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Original Research Article

Microbiology for Better Medical Care

Synergistic Effect of Antibiotics and Enzymes as Strategies for Combating Biofilm Formation by *Pseudomonas aeruginosa* PAO1

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Abstract: Biofilms are surface attached communities of bacteria, fungi, protozoa, and many other microorganisms. Potential biofilm formers are known as "ESKAPE" which includes *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter aerogenes* . Among them, *Pseudomonas aeruginosa* is considered as the most health injurious biofilm forming organism that causes nosocomial infections like ventilator associated pneumonia, chronic wound infections, chronic rhinosinusitis etc. Efficient removal of biofilm from medical devices is a big challenge to avoid hospital acquired infections as these devices are delicate and cannot be reprocessed using harsh chemicals or high temperature. Therefore, use of mild solutions for removal of biofilm is advisable. In the present study, *Pseudomonas aeruginosa* PAO1 acted as potential biofilm former with 10% inoculum size in TSB medium. The biofilm of *P.aeruginosa* PAO1 was studied microscopically and the results revealed that with time the number of cells increased and thick biofilm formation was observed with more Exopolysacharide production. Susceptibility of *P. aeruginosa* PAO1 against some antibiotics and enzymes were analyzed individually as well as in combinations using microtiter plate. The efficiency of antibiotics to eradicate biofilm was higher than the enzymes but the use of antibiotics alone required higher concentration to eradicate biofilm. While combination of enzymes and antibiotics can eradicate the biofilm at sub minimal concentration as well as that can minimize the load of antimicrobials in the environment. Therefore, in this study when combination of ciprofloxacin at sub MIC of 1.56 µg/ml was applied with lysozyme and protease, 68±0.5% and 56±0.6% biofilm eradication were observed, respectively but only 40±0.5% eradication was observed when treated with ciprofloxacin alone. Similarly, when combination of levofloxacin at sub MIC of 6 µg/ml was applied with DNase, 95% eradication was observed while only 32±0.8% eradication was observed when treated with levofloxacin alone.

Keywords: Biofilm, Antibiotic, Enzyme, Minimum Inhibitory Concentration (MIC), Drug resistance

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1. INTRODUCTION

In the majority of niches bacteria live in the form of biofilms on various support materials and the planktonic single cell state is considered as a transition phase. The shift of microorganism from the planktonic growth to the biofilm is dependent on the production of adhesions and extracellular matrix components[1]. The microbial cells are attached to self-produced extracellular matrices containing polymeric substances like polysaccharides, proteins, lipids and DNA[2]. The formation of biofilm begins with attachment to a suitable surface then followed by aggregation, multiplication, secretion of extra, intra and intercellular communication molecules, like quorum-sensing (QS) signaling molecules and cyclic-di-GMP, which starts extracellular polymeric substance (EPS) production[3-4]. Production of EPS gives mature and rigid biofilm. Due to biofilm formation different industrial, environmental and health problems occur which can lead to loss of product quality and shelf-life of products. In the industrial sector, biofilm formation can create many negative impacts like reduced operational efficacy in heat exchangers, increased operational pressure in desalination plants, increased energy consumption, increased metal corrosion and blockage of pipes in food and dairy industries[5-6]. In the medical sector, it was reported that about 65% of all bacterial infections are associated with bacterial biofilms[7]. Furthermore, biofilm communities' enhanced antibiotic resistance is 1,000-fold greater than those of planktonic cells[8-10]. According to CDC, the term "ESKAPE" was introduced, it includes six pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter aerogenes*) which escape the effects of antibiotics[11]. Among them, *P. aeruginosa* is a well recognized opportunistic pathogen in immunocompromised patients[12], and it gave a mortality rate as high as 50%[13]. In the 21st century, when rate of highly susceptible immunocompromised patients was increased in most of the countries, *P.aeruginosa* has played a leading role in hospital borne infections[14]. It is a motile, non-fermentative, gram negative bacteria, which can cause respiratory infections, urinary tract infections, gastrointestinal infections, keratitis, otitis media and bacteremia in patients with compromised host defenses such as cancer, burn, HIV and cystic fibrosis (CF), etc[14]. The biofilm matrix of *Pseudomonas aeruginosa* mainly consists of polysaccharides, proteins, extracellular DNA and lipids and the composition may differ from strain to strain, depending on the growth conditions and the age of the biofilm[15]. Furthermore, presence of extracellular DNA (eDNA) functions as an important matrix component in *P. aeruginosa* biofilms [1,16]. As a solution, disinfectants and antibiotics have been used vigorously to control and eradicate this bacterial biofilm in industries and hospitals. However, pathogens in biofilms, with their altered microenvironment, can easily resist conventional antibiotics and antibiofilm agents. Disinfectants and chlorine-based sanitizing agents are the common practices in food and dairy industries. Nevertheless, these chemicals secrete toxic residues to the environment and also cause metallic erosion of applied equipment and processing surfaces [17-18]. To overcome these problems and to increase the quality and safety of food products, researchers have studied the basic mechanism of biofilm dispersal. They observed that at the final stage of biofilm formation, the embedded cells return to their planktonic phase by self-denaturation through endogenous extracellular enzymes [19-20]. Thus, microbial derived enzymes can be useful in every stage of biofilm detachment and degradation[21]. Moreover, clinical and *in vitro* evidences suggest that certain antibiotics (trimethoprim sulfamethoxazole, ampicillin, nalidixic acid, gentamicin, nitrofurantoin, cephalexin [Kelfex], tobramycin, amoxicillin) eradicate the planktonic organisms from the urine but do not eradicate the biofilms that adhere to body cells and devices [22-23]. Therefore, as a solution, we have attempted to illustrate the enzymatic approaches used against pathogens with combination to antibiotics. In addition, we studied the synergistic effect of enzyme and sub- MIC of antibiotic for biofilm eradication of *Pseudomonas aeruginosa* PAO1 .

