a dose-dependent suppression of Klebsiella pneumoniae, demonstrating the produced NLCs' strong antibacterial activity. This strategy promises better therapeutic outcomes by addressing significant issues with traditional medicines, such as poor bioavailability and non-specific targeting. Phage enzyme-infused NLCs have the potential to be a unique, effective, and targeted therapeutic method for treating respiratory infections. The successful formulation and encouraging in vitro results highlight this promise, opening the door for further in vivo research and clinical applications.

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