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Biofilms are microbial aggregations that are attached irreversibly to a solid surface and are encased in a matrix secreted by the resident organisms themselves. Microorganisms in the biofilm are known to show variable gene expression within the biofilm and exhibit altered phenotypes compared with the corresponding planktonic cultures (Hall-Stoodley et al 2004). It has been speculated that more than 65% clinical infections are caused by bacteria in their biofilm state because this mode of growth not only provides a safe haven for the microbial population but also help bacteria in developing inheritable resistance to antimicrobials. Hence understanding biofilms in clinical settings is important since human micro flora such as that present on the human skin, oral cavity, genitourinary tract, gastrointestinal tract and external environment, or that is found in the infected tissue, may colonize the indwelling medical devices and form biofilms, especially in the immunocompromised hosts (Donlan and Costerton 2002).

Biofilm development occurs in different sequential phases. First and foremost is the conditioning of the surface. Surfaces of attachment are conditioned by adsorption of organic and inorganic nutrients that influence subsequent bacterial attachment (Donlan 2002). Initial bacterial attachment is reversible and is mediated by transport of bacterial cells to a surface by sedimentation and Brownian motion of the microbial cells, convection currents within the bulk liquid, transporting bacteria to the surface, active movement by motile bacteria, or by electrostatic and physical interactions between the bacterial cell surface and substratum (van Loosdrecht et al 1990; Flemming et al 1998). Further, cells attach irreversibly to the surface, secrete EPS and develop cell-to-cell bridges (Hall-Stoodley et al 2004). The final stage in biofilm establishment is the surface colonization (Zottola and Sasahara 1994).
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The organisms found most commonly in the biofilms include the coagulase negative staphylococci, Staphylococcus aureus, Enterococcus faecalis, Enterococcus faecium, Escherichia coli, Proteus mirabilis, Klebsiella pneumoniae, Viridans streptococci, Pseudomonas aeruginosa and Acinetobacter spp. (Donlan 2005 and 2008). Out of these, Klebsiella strains are important cause of nosocomial infections, particularly Klebsiella pneumoniae that is responsible for approximately 8% of all nosocomial infections (Emori & Gaynes 1993). K. pneumoniae infections can occur in almost all body sites, but the highest incidence is in the urinary and the respiratory tracts. The main populations at risk are neonates, immunocompromised hosts, and patients predisposed by surgery, diabetes, malignancy, etc. (Emori and Gaynes 1993; Hansen et al 1999; Herva’s et al 1993). The existence of multiple antibiotic-resistant K. pneumoniae strains is well known. These resistant strains of Klebsiella produce extended-spectrum beta-lactamses and infection caused by them lead to high mortality rates of about 15% (Jacoby 1994). Various virulence factors that are associated with Klebsiella pathogenesis include, production of capsule that mediates protection against phagocytosis and the bactericidal effects of serum (Domenico et al 1994), siderophores that capture the host iron and type 1 and type 3 fimbriae that allow the bacteria to bind to host structures (Tarkkanen et al 1997; Wurker et al 1990). Recently, biofilm formation has been demonstrated to be an important virulence factor that contributes substantially to its surface associated infections such as that of urinary tract (Jagnow and Clegg 2003).

The production of bacterial biofilms appears to be dependent on multiple genetic factors as the expression of some genes in sessile, biofilm-producing bacteria may be different to that of planktonic cells (Whitely et al 2001). Hence, surface components of the bacterial cell that increase the efficiency of biofilm formation are likely to play a significant role in the establishment of infection by pathogens (Jagnow and Clegg 2003). One such bacterial surface component is capsule. The vast majority of K. pneumoniae isolates express pronounced polysaccharide capsule covering the entire bacterial surface, resulting in a
characteristic mucoid phenotype, when grown on agar plates. The formation and maintenance of structured multicellular communities critically depends upon the production of extracellular substances that, in association with each other, constitute an extracellular matrix (Sutherland 2001). This ability to form the extracellular matrix appears to be a common feature of biofilms, though diversity in the means of production of these matrices may exist.

The existence within a biofilm represents a basic survival mechanism of microorganisms in which the organisms are protected not only from environmental onslaught such as drying and desiccation, but also from antimicrobials. Biofilms are inherently resistant to antimicrobial action as even the sensitive bacteria that do not have genetic basis for resistance can have profoundly reduced susceptibility when present in biofilm state (Anderl et al 2000). This fact is supported by the observation that biofilm bacteria once dispersed, readily regain the susceptibility to antibiotics (Anwar et al 1989; Williams et al 1997). Hence, it has been suggested that certain biofilm specific resistance mechanisms must be playing a role in antimicrobial resistance. Three mechanisms have been proposed to explain the general resistance of the biofilms to biocidal agents. The first is the barrier properties of the slime matrix. This mechanism is more relevant for reactive (superoxide), charged (metal) or large (immunoglobulin) antimicrobial agents that are either neutralized or bound by the matrix exopolysaccharide (EPS). As a result these antimicrobials are diluted to sublethal concentrations before they can reach the individual bacterial cells within the biofilm. However, certain antimicrobials of the size of antibiotics are shown to diffuse freely through the EPS, provided these are not inactivated by the matrix (Anderl et al 2000) suggesting some other methods employed by the biofilm for antimicrobial resistance.