2. MATERIALS AND METHODS

Tryptic Soy Broth (TSB), Luria Bertani (LB) and Nutrient Broth (NB) media were used in the study, Antibiotics- Ciprofloxacin, Levofloxacin, Ofloxacin, Ceftriaxone and Chloramphenicol were selected on the basis of their broad spectrum nature and effectiveness on gram negative bacteria. Enzymes- Protease, Lysozyme, Amylase and Lipase were procured from Hi media Labs., Mumbai, India. Reagents: Crystal Violet, Methanol, Glacial acetic acid were procured from Loba Chemie, Mumbai, India. All the chemicals are of analytical grade.

2.1 Bacterial Strains

The bacterial strain *Pseudomonas aeruginosa* PAO1 (MTCC 3541) was procured from MTCC Chandigarh.

2.2 Optimization of inoculum size for biofilm formation

To optimize inoculum size for biofilm formation, *Pseudomonas aeruginosa* PAO1 was grown in TSB media. The actively growing culture having 0.5 O.D at 600 nm was inoculated with variable inoculum size ranging from 2%, 4%, 6%, 8%, 10%, 12% in TSB broth in each well. The microtiter plate was incubated for 24 h at 37 °C and the biofilm was formed on 96 well polystyrene microtiter plates in triplicates. After the aspiration of planktonic cells, biofilms were washed twice with phosphate buffer saline and air-dried. Then, fixed with methanol and 200 µl of crystal violet solution (1%) was added to all wells. After 15 min, the excess crystal violet was removed and plates were washed twice and air-dried. Finally, the cell-bound crystal violet was dissolved in 33% acetic acid and biofilm potential was measured at O.D 595 nm using microplate reader EPOCH 2c (Biotek Vermont USA)[24].

2.3 Determination of biofilm forming potential using different media

In 96 well polystyrene microtiter plates, 200µl sterile NB, LB, TSB media were added in triplicates. Media containing each well was inoculated with 10% *Pseudomonas aeruginosa* PAO1 culture having 0.5 O.D at 600nm and incubated at 37 °C for 24 h. The abiotic controls were also maintained parallelly. After incubation, staining procedure was carried out using methanol as fixative followed by washing with phosphate buffer saline and at last staining with 1% crystal violet; as described earlier. Cell density was measured at 595 nm in ELISA reader by adding 33% glacial acetic acid[24].

2.4 Macroscopic and microscopic observation of P.aeruginosa PAO1 biofilm on glass slide

Four sterile glass bottles were filled with 200 ml sterile TSB medium containing one sterile glass slide in each bottle. These glass bottles were inoculated with 10% of actively growing culture and incubated at 37 °C for 24 h under shaking condition at 100 rpm. At every 24 h time interval glass slide from one bottle was removed under aseptic condition with the help of sterile forceps. Then the biofilm which were developed on the glass slides were stained as per the method described earlier[24]. The stained slides were visualized directly and photographs were taken and further they were observed under light microscope at 100X magnification.

2.5 Determination of minimum inhibitory concentration of antibiotics

The Minimum Inhibitory Concentration (MIC) of antibiotics was determined by the tube dilution method as previously described by the Clinical and Laboratory Standards Institute[25]. To determine MIC of antibiotics, 2 ml of sterile TSB medium was filled in each text tube and inoculated with 10% actively grown culture of *P.aeruginosa* PAO1 having 0.5 O.D at 600 nm (App. 1*107 CFU/ml). As per CLSI guideline, ciprofloxacin, levofloxacin, ofloxacin and ceftriaxone stock concentrations were prepared and they were serially diluted till least concentration. The tubes were then incubated at 37°C for 24 h and then further examined for inhibition of growth at Minimum Inhibitory Concentration and Sub MIC concentration[26].

2.6 Determination of Minimum Biofilm Inhibition Concentration (MBIC) of Enzymes

Minimum Biofilm Inhibition Concentration (MBIC) is the lowest concentration of the agent which can damage the biofilm structure. To determine the MBEC concentration, 96 well microtiter plates filled with 200 µL of TSB media were further inoculated with 10% of actively grown culture of *P.aeruginosa* having 0.5 O.D at 600 nm. Different enzymes (protease, lysozyme, amylase and lipase) stock samples were prepared at a concentration of 1000 µg/ml while for DNase stock concentration was 40 µg/ml. They were serially diluted and then added to each well at a volume of 200 µL. The plates were incubated at 37 °C for 24 h. After incubation, the wells were washed with sterile phosphate buffer saline to remove non-adherent cells. Further, it was treated with methanol and stained with crystal violet. Culture suspension and medium served as the positive control. The cell density was measured at 595 nm in the ELISA reader by adding 33% glacial acetic acid[24].

