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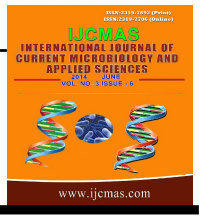


**Original Research Article**

# Biofilm dispersal activity of DNase produced by *Serratia sps YAJS*

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**Keywords**

Microbial biofilms, *Serratia sps YAJS*, extracellular DNase, biofilm, dispersal activity,

**A B S T R A C T**

Microbial biofilms constitutes a hydrated matrix of biopolymers like polypeptides , polysaccharides and extracellular DNA or eDNA. They pose problems in industrial settings like biofouling , and in clinical samples where they cause antibiotic resistant infections. We report the biofilm dispersal activity of extracellular Dnase produced by the *Serratia* isolate on various clinical pathogens. As most of the natural isolates were known to produce very low amounts of extracellular enzymes, initial work has been focused to improve the extracellular DNase production by *Serratia sps YAJS.* After achieving an 10 fold improvement in the enzyme production, *Serratia* culture supernatants were used to check for the biofilm dispersal activity. Present results are in support that extracellular Dnase of *Serratia* could effectively disperse the biofilms of all clinical pathogens tested both gram positive and gram negative bacteria. Our results indicate that the dispersal of biofilms by treatment with Dnase from *Serratia* leads to decrease in strength of biofilm matrix and as a result make them more sensitive to the antibiotic treatment.

## Introduction

Most bacteria grow in natural environment as biofilms. Microbial biofilms are composed of a hydrated matrix of biopolymers like polypeptides, polysaccharides and nucleic acids in the form of extracellular DNA or eDNA (Karatan, E & Watnick, P., 2009). Bacterial biofilms pose problems in industrial settings, where they contribute to biofouling ,and in clinical samples where they cause antibiotic resistant infections. The visoelastic and adhesion properties of biofilms are mainly due to influence of polysaccharides in the matrix (Lau PC, et al., 2009). eDNA or linear high molecular weight DNA likely to contribute to the viscoelastic and adhesion properties of the biofilm matrix (Uhlenhopp EL 1975).

Catlin, BW (1956) demonstrated viscosity of bacterial biofilms was significantly reduced by adding Bovine DNase. Whitchurch, CB et al., (2002) proved the role of DNA in biofilm formation and bovine DNase I inhibits biofilm formation for up to 60 hours after the biofilm growth is initiated. Suri, R. (2005) used commercial bovine and recombinant human DNase I in the

disruption medically important biofilms like cystic fibrosis. Treatment of antibiotic resistant biofilms with Dnase I has been shown to increase in antibiotic susceptibility.

Bacteria are known to modify the structure of their own biofilms as part of their life cycle by secreting matrix degrading enzymes like proteases and amylases or dispersin. Nijiland, B.R. ( 2010) reported that secreted bacterial nucleases can be employed to control the development and dispersal of bacterial biofilms. We report the biofilm dispersal activity of extracellular Dnase produced by the *Serratia* isolate on various clinical pathogens.

## Materials and Methods

### Media employed

Nutrient broth (NB)and agar media, Luria Bertani broth(LB) and agar media, DNase agar medium were employed in study. These were prepared by using Himedia chemicals and sterilized at standard autoclaving conditions.

### DNase production by plate based screening

Enzyme production was tested on DNA agar by inoculating the culture as a thick band at the center of the plate. The plate was incubated at 32$^{o}$C overnight. After incubation the plate was flooded with 2N Hydrochloric acid. DNase production was identified by a halo zone of clearance (DNA degradation) around the culture streak (Blair EB, et al., 1957).

### Enzyme activity determination Spectrophotometric method

DNase activity was routinely determined by the Kamekura & Onishi (1974) method. The assay mixture contained 0.5ml of DNA (1mg/ml) , 0.4ml of 0.1 M Tris – hydrochloric acid (pH8) containing 4M NaCl, 0.05ml each of 0.2M $MgSO_4$ and 0.2M$CaCl_2$ and 0.1ml of enzyme solution. After 10min at 0$^{0}$C, the precipitate was removed by centrifugation, and the absorbance of the supernatant was measured with a spectrophotometer at 260nm. One enzyme unit of the DNase activity was defined as the amount of enzyme catalyzing an increase of 1.0 in absorbance at 260 nm under the above condition.

