Traditionally, study of microbial cells has revolved around planktonically suspended free-swimming cells. These planktonic cells have remained as model for many microbiological studies till date. It was only in late 1980s that scientists began to appreciate the importance of a rather very old phenomenon; microbial adherence to surfaces and their tendency to organise into multicellular microbial aggregations called biofilms. This surface associated microbial growth represents an altogether different niche in which microorganisms differ drastically in their properties than their planktonic counterparts. Hence, studies carried out on planktonic cultures provided a biased view of microbial life. Though biofilms have been spotted in every sphere of life but their association with clinical environments has been of special interest as more than 65% of infections are biofilm associated (Mah and O'Toole 2001) and biofilm enclosed microorganisms have the ability to resist 100-1000 times higher antibiotic concentrations (Gilbert et al 2002).

*Klebsiella* species account for up to 8% of all nosocomial infections (Podschun and Ullmann 1998). The most important species in the genus is *Klebsiella pneumoniae*. It is an opportunistic pathogen that is associated with hospital acquired urinary tract infections (UTI), pneumonia, septicaemia and wound infections. Individuals most at risk are infants, the elderly and those with immunocompromised status. Various virulence factors that contribute to *Klebsiella pneumoniae* pathogenesis include production of antiphagocytic capsule, serum resistance due to lipopolysaccharide (LPS) antigen (Podschun and Ullmann 1998), siderophores (Reissbrodt and Rabsch 1988) and type 1 and 3 fimbriae. Fimbriae help bacteria to bind to biological as well as non-biological surfaces and form biofilms (Tarkkanen et al 1997). Recently, biofilm formation by *Klebsiella pneumoniae* has been suggested to be an important virulence factor.
(Jagnow and Clegg 2003) as bacteria growing within biofilms are more resistant to antibiotics than their planktonic counterparts (Mah and O'Toole 2001). Moreover elaboration of extended spectrum beta lactamase by *Klebsiella* species has further engraved the scenario of antibiotic resistance. Hence an antimicrobial that can be used effectively against biofilms of *Klebsiella pneumoniae* is needed.

Use of bacteriophages as an antibacterial agent has come a long way since its first discovery by d'Herelle in 1920s. But due to unequivocal outcomes of the treatment and discovery of wonder drugs, the antibiotics, use of bacteriophages as antibacterial agents was curtailed, at least in the greater part of the world. However due to widespread documentation of bacterial resistance to antibiotics and establishment of biofilm concept in clinical infections, interest in bacteriophages as therapeutic agents has rekindled the interest of scientific world in this area.

One of the reasons of failure of bacteriophage therapy in earlier days was the application of uncharacterized bacteriophages. Hence in the present study the emphasis was on the isolation and characterization of lytic bacteriophage against *Klebsiella pneumoniae*. Mode of bacteriophage replication is very important in its consideration as therapeutic agent. Bacteriophages multiply by lytic or lysogenic mode. In lysogenic cycle host bacterium is not lysed and phage DNA is replicated along with bacterial DNA. However, in lytic cycle phage multiplication is associated with cell lysis, ultimately killing the bacterium. Hence bacteriophages selected for therapeutic use should be lytic in nature, which due to their cidal effect, can be effective in arresting the growth of the pathogen. Of the bacteriophages isolated in the present study KPO1K2 was selected for therapy as it was found to be a lytic phage having a triple-zone plaque morphology indicating its ability to produce depolymerase in copious amount. Previously also bacteriophages with such plaque morphology have been demonstrated to carry associated depolymerase activity with them (Geyer et al 1983). Major function of depolymerase enzyme is the degradation of the bacterial capsule helping the bacteriophage reach its primary receptors. The first step in the lytic cycle of bacteriophage, termed adsorption, is facilitated by tail fibres that
Discussion

bind to specific molecules, called phage receptors, on the bacterial cell surface. The specificity of receptors for a single phage strain determines its host range; some phages have specificity at strain level while others at species level. Bacteriophage selected for the present study was found to infect not only several strains of *Klebsiella pneumoniae* but also *Escherichia coli* ATCC 25922 and one of its five clinical isolates. Finding bacteriophages with broader host range is not uncommon (Goodridge et al. 2003) and previously also isolation of lytic bacteriophage infecting multiple capsular types has been documented (Scholl et al. 2001). Isolation of a bacteriophage infecting both *Klebsiella* and *Escherichia coli* species is of added interest as both these pathogens are causative agents of hospital acquired catheter associated urinary tract infections (UTIs) and a single phage infecting both the bacteria might prove to be of extra help for the clinicians.

Major criteria used for bacteriophage classification is its morphological, structural, growth and genetic characteristics. Morphology as seen under transmission electron microscope (TEM) revealed a bacteriophage with icosahedral symmetry and pentagonal outline. The presence of a small non-contractile tail indicated towards inclusion of this bacteriophage into family *Podoviridae*. Moreover, in accordance with the previous studies (Ackermann 2005) head and tail size of KPO1K2 matched with the size range of the T7-like lytic bacteriophages suggesting a close resemblance of the isolated phage with T7 phages. The structural protein pattern of this bacteriophage also resembled with that of the known T7 proteins. Presence of two prominent protein bands of 75 and 43 kDa on SDS-PAGE were suggestive of major tail and head proteins, respectively, as has been shown for T7-like bacteriophage (Sillankorva et al. 2008).