2.7 Synergistic biofilm inhibitory assay

Interference of *Pseudomonas aeruginosa* PAO1 biofilm formation upon treatment with antibiotics alone or in combination with enzymes was performed as the method described earlier, with minor modifications[27] The 200µl TSB is supplemented with antibiotics (ciprofloxacin, levofloxacin, ofloxacin, ceftriaxone), with selected enzymes (protease, lysozyme and DNase) and with combination of antibiotics and enzymes at their sub-MIC levels. In this study, among five three enzymes (protease, lysozyme and DNase) were selected on the basis of their biofilm inhibition efficacy. The actively grown culture having 0.5 O.D at 600 nm was inoculated (10%) to the broth. Further, the microtiter plate was incubated at 37°C for 24 h. After incubation, the plate was washed, stained with 1% crystal violet as described above and then the absorbance at 595 nm was measured by ELISA reader. Further, percent biofilm reduction was calculated.

3. STATISTICAL ANALYSIS

All sets of experiments were performed three times, with triplicate samples per trial and results were calculated as average, standard deviation using Microsoft excel programme (Microsoft office 2007), data was analyzed using student's t-test and statistical significance was calculated as $p< 0.05$.

4. RESULTS AND DISCUSSION

4.1 Determination of biofilm formation potential of P.aeruginosa PAO1 at variable inoculum size

The biofilm potential of *Pseudomonas aeruginosa* was assessed using 96-well microtiter plate. Upon 24h incubation, with increase in inoculum size, cell density was increased and it was observed that biofilm forming potency was observed maximum at 10% inoculum size. However, with further rise in inoculum size, no rise in biofilm formation potential observed (Figure 1). In order to determine biofilm formation potential, crystal violet dye is used, which is basic in nature and that binds non-specifically to negatively charged surface molecules such as cells, polysaccharides, LPS, environmental DNA in the extracellular matrix. Therefore, this basic dye is used to evaluate biofilm formation[28].

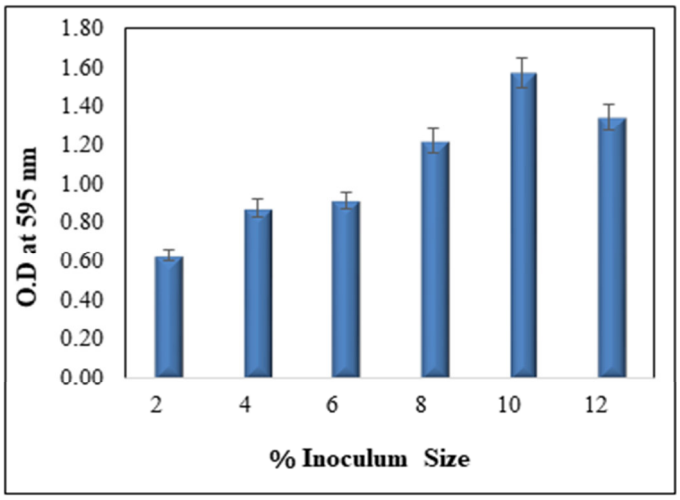

Fig 1. ***Determination of Inoculum Size for Potential Biofilm Formation***

4.2 Determination of biofilm forming potential of P. aeruginosa PAO1 using different media

The biofilm forming potential of *Pseudomonas aeruginosa* PAO1 was assessed using different media such as LB, NB and TSB. Among them, with 10% inoculum size maximum biofilm formation was observed in TSB medium while moderate turbidity was observed in LB and NB media (Figure 2). Similarly, *P. aeruginosa* randomly selected 45 isolates were screened for their ability to form biofilms using 96-well microtiter plates with four different media namely TSBG, Mueller–Hinton broth (MHB), brain-heart infusion broth (BHIB) and LB. Among them 44% isolates have shown strong biofilm formation in TSB media[29]. Likewise, *Pseudomonas aeruginosa* growth was observed stable and maintained at 10^{10}–10^{11} CFUs/mL in the LB, TSB and Dulbecco's Modified Eagle's Medium (DMEM) tested except for Synthetic Cystic Fibrosis Sputum Medium 2 (SCFM2), in which the strain reached to maximal growth at 10^{9} CFUs/mL[30]. In contrast, highest biofilm formation was found in Luria bertani broth (LB) in comparison to Nutrient broth (NB) and Tryptone water with 10% inoculum size in case of *S. aureus*[31]. Similarly, the biofilm formation was also studied using Luria bertani broth (LB) for different pathogenic strains of *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 6358P, *Pseudomonas aeruginosa* ATCC 15442, *Bacillus cereus* ATCC 11770, *Listeria monocytogenes* MTCC 1143[32].