The second protective mechanism involves the physiological state of biofilm organisms. The creation of starved, stationary phase dormant zones in the biofilm has been documented to be a significant factor in the resistance of biofilm bacterial population to antimicrobials (Spoering and Lewis 2001; Walters et al 2003). This is true for antibiotics that act on cell wall, though almost all
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Antibiotics need some bacterial metabolic activity to be effective. The β-lactams that act by affecting the cell wall synthesis are especially affected by the starved conditions in the biofilm, as these conditions make the bacteria docile and hence refractory to antibiotic. Third and most speculative mechanism of antibiotic resistance is the presence of a subpopulation of micro-organisms in a biofilm that form a unique and highly protected phenotypic state that persist in the biofilm even after prolonged and continued exposure to the antibiotic (Stewart and Costerton 2001).

Though, due to various mechanisms of antimicrobial resistance, eradication of biofilms is extremely difficult, still four main interventions in clinical practices for biofilm-associated infections have been suggested (Aslam 2008). The first strategy is to prevent initial device contamination by maintaining optimal aseptic techniques. Second step involves the minimization of initial microbial cell attachment, for example, by use of antimicrobial coated catheters (Darouiche 1999). Third strategy is for established infections where agents that can penetrate the biofilm matrix and kill the embedded organisms are used. Last resort is the removal of the colonized device or surgical intervention (Aslam 2008) both of which are traumatic to the patient.

All the mechanisms of biofilm resistance depend on its multicellular nature (O'Toole et al 2000); hence dispersion of multicellularity of the biofilms can have a detrimental effect on the antimicrobial recalcitrance of the biofilms (Stewart and Costerton 2001). Since, antibiotics are not capable of breaking the biofilm structure; other strategies have been looked into. One strategy with promise is the application of bacteriophages. Bacteriophages or simply phages are obligate bacterial parasites that use host machinery for its own replication resulting in ultimate lysis and hence killing of the bacterial host. Lytic bacteriophages have been demonstrated variously to be used for the eradication of bacterial infections in plants (Fox 2000) and animals (Barrow et al 1998; Chhibber et al 2009; Kumari et al 2009; Malik and Chhibber 2009). Bacteriophage therapy has also been performed in humans for the treatment of different infectious diseases caused by various organisms such as *Staphylococcus*, *Streptococcus*, *E. coli*. *P.*
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*aeruginosa, Shigella* and *Salmonella* (Sulakvelidze 2001). Moreover, use of bacteriophages for the treatment of bacterial infections could reduce the use of antimicrobial agents thereby limiting the spread of antimicrobial resistant organisms as indiscriminate application of antimicrobials in healthcare has selected for resistant organisms (Tenover et al 2001; Sieradzki et al 2003).

There is also an evidence for the potential of bacteriophage for biofilm control (Hughes et al 1998; Donlan 2009). Bacteriophages have been demonstrated to be effective under all conditions of bacterial physiology that otherwise tend to be inhibitory for the antimicrobial action against biofilms (Stewart et al 2004; Ito et al 2009) and if at all effective, very high concentrations of antimicrobials are required for prolonged periods to achieve biofilm reduction or eradication (Ceri et al 1999). On the other hand it has been demonstrated (Doolittle et al 1996) that a single dose of bacteriophage could treat a biofilm-related infection. Moreover age related biofilm recalcitrance does not affect the action of bacteriophage (Hanlon et al 2001).

Furthermore, bacteriophages produce polysaccharide depolymerases that have the potential to degrade the biofilm EPS matrix. Few studies have demonstrated the effect of depolymerase on biofilms. Hanlon et al (2001) reported the diffusion of a *P. aeruginosa* bacteriophage through alginate and that phage-free suspension containing only depolymerase reduced the viscosity of the alginates and EPS of *P. aeruginosa*. Also, Lu and Collins (2007) demonstrated the efficient removal of the biofilms by depolymerase containing bacteriophage and not by the non-depolymerase bacteriophage, thus highlighting the important role played by depolymerase in bacteriophage infection and biofilm eradication. Moreover, the grand majority of bacteria living in our bodies are beneficial or innocuous and these bacteria are killed indiscriminately by antibiotics, exposing tissues to colonization by potentially harmful bacteria. Therefore, the ability of bacteriophages or their components to selectively target pathogenic species of bacteria represents an important advantage over the antibiotics.
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Some of the most important obstacles to biofilm eradication by antimicrobial agents, such as antimicrobial tolerance, extracellular matrix of biofilm, and age related antimicrobial resistance shown by biofilm might be overcome by the application of bacteriophage. However, there are several issues that need to be addressed before considering application of bacteriophages or their components for treatment of medically important pathogens in clinical settings. Some of these concerns involve the lack of information about the *in vivo* kinetic of the bacteriophage action, the potential of development of host resistance to bacteriophages, whether bacteriophages can be applied synergistically with antibiotics and if so what is the mechanism of synergism between the bacteriophage and the antimicrobial agent.

To address some of these issues, present study was undertaken with the following aim and objectives.

**Aim:**

Production, Purification and Characterization of depolymerase enzyme produced by a *Klebsiella pneumoniae* specific bacteriophage and its potential in eradicating biofilms of *Klebsiella pneumoniae*.

**Objectives:**

1) Isolation and selection of appropriate depolymerase producing bacteriophage.

2) Characterization of bacteriophage on the basis of:
   a) Structural protein analysis.
   b) Ultra microscopic structure.
   c) Genetic characterization.

3) Production and purification of specific enzyme.

4) Biophysical and Biochemical characterization of purified enzyme.

5) Development and standardization of biofilm assay for *Klebsiella pneumoniae*.

6) Demonstration of efficacy of enzyme alone or in conjunction with phage and/or antibiotic in eradicating the biofilms.