Further evidence to show KPO1K2's closeness to T7 bacteriophage came from its genome characteristics. Digestion of bacteriophage nucleic acid revealed the genome to be dsDNA. Moreover, bands produced after digestion with *Sau3AI* indicated a genome size of 42 kbps that was found to be close to the genome
Discussion

size of 42,519 bp of T7 phage of *Pseudomonas aeruginosa* (Lavigne et al 2003) and other T7-like bacteriophages (Ackermann 2005). Stability of bacteriophage following long term exposure to chloroform and ether indicated that this bacteriophage could be stored over these agents for longer duration and that this bacteriophage did not contain any lipid in its capsid, as suggested by Jarrell et al (1997). Furthermore, bacteriophage, KPO1K2, was found to be stable over a wide range of pH from 4-11 as after an exposure of 1 hour at respective pH no significant decrease in bacteriophage activity was noticed. All the stability studies carried out in the present study confirmed the applicability of this bacteriophage as a therapeutic agent, the aim with which this phage was isolated in the present study. Since the bacteriophage did not lose its activity at even extremes of pH, hence this bacteriophage can be conveniently used for the impregnation of urinary catheters for the prevention of bacterial biofilms as suggested elsewhere (Danese 2002; Johnson et al 2006). Moreover, in line with the previous study (Jarrell et al 1997) KPO1K2 lost 27% activity when exposed to 56°C for an hour indicating its relative heat stability. This observation supports the predisposition that slight exposure of phage impregnated catheters to elevated temperatures would not affect the activity and quality of the catheter. Moreover, since 37°C was found to be the optimum temperature for bacteriophage activity, the phage impregnated catheter would be highly effective when placed inside the body.

Application of bacteriophages for treating bacterial infections has been deterred due to poor understanding of bacteriophage kinetics (Dabrowska et al 2005). Bacteriophages as self replicating pharmaceuticals substantially differ from other chemical pharmacological agents. Hence, kinetics of bacteriophage survival in mouse model was performed to estimate the stability of bacteriophage in various organs and its subsequent elimination from host system. As has been noted earlier by Dabrowska et al (2005), intraperitonial (i.p.) injection was found to be very effective in introducing bacteriophages into the blood stream and peritoneum-blood penetration appeared to be very fast and facile (Bogozova et al 1991; Merril et al 1996). In the present study when mice were inoculated i.p. with bacteriophage, appreciable phage titers were seen in blood as well as in lungs.
Discussion

after one hour. Titer in all the organs was found to be higher than the titer in blood which is in contrast to the previous observation (Bogozova et al 1991). In the present study the highest concentration of bacteriophage in all organs, including blood, was seen after 6 hour post-inoculation. Contrary to our results Cereveny et al (2002) found peak bacteriophage concentration in various organs at 1 hour post inoculation. However, the difference in bacteriophage titer at 1st and 6th hour was not significant. The difference observed in the two studies may be due to the difference in the route of phage inoculation in mice. It was observed that when bacteriophage was given intravenously it accumulated to higher titers in blood in shorter durations than when given i.p. emphasising the importance of route of inoculation for the success of phage therapy in vivo. In the present study accumulation of bacteriophage in higher concentrations was observed in kidney and urinary bladder. Though its titer started declining after 12th hour of inoculation, but complete removal of bacteriophage from these organs occurred only after 36th hour of inoculation. In addition, in line with the previous observation (Keller and Engley 1958) highest titer of bacteriophage in liver and kidney was achieved after 6 hours of inoculation. In blood and lungs bacteriophage became undetectable at 36th hour post-inoculation while in kidney, urinary bladder and liver bacteriophage became undetectable only after 48th hour of inoculation. Since, without its host bacteriophage survived for appreciable duration, in vivo, hence, we speculate that it will act as an excellent antibacterial agent especially in the light of observation that bacteriophages are self replicating and become available in higher concentrations whenever its host is available. Hence, the data on bacteriophage characterization clearly indicated that the isolated bacteriophage KPO1K2 is a T7-like lytic bacteriophage and belonged to the family Podoviridae. Further, this data reinforces the idea of using bacteriophage as a therapeutic agent, since not only it kills the bacteria optimally at physiological conditions but also survives for longer periods both in vitro as well as in vivo.

Bacteriophages are obligate bacterial pathogens that replicate only inside their bacterial hosts implying that they must enter into the bacterial cell or at least
inject their genetic material into the bacterial cell for replication cycle to start. But complex nature of cell wall especially that of Gram-negative bacteria poses a hindrance for the entry of bacteriophage into the bacterial cell. To overcome this problem bacteriophages have developed certain structural units having enzymatic activity. Tail spike is the terminal part of bacteriophage structure that is protein in nature and has got depolymerase activity associated with it. Depolymerase is the enzyme that degrades the bacterial capsule, LPS or other cell wall components as demonstrated by Geyer et al (1983). Major use of depolymerase is to help bacteriophage reach its bacterial host by degrading the protective bacterial capsule. Capsule is a major virulence characteristic of the bacterial cell that helps the bacterium in adhering to surfaces and form biofilms reducing the bacterial susceptibility to antimicrobials such as phagocytes, complement and antibiotics. Since capsular matrix is the integral part of the biofilm produced by *Klebsiella pneumoniae*, its degradation will result in disintegration of biofilm and hence loss of recalcitrance mechanisms associated with it. Since, bacteriophage borne depolymerase degrades the bacterial capsule hence its use as an antibiofilm agent could be speculated.