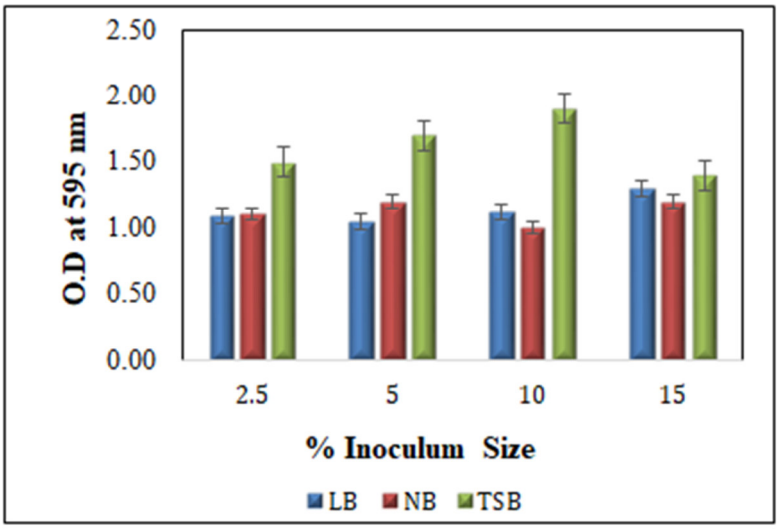

Fig 2. ***Biofilm Formation Using Different Media***

4.3 Macroscopic and microscopic observation of biofilm of P. aeruginosa PAO1 on glass slide

Pseudomonas aeruginosa biofilm formation was allowed to grow on the glass slide dipped in TSB medium. At different time intervals (24 h, 48 h, 72 h and 96 h), the glass slide was removed and stained with 1% crystal violet. The results have indicated that turbidity increased with time and density of biofilm formation was also increased with time and that is depicted in macroscopic observation of the slide (Figure 3). Likewise, biofilm formation of *Pseudomonas aeruginosa* was studied on the slide and staining was carried out with tetrazolium dye instead of crystal violet[33]. Similar line of work was reported for certain pathogenic strains which were grown on the silicon tubes and biofilm formation was evaluated using crystal violet dye[30]. The surfactant produced by *Pseudomonas putida* BD2 strain was evaluated to determine its anti-adhesive activity and for the same, bacterial biofilm was prepared and stained with crystal violet [34].

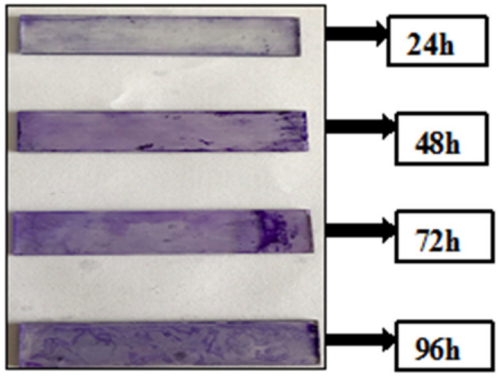

Fig 3. ***Macroscopic Observation of Pseudomonas aeruginosa* PAO1 *Biofilm on Glass Slide***

Pseudomonas aeruginosa biofilm formation was also observed microscopically under 100X magnification. As shown in Figure 4 bacterial biofilm formation was increased with time (24 to 96 h). The glass surfaces presented a high intensity of biofilm formation, compared to the stainless-steel surfaces, which may be due to higher electric charge of the glass surface[35].

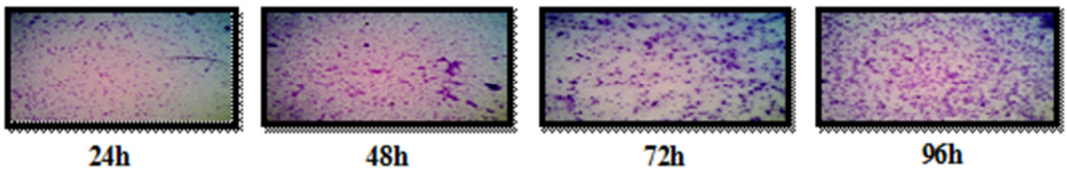

Fig 4. ***Microscopic Observation of Pseudomonas aeruginosa* PAO1 *Biofilm (100X Magnification)***

4.4 Determination of minimum inhibitory concentration of antibiotics

MIC determination against *Pseudomonas aeruginosa* PAO1 was carried out using tube dilution method. The MIC of ciprofloxacin and ceftriaxone was 3.12±0.05 and 3.12±0.25µg/ml respectively. MIC of ofloxacin and levofloxacin was 12±0.15 and 12±0.50µg/ml respectively while MIC value for chloramphenicol was 50±0.15µg/ml (Table I). Similarly, forty different strains of *Pseudomonas aeruginosa* were studied against different antibiotics and MIC of ciprofloxacin, levofloxacin and moxifloxacin was 6 µg/ml, 1.5 µg/ml and 4 µg/ml respectively[36].