DNase activity was also confirmed by incubating purified plasmid DNA with the DNase containing fractions for 30min at 37$^{o}$C. The samples were run on a 1% agarose gel containing ethidium bromide to visualize DNA degradation.

### Media Optimization for the growth and DNase production

The nutritional requirement of *Serratia* isolate for DNase production was studied by growing the isolate in different media like nutrient broth, luria broth, peptone glycerol medium and M9 (Minimal medium). 25 ml of respective media broths in 150ml conical flasks were inoculated with 0.1 ml of *Serratia* culture with 0.6 OD and incubated at 30$^{0}$C for growth monitoring. Absorbance at 600 nm data were collected and analyzed by plotting. Effect of NaCl supplementation and induction of DNase production by addition of standard DNA in the production medium was tested at various concentrations.

### SDS – PAGE Analysis

Standard SDS-PAGE of 12% was performed as described by Laemmili (1970). To get a clearly focused protein pattern a phenol extraction on bacterial extract was performed as described by Hanna et al,

2000. In a 2ml screw cap tube, 1ml aliquots of bacterial extracts and 1ml phenol were mixed thoroughly and incubated for 10min at $70^0$C. The sample was cooled on ice for 5 min and the phases were separated by centrifugation for 10mins at 5,000g. The top aqueous phase was discarded and 1ml of distilled water was added. After vortexing and incubation for 10min at $70^0$C, the sample was cooled and the phases were separated. The aqueous phase was discarded and the proteins were separated by adding 1ml ice cold acetone. The sample was pelleted by centrifugation for 20 min at 10,000g after a final centrifugation step. These samples were used for SDS-PAGE analysis.

**Biofilm dispersal activity screening**

Biofilm dispersal activity was screened using clear 96 well flat bottom polystyrene tissue culture plates. Clinical bacterial strains were grown for 48–96 h and diluted 1:100 in fresh LB. 200 µl of this culture was added to every well of a microtiter plate. Extracellular Dnase of *Serratia* was added after 96 hr of growth and biofilm development and incubated for 1 h at 37°C for assay. Then all non-attached cells were removed by discarding the culture medium and rinsing the plate in a container by immersing and agitating gently four times in tap water. Attached biofilm material was stained by addition of 250 µl of 0.5% crystal violet solution (CV) to each well of the plate for 10 min. Unbound CV stain was removed by aspiration and the plate was rinsed again in tap water until no more CV was observed to dissolve in the water. The plates were air dried and photographed. Subsequently, 250 µl of 96% ethanol containing 2% acetic acid (v/v) was added to each well. Adsorption at 595 nm was measured using a ELISA plate reader with appropriate controls (Reindert Nijland, et al., 2010).

**Isolation and Bioassay guided fractionation of proteins from the supernatant**

The proteins in the *Serratia* culture supernatant were concentrated 50 fold by precipitation with trichloroacetic acid (TCA) as follows: The supernatant of several cultures was pooled, and 6.1 M TCA solution was added to give a final concentration of 0.9 M TCA. This solution was kept on ice for 30 minutes to allow for protein precipitation and the precipitated protein was collected through centrifugation (10 min at 7800 rpm). The protein containing pellets were washed twice using ice cold 96% ethanol, and air dried for 30 min at 45°C. Each pellet was dissolved in $1:50^{th}$ of the original volume with 0.05 M Tris-HCl buffer (pH 7.0). This concentrate was fractionated using a Sephadex gel filtration column (Bangalore Genei Kit) using ultra pure water as the mobile phase and fractions of 1 ml each were collected. The fractions were pooled and concentrated by TCA treatment for biofilm dispersal activity (Reindert Nijland, et al., 2010).