In the present study, from the characterized T7-like lytic bacteriophage, KPO1K2, depolymerase was isolated, purified and characterized with the intention of checking its antimicrobial potential against biofilms of *Klebsiella pneumoniae*. Infection of bacterial cell is associated with the production of depolymerase that exists in two forms; phage bound and free, major portion being in free form (Yurewicz 1971). For each depolymerase molecule incorporated into the bacteriophage about 1000 molecules are produced into the suspension (Eklund and Wyss 1962). Bacteriophage bound depolymerase adheres tightly to the phage, making its separation difficult. Methods of protein precipitation such as ammonium sulphate and ethanol precipitation, tried in the present study, did not work well for this protein as enzyme denaturation was observed after application of these techniques leading to decreased enzyme activity. However, as suggested earlier (Rieger et al 1975) treatment of bacteriophage suspension with mild acid led to specific bacteriophage
Discussion
denaturation, retaining the depolymerase activity. Hence high tittered bacteriophage suspension was exposed to mild acid treatment for 5-8 min leading to production of crude enzyme lysate having little bacteriophage activity associated with it. This crude enzyme lysate was further purified by ion-exchange and size exclusion chromatography. Purification of crude lysate, containing depolymerase, using chromatographic techniques resulted in an enzyme preparation having a specific activity of 258 units (U)/mg of protein with a recovery rate of 50% in comparison to crude lysate. Moreover, over the various purification steps the enzyme was purified 16 fold with respect to crude lysate. The total units obtained were slightly higher than the total depolymerase units (DU) obtained by Rieger et al (1975) from the disrupted bacteriophage. However the DU obtained were less than that obtained by Vandenbergh et al (1985). It seems that ammonium sulphate precipitation works for depolymerase of *Erwinia amylovora* and not for *Klebsiella pneumoniae* (Vandenbergh et al 1985), though the folds of purification achieved in the present study was higher compared to earlier studies.

For checking the homogeneity of the enzyme preparation, protein at each step was subjected to polyacrylamide gel electrophoresis. It was observed that with each purification step purity level increased and ultimately a single protein band of 30 kDa was obtained. The low molecular weight of the purified depolymerase observed in the present study was in contrast to the molecular weight of other depolymerases (Bartel et al 1968; Yurewicz et al 1971; Higashi and Abe 1978; Tanio et al 1982). However, depolymerases with low molecular weight have also been reported in the past. Vandenbergh et al (1985) reported depolymerase from *Erwinia amylovora* bacteriophage with a molecular weight of 21 kDa while that reported by Mohammed et al (2006) was 31 kDa. Molecular weight of protein depends on its association with other structural proteins of bacteriophage. It was observed that when bacteriophage proteins were precipitated using (NH₄)₂SO₄, molecular weight of enzymatically active fragment was ~208 kDa. While following mild acid treatment method, a low molecular weight protein having associated depolymerase activity was obtained. These
Discussion

results explain why depolymerase of low molecular weight was obtained in the present study as method of acid denaturation was applied for enzyme extraction.

Enzyme activity is known to be influenced by its environment. In the present study an attempt to standardize the optimum conditions for depolymerase activity was made. In contrast to earlier studies (Iwashita and Kanegasaki 1976), depolymerase in the present study was found to be heat labile as considerable loss in enzyme activity upon increasing the temperature was observed. However, optimum temperature for depolymerase activity was found to be around 37°C and with increase in temperature of incubation, enzyme activity decreased. This observation is in contrast to the temperature optimum for depolymerase of *Pseudomonas aeruginosa* reported by Bartell et al (1966). Moreover, the optimum pH for the activity of depolymerase enzyme was found to lie between pH 7 and 8 that was in accordance with the pH optimum for depolymerase of *Pseudomonas aeruginosa* reported earlier (Bartell et al 1966). Also, as seen earlier, a slight decrease in pH resulted in loss of enzyme activity (Eklund and Wyss 1962) though slightly elevated pH was tolerated for at least 20 min of incubation. In contrast to earlier observations reported in literature (Bartell et al 1966), an increase in substrate concentration did not lead to increase in enzyme reaction. In fact a concentration of more than 1 mM was found to be inhibitory for the enzyme reaction. Moreover, when the substrate concentration was less than 1 mM of polysachharide sugar, a gradual decrease in enzyme reaction was observed. All these observations emphasized the importance of standardization of optimal conditions for enzyme activity.

Presence of metal ions and chemicals produced mixed effects on enzyme activity. Decrease in depolymerase activity with increase in metal ion concentration was observed as metal ions are known to affect the enzyme activity. Moreover, as reported earlier, enzyme was found to be insensitive to many chemical agents including SDS, Triton-X and Tween-20. Absence of any effect of reducing agents on enzyme activity hinted towards absence of sensitive reducible bonds in enzyme structure, especially the disulphide linkages as
depolymerase retained its activity even after SDS treatment. The latter is known
to reduce the disulphide linkages and bring the enzyme to primary structure level.
Moreover, like the enzyme from \textit{R. trifolii} (Higashi and Abe 1978), EDTA was
found to inhibit depolymerase activity partially. Furthermore, in comparison to its
parent bacteriophage, purified depolymerase showed higher degree of cross
reactivity and broader host range that could be due to the fact that bacteriophage
bound depolymerase cannot function once phage gets adsorbed to its host
(Iwashita and Kanegasaki 1976). Moreover lesser number of moieties comprising
the capsular material may be another reason for broader host range exhibited by
the free depolymerase (Rieger-Hug and Stirm 1981). Enzyme having the ability
to depolymerise capsular polysaccharide from different sources at physiological
conditions (37°C and pH 7) indicated the prospect of it being used for therapeutic
purposes, at least for topical application.