Table I *MIC of Antibiotics*

Antibiotic	MIC (µg/ml)
Chloramphenicol	50±0.15
Ciprofloxacin	3.12±0.05
Levofloxacin	12±0.50
Ofloxacin	12±0.15
Ceftriaxone	3.12±0.25

4.5 Determination of Minimum Biofilm Inhibitory Concentration (MBIC) of Enzymes

Inhibitory concentration of various enzymes (amylase, protease, lysozyme, DNase and lipase) against *Pseudomonas aeruginosa* PAO1 was determined in 96 well microtiter plate using microdilution method. *P. aeruginosa* biofilm inhibition was observed for enzymes amylase, protease, lysozyme and lipase at 500 µg/ml concentration and for DNase at 10 µg/ml concentration. Maximum eradication 66±0.6% was observed with protease enzyme, 59±0.5% was observed with DNase and moderate eradication 53±0.5%, 53±1% and 46±0.5% were observed with lysozyme, amylase and lipase, respectively (Figure 5 to 9). It was reported that proteolytic enzymes such as trypsin and papain gave 20% to 30% eradication of biofilm at concentration of 100µg/ml, whereas proteinase k has given 56% eradication at the same concentration[29]. Similar type of study was carried out for biofilm eradication of *Pseudomonas aeruginosa* and *Salmonella* Typhi, 43.83% and 61.68% biofilm eradication were observed respectively, using purified alpha-amylase enzyme[37]. On the similar lines, 90 to 95% *P. aeruginosa* biofilm disruption using *Oceanobacillus* spp. isolated lipase was reported[38]. Moreover, over expression of the extracellular lipases LipA and LipC, the esterase EstA and the proteolytic elastase LasB from plasmids revealed that some of these hydrolases affected the composition and physicochemical properties of the extracellular polymeric substances (EPS)[39]. These observations indicate different enzymes have different inhibitory concentration and they can eradicate biofilm of various microorganisms but their responses differ organism to organism. Moreover, use of high concentration of enzyme for biofilm removal is not economically favorable. Therefore, in the present study, sub MBEC concentration was considered as partial inhibitory concentration for DNase, lysozyme, and protease enzymes.

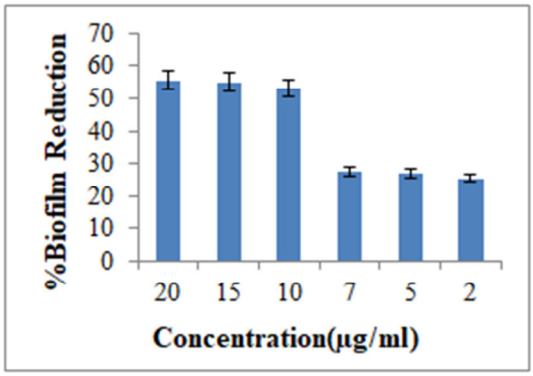

Fig 5. *Determination of Sub Inhibitory Concentration of DNase*

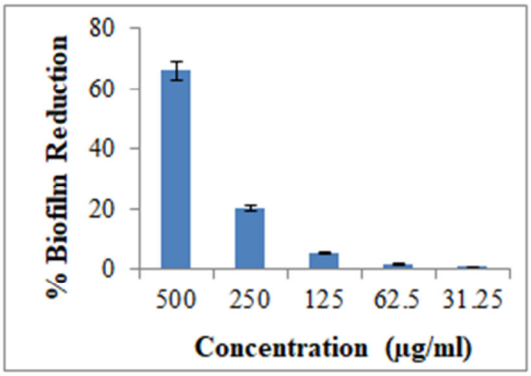

Fig 6 . *Determination of Sub-Inhibitory Concentration of Protease*

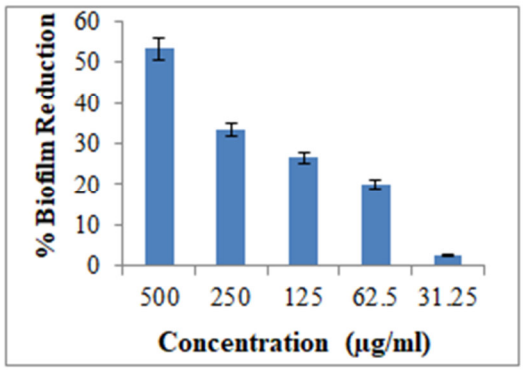

Fig 7. *Determination of Sub-Inhibitory Concentration of Lysozyme*

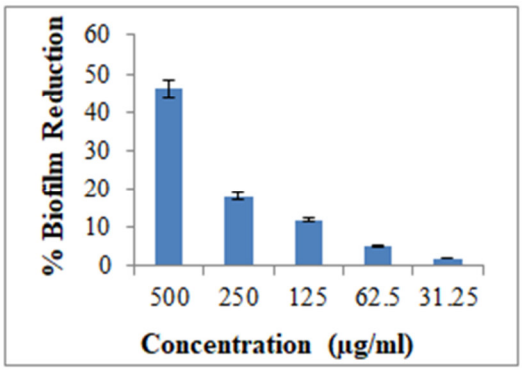

Fig 8. *Determination of Sub-Inhibitory Concentration of Lipase*

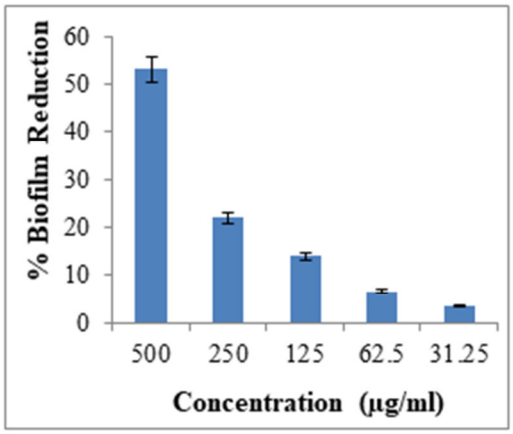

Fig 9. *Determination of Sub-Inhibitory Concentration of Amylase*

4.6 Synergistic biofilm inhibitory assay

Pseudomonas aeruginosa PAO1 biofilm eradication study was carried out using protease, DNase and lysozyme with different antibiotics. Selection of these three enzymes was based on their efficacy of biofilm removal among five different enzymes used in this study. Using partial inhibitory concentration of enzymes and sub-MIC concentration of different antibiotics in combination have shown efficient eradication of biofilm. In this study, biofilm removal was nearly 20±0.7% when treated only with protease enzyme. Whereas, when protease used in combination with chloramphenicol, ciprofloxacin, levofloxacin, ofloxacin and ceftriaxone antibiotics at their sub-MIC concentrations it has given 33±0.3%, 58±0.5%, 46±0.60%, 56±0.6% and 54±0.66% of biofilm eradication, respectively (Figure 10). The overall results suggest maximum eradication (58±0.5%) was observed in case of protease with ciprofloxacin. Similarly, the effect of α-mannosidase, β-mannosidase and trypsin enzymes on the degradation of *P. aeruginosa* biofilms was studied and simultaneously, the reduction of ceftazidime Minimum Biofilm Eliminating Concentration (MBEC) was also determined. The overall results have indicated that tested enzymes can destroy the biofilms and reduced the ceftazidime MBECs. However, only trypsin had no cytotoxic effect on A-431 human epidermoid carcinoma cell lines. This observation indicated that trypsin had better features than mannosidase enzymes and it can be a promising agent in combating *P.*

aeruginosa burn wound infections[40]. Likewise, protease (savinase, everlase and polarzyme) and amylase (Amyloglucosidase and Bacterial Amylase Novo) activity was tested on both biofilms of *Pseudomonas fluorescens* and on extracted EPS. After testing enzymes, biofilm integrity was evaluated by SEM, they found EPS composition consisted mainly of proteins and Everlase and Savinase were the most effective enzymatic treatments on removing biofilms and degrading the EPS[41]. Another study has reported 80-90% biofilm eradication of *S. aureus* using ciprofloxacin at MIC of 4 µg/ml with peptidase at 2500 µg/ml concentration[27]. Similarly, when Serratiopeptidase was used at 100 µg/ml with streptomycin at 25 µg/ml, they observed inhibition of *S. aureus* and *Klebsiella pneumoniae* biofilm[42]. Moreover, it was observed that co-administration of alginate lyase with gentamicin has increased the removal of biofilms of mucoid *P. aeruginosa* growing in respiratory tract[43]. Further, in our study when DNase was used in combination to chloramphenicol, ciprofloxacin, levofloxacin, ofloxacin and ceftriaxone antibiotics at their sub-MIC values it has given 81±0.8%, 90±0.7%, 95±0.5%, 79±0.5% and 91±1% of biofilm eradication, respectively. Whereas, only 59±0.5% biofilm removal was reported when biofilm of *P.aeruginosa* was treated with DNase alone (Figure 11).Therefore, the overall results suggest maximum eradication (95±0.5%) was observed in case of DNase with levofloxacin. Similar kind of observation using a combination of DNase and levofloxacin or rifampin was reported in various gram-positive and gram-negative bacteria and biofilms were reduced by 43 to 64%[44]. In one of the studies, the efficacy of trypsin, β-glucosidase, and DNase I enzymes on the degradation of dual-species biofilms of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in a wound-like medium was studied. The reduction of minimum biofilm eradication concentration (MBEC) of antibiotics, meropenem and amikacin was evaluated when combined with enzymes. The minimum effective concentrations of trypsin, β-glucosidase, and DNase I enzymes to degrade biofilms were 1 µg/ml, 8 U/ml, and 150 U/ml, respectively. Combination of 0.15µg/ml trypsin and 50 U/ml DNase I had a significant effect on *S. aureus*-*P. aeruginosa* biofilms which resulted in the dispersal and dissolution of all biofilms[45]. Similarly, DNase1L2 was found to be present in an enzymatically active form in the stratum corneum of human skin. In an *in vitro* assay, purified recombinant DNase1L2 efficiently suppressed the formation of biofilms by *P. aeruginosa* and *S. aureus*[46]. Similarly, in this study when *P. aeruginosa* biofilm was treated only with lysozyme enzyme, nearly 33.3% removal was observed. Whereas, when lysozyme used in combination with chloramphenicol, ciprofloxacin, levofloxacin, ofloxacin and ceftriaxone antibiotics at their sub-MIC values it has given 60±0.5%, 68±0.7%, 33±0.3%, 49±0.5% and 52±1% of biofilm eradication respectively (Figure 12). So, maximum eradication (68±0.7%) was observed in case of lysozyme with ciprofloxacin. Similarly, the effect of lysozyme on biofilm formation capacities of 16 strains of selected microorganisms (*Staphylococcus aureus, Streptococcus pyogenes, Pseudomonas aeruginosa* and *Gardnerella vaginalis*) was investigated. A lysozyme concentration of 30 µg/ml indicated to have the highest inhibiting effect on all tested microorganisms. In the same way the exposure of 24-h-old biofilms of *P. aeruginosa* isolates to lysozyme (30 µg/mL) and 50 times MICs of ceftazidime or cefepime resulted in a significant reduction in biofilm (49.3 %) as compared with the exposure to lysozyme or either antibacterial agent alone[47]. Comparable observation was reported in case *S. aureus* where 70-80% biofilm eradication observed when ciprofloxacin at MIC of 4 µg/ml used in combination with lysozyme at 2500 µg/ml concentration[27]. In contrast, when lysozyme was used at concentration of 5 mg/ml with Gentamicin at 0.01 µg/ml, they observed no inhibition of *Klebsiella pneumoniae* biofilm[42]. Finally, all these observations indicate that the synergistic effect of enzyme and antibiotic on the biofilm eradication is efficient and significant. Maximum eradication was observed in case of DNase with levofloxacin. This kind of observation is may be due to DNase disrupts the biofilm structure and levofloxacin antibiotic inhibits topoisomerase activity which is involved in DNA replication of cells.