\textit{Klebsiella pneumoniae} is able to adhere to inanimate surfaces and form
biofilms, which is an important feature in the pathogenesis of its surface
associated infections such as UTI (Jagnow and Clegg 2003; Langstraat et al
2001). Biofilm on surfaces is formed in several distinct steps that range from non­
specific reversible adherence to specific irreversible attachment followed by
micro colony formation. This procedure leads to formation of biofilms, microbial
load of which increase with time. In the present study also a gradual increase in
bacterial load of the biofilm was observed with time, attaining a maximum load on
4\textsuperscript{th} or 5\textsuperscript{th} day of biofilm formation. Thereafter a gradual decrease in bacterial
numbers was seen. Protocol employed for biofilm development is important as
the conditions of biofilm formation are known to affect its architecture as well as
microbial physiology. Static growth techniques allow the culture to grow in media
where there are few mechanical or thermal fluctuations (Peng et al 2002).
However, under such static conditions nutrients become depleted over time,
affecting biofilm growth. Hence, in the present study though biofilms were grown
under static conditions, growth medium was replenished every 18- 24 hours so
that nutrient depletion did not become a limiting factor for biofilm development.
Discussion

This method helped in generating homogenous biofilms of adequate density as observed under electronmicroscope.

Bacteria respond to changes in internal and external pH by adjusting the activity and synthesis of proteins associated with many different cellular processes (Olsen 1993). There exist mechanisms that allow bacterial population to adapt to small micro-environmental changes in pH. However, there are certain cellular processes that do not adapt to pH fluctuations. One such process is the excretion of exopolysaccharide substances. Optimum pH for polysaccharide production depends on the individual species, but it is around 7 for most of the bacteria (Oliveira et al 1994). Since the primary aim of the present study was to evaluate the potential of bacteriophage and its associated depolymerase in disintegrating the biofilm brought about by polysaccharide digestion, hence, biofilm was grown at pH 7 so that capsular polysaccharide was formed at its optimum level. Moreover, as biofilm disintegration in this study was targeted from therapeutic point of view hence, physiological temperature of 37°C was chosen for biofilm production.

Antibiotic susceptibility of planktonic bacteria and resistance of corresponding biofilm cells is a well-established phenomenon. In most cases, treatment with antibiotics slows down biofilm progression by eliminating planktonic cells and interfering with biofilm metabolism (Tenke et al 2006). In the present study as well, when planktonic cultures of *Klebsiella pneumoniae* were subjected to treatment with antibiotic, within hours, a significant reduction in bacterial numbers was observed (P<0.05). Similarly, a significant reduction of viable bacteria in planktonic culture upon exposure to bacteriophage was observed (P<0.05). However, the reduction after antibiotic treatment was greater, which could be attributed to even distribution of antibiotic around bacteria in planktonic culture.

Bacteria in biofilms differ physiologically as well as metabolically from its planktonic counterpart (Evans et al 1991) due to which biofilm bacteria show an increased resistance to antimicrobial treatment (Stewart and Costerton 2001).
Though bacteria have evolved various mechanisms to thwart antibiotic action, yet antibiotics remain the drug of choice against clinical infections. In the present study when biofilms were subjected to antibiotic treatment, it was observed that ciprofloxacin alone was able to eradicate the young biofilms of *Klebsiella pneumoniae* significantly (P<0.05) though with increase in age, the ciprofloxacin treatment of biofilm became completely ineffective. Age of biofilm plays a very important role in recalcitrance of biofilm towards various antimicrobials. It has been documented previously that with age, the heterogeneity of biofilm increases (Laspidou and Rittmann 2004), implying that the cells despite being descendents of same parent and having the same genetic information show varied characteristics (Stewart and Costerton 2001). Stress induced antimicrobial resistance is one such characteristic (Sedlacek and Walker 2007; Ito et al 2009) due to which the number of recalcitrant bacteria in older biofilm is more compared to the young biofilm. This explains why in the present study ciprofloxacin treatment of the older biofilms was found to be ineffective, though the treatment of younger biofilm with ciprofloxacin alone was effective. Moreover, increase in exposure time to antibiotic treatment had little effect on the outcome of therapy suggesting presence of recalcitrance mechanisms that could work for longer periods.