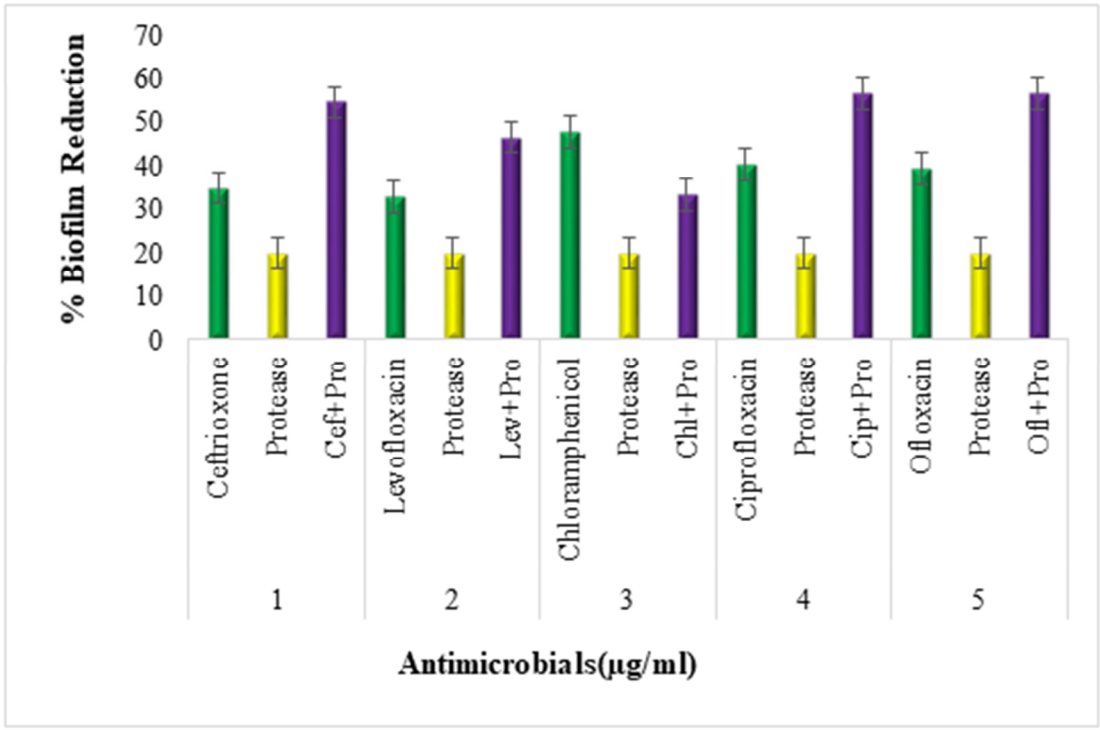

Fig 10. ***Determination of Antibiotics Efficiency with Protease for biofilm eradication***