The familiar mechanism of antibiotic resistance, such as efflux pumps, modifying enzymes, and target mutations (Walsh 2000) do not seem to be responsible for the protection of bacteria in biofilms (Stewart and Costerton 2001). Even sensitive bacteria that do not possess known genetic elements for resistance can show profound reduction in susceptibility when present in biofilm state, indicating biofilm specific resistance mechanisms. Though the full spectrum of mechanisms that might be at play in contributing towards antimicrobial recalcitrance of biofilms is not known, still the data accumulated over the past two decades hints towards three main hypotheses. The first hypothesis is the slow or incomplete penetration of antimicrobials into the biofilm. As early as 1981, Costerton et al (1981) have proposed that the EPS secreted by the biofilm cells may protect the cells from antimicrobial agents either by creating...
Discussion

diffusion barrier or by reacting chemically with the antimicrobial (Dodds et al 2000). Although, the matrix may not inhibit the penetration of antibiotics into the biofilm altogether, it may retard the rate of penetration enough to induce the expression of genes within the biofilm that mediate resistance (Jefferson et al 2005). However, measurement of antibiotic penetration into the biofilms in vitro has shown that some antibiotics penetrate readily into the bacterial biofilms (Anderl et al 2000) but still do not eradicate the biofilm bacteria effectively; emphasizing the importance of some other mechanisms that might be at play.

The second hypothesis depends on the altered chemical microenvironment within the biofilm involving the physiological state of organisms. The creation of starved, stationary phase dormant zones in the biofilms is considered to be a significant factor in the resistance of biofilm bacterial population to antimicrobials (Anderl et al 2003, Walters et al 2003). Though the worst affected antibiotics due to the slow bacterial growth are the ones that attack the bacterial cell wall, yet all the antibiotics require some metabolic activity for their efficacy as these act by disrupting the microbial processes. Hence, pockets of cells in a biofilm that are present in stationary phase might represent a general mechanism of antibiotic resistance. Third hypothesis emphasises the presence of specialised cells in the bacterial population that are differentiated into a highly protected phenotypic state and coexists with antibiotic sensitive neighbours. These cells are called persisters and comprise a small fraction of total biomass in the biofilm (Stoodley et al 2004).

Since biofilm resistance depends on aggregations of bacteria in multicellular communities, it was speculated that disruption of multicellular structure of biofilm would result in restoration of efficacy of not only antibiotic but also host defences (Stewart and Costerton 2001). Hence, one strategy might be to develop a therapy that disintegrates the biofilm and degrade the matrix as it is primarily responsible for biofilm multicellularity. Potential therapies include agents that dissolve matrix polymers, chemical reactions that block biofilm matrix synthesis and blockade of cell-to-cell communication required for normal biofilm
Bacteriophages are bacterial viruses that are known to be specific for their bacterial host and have been demonstrated to eradicate bacterial infections in humans (Inal 2003, Fortuna et al 2008); animals (Chhibber et al 2009; Kumari et al 2009; Malik and Chhibber 2009) and food (Garcia et al 2008). Recently, use of bacteriophages as one of the methods for controlling bacterial biofilms has been proposed (Curtin and Donlan 2006; Donlan 2009). In the present study phages were able to eradicate the biofilm substantially and younger biofilms of *Klebsiella pneumoniae* were highly sensitive to the bacteriophage action. To check and compare the efficacy of bacteriophage as an antimicrobial, in comparison to antibiotic, bacteriophage was also applied for the eradication of older biofilms. Bacteriophage could eradicate the biofilm very effectively up to 4th day, after which its efficacy against the biofilm was slightly diminished, though reduction in bacterial numbers after bacteriophage treatment compared to antibiotic treatment remained significant indicating its supremacy over the antibiotic.

Bacteriophages are obligate parasites and virulent phages that multiply by means of a lytic cycle, first adsorb on to the host bacterial cell surface, inject its genetic material into the cell, take over the host machinery and make copies of itself. Ultimate release of the newly assembled bacteriophage particles is brought about by lysis of the host bacterial cell. In this bacteriophage lytic cycle, infection of a bacterial cell with a single virion results in production of progeny phage, depending upon the burst size of the bacteriophage strain and this progeny phage is available for further infection of the bacterial cells making the bacteriophage, unlike antibiotic, a self replicating antimicrobial. Moreover, unlike antibiotics, even under conditions of nutrient limitation, bacteriophages have been shown to be effective against their bacterial host cells. Corbin et al (2001) reported a T4 bacteriophage that was effective against *E. coli* biofilms in a glucose limited chemostat, although the rate of phage synthesis and assembly were directly proportional to the amount of protein synthesis in the host cell. Moreover, higher nutrient levels result in increased cell growth that in turn result
in increased burst size and decreased eclipse and latent periods (Hadas et al 1997).