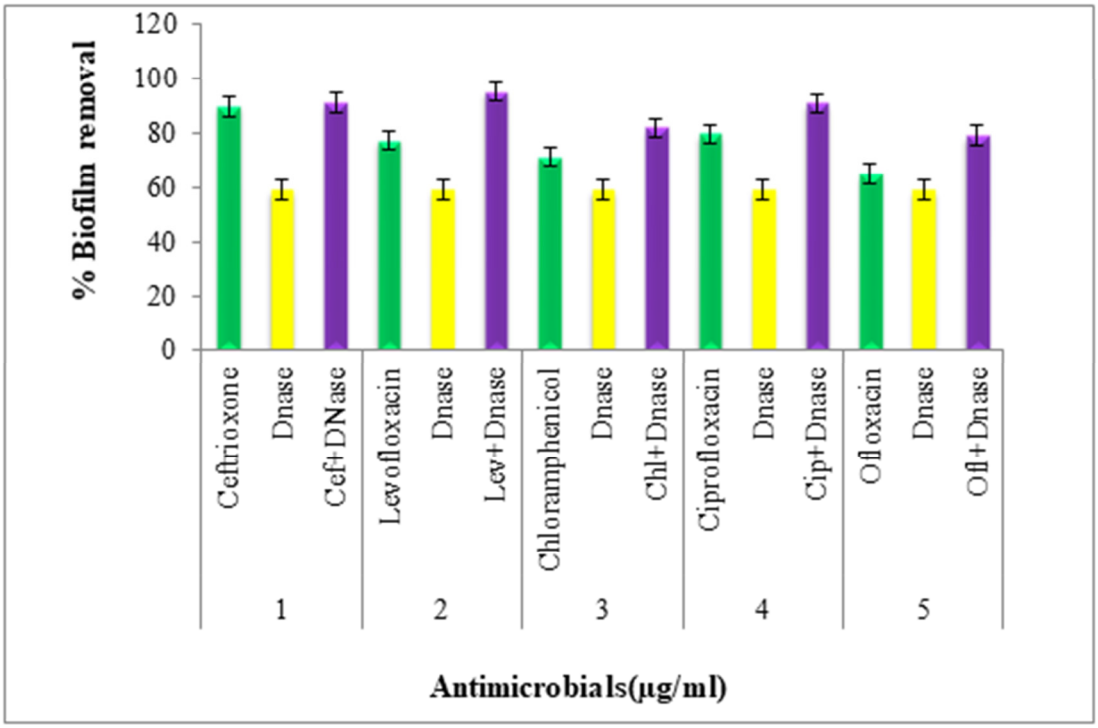

Fig 11. ***Determination of Antibiotics Efficiency with DNase for biofilm eradication***

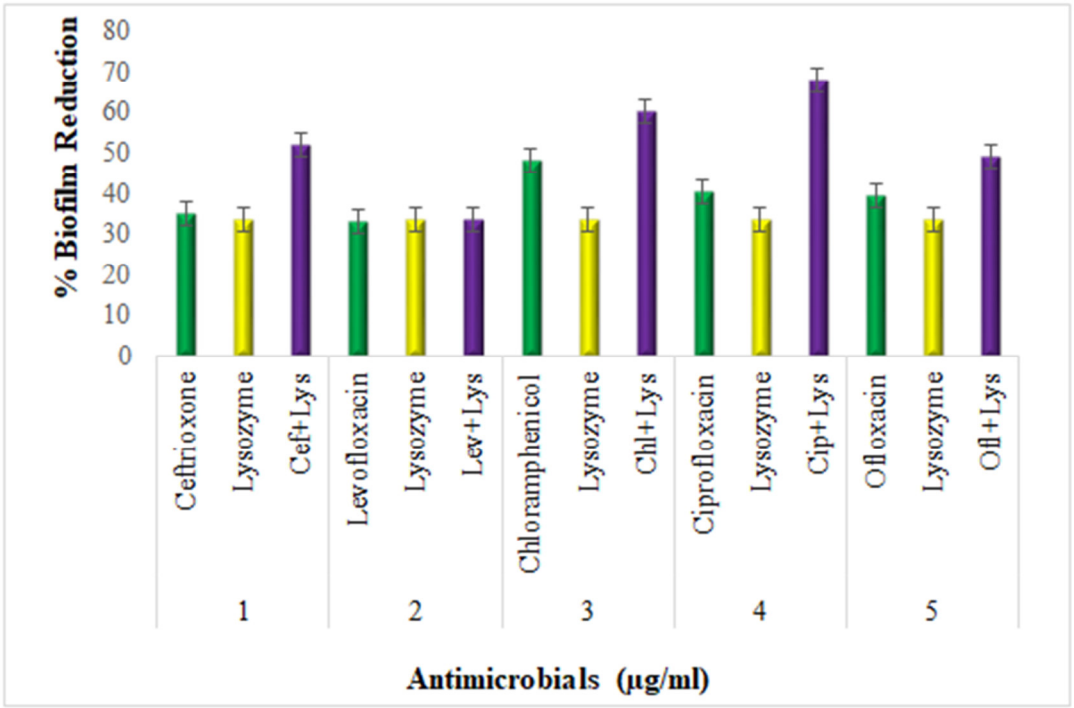

Fig 12. ***Determination of Antibiotics Efficiency with Lysozyme for biofilm eradication***

5. CONCLUSION

The treatment of biofilm-associated infections has become a challenge for clinicians owing to persistence of biofilms. These biofilms hamper the penetration of antimicrobials to the pathogenic microbes. In this present work minimum inhibitory concentration of enzymes and antibiotics were determined. Further, sub-MIC concentration of enzymes, antibiotics and their combinations were analyzed to eradicate *Pseudomonas aeruginosa* biofilm. Among different enzymes lysozyme, protease and DNase gave significant biofilm eradication. DNase with levofloxacin antibiotic has given maximum 95±0.5% eradication. Similarly, lysozyme and protease enzymes with combination to ciprofloxacin antibiotics have given 68±0.7% and 58±0.5% biofilm eradication, respectively. But biofilm removal was not significant when enzymes and antibiotics were utilized individually at their sub MIC concentration. Therefore, combination of enzymes and antibiotics has

shown synergistic effect to eradicate biofilm at significant level.

6. ACKNOWLEDGMENT

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7. AUTHOR CONTRIBUTION STATEMENT

Ms. Komal Sharma conceptualized and gathered the data with regard to this work. Ms. Mansi Shukla and Komal designed the experiments and Mr. Abhishek Prajapati and Komal performed the experiments. Dr. Shilpa Gupte analyzed these data and necessary inputs were given towards the designing of the manuscript. All authors discussed the methodology and results and contributed almost equally to the final manuscript.

8. CONFLICT OF INTEREST

The authors hereby declare that there is no conflict of interest whatsoever in this publication.

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