One of the drawbacks of antibiotic usage is the need for higher concentrations of drug to inactivate or eradicate the biofilm cells especially as the biofilm becomes old (Anwar et al 1992; Amorena et al 1999). In contrast, as also observed in the present study, it has previously been demonstrated that biofilm age does not significantly affect the susceptibility of the biofilm bacteria to its bacteriophage (Hanlon et al 2001). Nonetheless, a little decrease in efficacy of bacteriophage observed in the present study could be due to the combined effect of all the resistance mechanisms elaborated by the mature biofilm. However, decreased ability of bacteriophage alone in eradicating the older biofilm could be restored by concomitant application of antibiotic. When the two agents were applied in conjunction, within a period of 3 hours a significant reduction of greater than 3 logs was observed that increased to greater than 4 logs when the exposure time was increased to 6 hours. Further increase in exposure time did not show any impact on the efficacy of combined treatment. Concomitant application of various chemical antimicrobials has been used for eradication of bacterial infections (Kacmaz and Sultan 2007), though their use in combination has been deterred due to immense recalcitrance shown by the biofilms to chemical antimicrobials (Aaron et al 2002). Since, very high concentration of antimicrobials is required for the effective eradication of biofilms; hence, application of bacteriophages along with antibiotics has been suggested. This combination is not endowed with any side effects. The lytic bacteriophages not only show bactericidal effect but also tend to augment the antibiotic action against the biofilm (Hagens et al 2006). Lu and Collins (2009) have demonstrated that when antibiotic and lytic bacteriophage was applied in conjunction, the extent of biofilm eradication was more than when either of the antimicrobials was applied alone. In the present study as well pronounced eradication of biofilm, in comparison to either of the antimicrobials, was observed when the lytic bacteriophage and ciprofloxacin were applied in combination.
The possible reason for this accentuated effect could be the bacteriophage associated depolymerase enzyme that might have helped the ciprofloxacin to have an access to its target. Certain bacteriophages carry tail spikes that are enzymatic in nature and have associated polysaccharide depolymerase activity degrading the biofilm EPS matrix thus helping the bacteriophage in reaching the bacterial cell surface and inject its nucleic acid into its host for the initiation of lytic cycle. The degradation of the biofilm EPS facilitates deeper penetration of antimicrobials into the biofilm, besides resulting in aeration and replenishment of biofilm interiors (Hughes et al 1998), thus making the resident bacterial population metabolically active (Ahn et al 2009). Since, presence of biofilm cells in stationary phase has been demonstrated to be one of the reasons for biofilm recalcitrance to antimicrobials; hence regaining their metabolic activity will make the bacteria susceptible to antibiotics. This possibly explains the increased efficacy of antibiotic in presence of bacteriophage, observed in the present study.

The importance of bacteriophage associated depolymerase in infection of bacterial cells has been highlighted earlier (Adam and Park 1956). However, very few studies are available that have delineated the importance of phage associated depolymerase in eradicating the biofilms. Lu and Collins (2007) reported that a lytic bacteriophage, engineered to produce depolymerase, could eradicate the biofilms of *E. coli* effectively, in comparison to non-depolymerase producing bacteriophage. In order to check the significance of bacteriophage associated depolymerase in eradicating biofilms of *Klebsiella pneumoniae*, in the present study, a non-depolymerase bacteriophage was also isolated and applied for biofilm eradication. The biofilm eradicating potential of non-depolymerase bacteriophage was far less than depolymerase producing bacteriophage. Further, restoration of the efficacy of non-depolymerase bacteriophage in eradicating the biofilm of *Klebsiella pneumoniae* by concomitant application of purified depolymerase enzyme retrospectively confirmed the major role played by bacteriophage borne depolymerase in biofilm disintegration by the bacteriophage.
Discussion

The role of bacteriophage borne enzymes has been envisaged variously. Antibacterial enzymes such as bacteriophage encoded endolysins have been tried successfully for resolution of bacterial infections (Fischetti 2005). These enzymes not only kill bacteria but also decrease the minimum inhibitory concentration of antibiotics (Hagens et al 2006). Endolysins act by targeting one of the four major bonds in peptidoglycan of bacterial cell wall (Young 1992). For these enzymes to be effective, a direct contact between endolysins and bacterial cell is required. Hence, these can be used as exolysin only against Gram-positive bacteria. Presence of capsule and outer membrane in Gram-negative bacteria dissuades the endolysin action (Fischetti 2005, Loessner 2005). Hence, for an enzyme to be effective against Gram-negative bacteria, it must degrade the bacterial capsule and reach the bacterial cell surface, in process making the bacteria susceptible to antimicrobials. Bacteriophage borne depolymerase is the enzyme produced in addition to classical endolysins that are synthesized during bacteriophage infection. Since, depolymerase can work from outside, it can be a potential candidate for degrading the bacterial capsule of Gram-negative bacteria, thus altering its susceptibility to antibiotics and macrophages.

Bacteriophage borne depolymerase has been used for different purposes. In one study it was applied for the elucidation of the structural components of the polysaccharide (Sutherland 1967) while in other study it was used for the improvement of pear by transgenic expression against the bacterium causing the fire blight (Malnoy et al 2005). In this study we concentrated on the ability of depolymerase to disintegrate the biofilm. The action of depolymerase against biofilm was found to be both concentration as well as time dependent. At lower concentrations of 20 µg/ml, even after 120 min of treatment an insignificant reduction in biofilm bacterial load was observed though after increasing the concentration to 37 µg/ml of enzymatic protein the treatment became significant within 60 min of application. However, further increase in enzyme concentration did not offer any advantage; in fact it showed a negative effect.
Enzymatic degradation of matrix resulted in effective disruption of bacterial biofilm. However, taking note of the previous suggestions, purified depolymerase was applied in conjunction with ciprofloxacin to see if some added advantage of concomitant application was observed. Surprisingly, enzyme and antibiotic, when applied simultaneously, did not eradicate the biofilm effectively and reduction in bacterial numbers, with respect to untreated biofilm, remained insignificant ($P<0.05$). However, significant eradication of biofilm could be achieved upon application of depolymerase and antibiotic in sequence, addition of ciprofloxacin after enzyme treatment. Different outcomes of the concomitant and sequential application of ciprofloxacin may be attributed to the anoxic and acidic foci that are produced inside biofilms as a result of antibiotic activity, leading to diminished enzyme activity and hence less biofilm disruption (Stoodley and Stoodley 2009).

Unlike endolysin, depolymerase does not kill the bacteria but only digests its outer capsule (Lu and Collins 2007). In the present study as well, when the biofilm in the wells of microtiter plate was treated with depolymerase, though disintegration of biofilm was observed, but the bacterial counts in the suspension remained high since dislodged biofilm bacteria were not killed. When these bacteria were passaged in normal media, after 2-3 subcultures bacterial cells produced colonies with normal morphology having abundant slime secretion around it, thus suggesting the temporary nature of the change induced by the enzyme. As noted earlier, dislodged biofilm bacterial cells can be a source of secondary infections, especially in the immunocompromised host (Ymele-Leki 2007). Hence, as suggested for endolysins (Fischetti 2005), for depolymerase also, an adjunct antimicrobial therapy is required that takes care of the dislodged bacteria. In the present study ciprofloxacin was applied in conjunction with bacteriophage depolymerase. When the antibiotic was applied concomitantly with the enzyme, slight increased ($10^4-10^5$ log cfu/mL) bacterial titers in suspension was observed, though, as expected these titers were less than the titer observed after depolymerase treatment alone ($\sim 10^8$ cfu/mL). However, when ciprofloxacin was applied after depolymerase, reduced titer of $10^2-10^3$ log cfu/mL was obtained in suspension hinting towards the increased sensitivity of dislodged bacteria to
Discussion

antibiotic. These results are in agreement with previous findings where the interaction *P. aeruginosa* with a bacteriophage has been demonstrated to reduce the effective dose of several antibiotics (Hagens et al 2006). The increased sensitivity of dislodged bacteria may be due to the production of aqueous channels in the bacterial cell envelope by the bacteriophage protein (Russel 1995) through which antibiotic could travel and get accumulated in cell interiors.

As noted earlier, one very important aspect of antibiotic treatment of biofilms is the induction of resistant variants that do not contribute substantially to the biofilm structure, but may help the bacteria to survive the unfavourable conditions such as presence of an antimicrobial. No substantial data are available that indicate the pathogenic potential of these resistant variants *in vivo*. Nonetheless, production of the resistant variants is highly undesirable as these may act as an extended source of infection and further diminish the usefulness of antibiotics. In the present study, exposure of *K. pneumoniae* biofilm to ciprofloxacin caused a high frequency of resistant variant production, despite a significant decrease in log bacterial counts. This phenomenon of production of resistant variants was not only confined to antibiotic treatment but was also observed with bacteriophage treatment. The frequency with which bacteriophage-resistant variants were produced was high, though lower than the frequency for antibiotic-resistant variant production. Hence, as suggested previously (Schoolnik et al 2004) both the treatments were applied in combination. After combination treatment, the production of resistant variants was significantly less compared to individual treatment. This observation is of special interest, as it endorses our previous suggestion (Bedi et al 2009) that the combination of an antibiotic and bacteriophage works better than the two antimicrobials alone. The bacteriophage-resistant variants produced during the treatment were eliminated by the antibiotic, while the antibiotic-resistant variants were susceptible to the bacteriophage action. This is in agreement with a similar observation made by Lu and Collins (2009) wherein the use of an engineered bacteriophage, in combination with an antibiotic, led to decreased frequency of resistant variant production.
As demonstrated previously (Anderl et al 2003) the trait of antibiotic resistance was not permanent, as cells regained susceptibility to ciprofloxacin after subculture. In the present study permanent resistance was achieved only after repeated exposure to the antibiotic. In agreement with previous observations, (Fung-Tome et al 1993, Drenkard and Ausubel 2002), the antibiotic-resistant variant (KPARV) thus generated showed increased resistance not only to ciprofloxacin but also to other antibiotics. Hence, treatment of biofilm-associated infections using an antibiotic alone is not advisable as it selects resistant variants, increasing the microbial load in nosocomial settings. However, in case of bacteriophage resistant variant (KPØRV), the trait of resistance was permanent and remained so even after repeated subcultures. The bacteriophage-resistant variant (KPØRV) however showed an increased susceptibility to all the antibiotics tested. Moreover, the potential of cell variants (KPARV and KPØRV) to produce biofilms was significantly less than the parent bacteria. The reason for this anomaly could be the presence of smaller amounts of cell-associated CPS, which, in the case of *K. pneumoniae*, acts as an initial surface binder that helps the organism to cling to surfaces and form biofilms (Balestrino et al 2008). Due to the difference in the CPS content of the resistant variants, an altered morphology was observed under electron microscope. SEM demonstrated a drastic change in the extracellular polysaccharide substance associated with the resistant variants. The change was observed not only with CPS but also with the OMP profile. No significant difference in the OMP pattern of KPØRV and KPB5055 was observed. However, the OMP profile of KPARV was drastically different compared to that of KPB5055 and KPØRV.

When the resistant variants isolated during the study and bacterial culture, obtained after depolymerase treatment, were subjected to phagocytosis, a significantly higher rate of killing was observed compared with the parent strain. The increased phagocytic uptake and killing could be attributed to the loss of the bacterial capsule (Chhibber et al 2003). The depolymerase enzyme secreted by the bacteriophage is known to degrade the bacterial capsule, thus making it susceptible to phagocytic attack, which in turn may prevent the formation of
resistant variants during combination treatment, \textit{in vivo}. This underlines the importance of selecting bacteriophages for therapeutic purposes with utmost care. Only lytic bacteriophages whose safety and efficacy has been demonstrated should be selected. The presence of bacteriophage-associated depolymerase enzyme would be an added advantage. The enzyme may facilitate the spread of bacteriophage and aeration of the interiors of biofilm, increasing the efficacy of combination treatment and decreasing the emergence of resistant variants.

Though efficacy of bacteriophage against biofilm has been documented variously, but the mechanism behind bacteriophage action still remains obscure. Not many techniques are available that can demonstrate the interaction between biofilms and bacteriophages. Culturing of dispersed biofilm cells and viability staining are the most widely used methods employed for evaluating the effect of antimicrobials on biofilm (Briandet et al 2008). However, from these techniques the mechanism of action of antibiofilm agents cannot be deduced. Visualization of biofilm, in-situ, during antimicrobial treatment would provide a valuable information about the mechanism of antimicrobial action. Confocal scanning laser microscope is a technique that has the advantage of processing wet samples in their native form, making the examination of antimicrobial-pathogen interaction possible (Stoodley and Stoodley 2009). In the present study an effort in combining the advantage of CSLM, to visualize the biofilm non-destructively; along with the viable cell count method to assess the adjuvant effect of bacteriophage and ciprofloxacin, was made. An unspecific stain such as FITC, labels organic matter in general (Schmid et al 2003) making the florescence from the stained biofilm preparation an indicator of the microbial concentration on a given surface (Surmana et al 1996). With increase in age an increase in extracellular matrix associated with bacterial colony and a corresponding increase in its surface area, was observed. However after treatment with bacteriophage, degradation of biofilm and reduction of microcolony size was observed. Similar results were obtained in the present study after biofilm was stained with crystal violet. The two types of cells observed after crystal violet
staining of the phage treated biofilm were of variable viability. Majority of the cells were light blue while a fraction of cells were stained dark violet. Upon bacteriophage action cell wall of bacteria is disrupted and its contents comes out due to which cells do not take-up the stain properly and are stained light compared to viable cells that stain dark violet.

With age biofilm heterogeneity increases that may be responsible for ineffectiveness of ciprofloxacin treatment, leading to non-significant decrease in bacterial numbers. However, using CSLM we could demonstrate the breakup of matrix with depolymerase producing bacteriophage. Hence, increased efficacy of ciprofloxacin obtained upon combination with bacteriophage suggested a mechanism where antibiotic is able to reach the susceptible cells through the channel formed in the matrix by the bacteriophage, as has been suggested earlier (Hughes et al 1998).

Hence, present study, in line with other studies clearly indicates that some of the most important obstacles to biofilm control by antimicrobial agent, such as antibiotic tolerance, presence of biofilm extracellular matrix and effect of biofilm age might be overcome by bacteriophage treatment. However, there are several important characteristics of bacteriophages that should be considered carefully before application of bacteriophage to control clinically relevant biofilms. The high degree of specificity shown by bacteriophages can be a drawback for the bacteriophage therapy. However, reports of bacteriophages infecting multiple bacterial strains and manipulation of phage genes to express tail spikes that can detect multiple receptors on different bacterial surfaces has diminished the concern of narrow host range in bacteriophages. Moreover, application of bacteriophage will not eradicate the normal flora, a characteristic lacking in antibiotic therapy due to which a faster recovery after bacteriophage therapy, especially in immunocompromised host is anticipated.

Further, bacteria and their corresponding lytic phages co-evolve and establish equilibrium between their respective populations. However, rates of bacterial phage-resistance are low and usually range between $10^7$ per cell.
Discussion

(Lenski 1988). In the present study also the extent of production of phage resistance was far less than the antibiotic resistance. Moreover, considerably less population of phage resistant variants that appeared during phage treatment could be eradicated by concomitant application of antibiotic to which the resistant variants showed increased sensitivity. Besides, the results of the present study also support a previous observation where phage resistant variants showed slower multiplication rates than the sensitive population and soon the resistant variants got eliminated by the overgrowth of sensitive population (Lenski 1988). Another important question about bacteriophage therapy is the immune response elicited by the host system as phages are immunogenic in nature. However, several studies indicate that the antibody response to phage is very weak except where high serum antibody titers were present before bacteriophage treatment (Kallings 1961; Kucharewicz and Slopek 1987). Nonetheless, it might be possible to design mutant phages that resist the clearance by the host immune system (Donlan 2009). Moreover, as phages have been isolated from human gut (Gorski and Weber-Dabrowska 2005) and as humans are continuously exposed to bacteriophages in the environment (Sulakvelidze and Kutter 2005); there is no doubt about safety of phages for human use.

The persistence of healthcare-associated infections, many of them device associated, along with rise in antimicrobial resistance continue to guide the chase for development of newer antimicrobials especially against the devise associated biofilm related infections. The biofilm associated infections require the most effective treatment that will not only prevent the biofilm formation but also eradicate the already formed biofilms. Further requirement for antibiofilm agent will be its ability to target extracellular matrix component of the biofilm, effectiveness against cells of different ages and help to avoid the overuse of chemical antimicrobials. Bacteriophages can potentially meet these criteria. Phage cocktails and its combinations with antibiotics, can be developed that will help in restricting the emergence of resistance among bacterial population, as has been documented in the present study where combination of bacteriophage and antibiotic most significantly reduced the emergence of resistant variants in comparison to individual treatments. Moreover, new bacteriophages can be readily isolated from the environment and phage could be engineered genetically for greater efficacy against biofilms.