## Results and Discussion

Soil isolate obtained in the laboratory was observed to be rod-shaped, gram negative, (Fig.1) facultative bacterium belonging to the Enterobacteriaceae family and characterized by its ability to produce the red pigment prodigiosin (Fig 2). This isolate was identified to produce extracellular DNase when tested on DNA agar (Fig.3).

As few strains are known to produce extracellular DNase, this isolate was subjected to molecular characterization by 16srRNA analysis. The sequenced sample was analyzed for nucleotide matching from gene bank by the nucleotide blast. The strain showed 95% sequence similarity with

*Serratia marcescens strain SB08* and was labeled as *Serratia sps YAJS*.(Y.Aparna & J.Sarada 2012). As most of the natural isolates were known to produce very low amounts of extracellular enzymes, initial work has been focused to improve the extracellular DNase production by figuring out the nutritional requirement of the *Serratia sps YAJS*.

**Media Optimization**

The nutritional requirement of *Serratia* isolate was studied by growing the isolate in nutritionally different media like nutrient broth (NB), luria broth (LB), peptone glycerol medium (PGP) and M9 (Minimal medium). 25 ml of respective media in 150ml conical flasks were inoculated with 0.1 ml of *Serratia* culture with 0.6 OD and incubated at $30^0$C for growth and DNase production. Growth of *Serratia* was monitored as Absorbance at 600nm. DNase activity was monitored by Kamekura & Onishi (1974) method. Samples were collected at 24, 48, 72 and 96 hrs of incubation from each flask to estimate the DNase production. The data obtained during these experiments were represented in Graph 1.

From the graph it was evident that DNase production was stable in L.B broth ,where the enzyme production started by 48hr of incubation and was observed till 96hrs. Maximum DNase activity 4.8 units /ml was reported at 72 hrs of incubation in this medium. In Nutrient broth (NB), the DNase activity was maximum (3.9units/ml) by 48hrs of incubation and later it decreased to 2.2 units /ml by 96hrs. Peptone glycerol broth (PGP) could support the DNase production by 48 hrs which recorded maximum activity as 5.2 units /ml, however the DNase activity dropped drown drastically to less than 1 unit /ml by 72 hrs and beyond. M9 or minimal medium was not supporting the enzyme production as the DNase activity was only 1.1uints/ml by 72 hrs of incubation. Luria Bertani (LB) broth contains Tryptone 1.0%, Yeast Extract 0.5%, Sodium Chloride 0.5%, which were supporting stable production of DNase from 48 hrs to 96 hrs of incubation, hence this medium was employed for DNase production.

Sodium Chloride, was reported to be essential for supporting higher yields of enzyme in the production medium for DNase activity. Experiments were conducted to figure out the required concentration of NaCl in the medium at range of 250, 500, 750 and 1000 milligrams in each flask. LB broth without NaCl was treated as control for investigating the effect of NaCl. The data obtained during these experiments were represented in Graph 2 & 3.

NaCl supplementation in the LB broth had supported good growth in the flask (Graph 2). It was noticed from the results that maximum growth recorded at 250mg concentration and on $2^{nd}$ day of incubation or 48hrs. At higher concentration, though growth was observed, but it was decreasing in comparison with flask containing 250mg concentration. Hence supplementation of NaCl at 250 mg concentration was fixed for further study.

DNase activity in LB broth supplemented with NaCl revealed an interesting observation that by 24hrs of incubation, DNase activity was recorded in culture supernatant. There was an improvement in the enzyme yield i.e 11 units /ml. against the control flask (without NaCl) 1.9units/ml. (Graph 3). DNase activity at higher concentrations were observed to be lesser than at 250mg.

**Induction of DNase enzyme by adding DNA**

Most of the extracellular enzymes respond to the substrate addition in the production medium. For example, β-galactosidase enzyme production was recorded to be enhanced by addition of either lactose or artificial inducer like IPTG. Generally low concentration of the substrate in the production medium will stimulate or trigger the production of the enzymes. Experiments were made to understand these phenomena of induction by adding calf thymus DNA (Commercially available pure form of DNA, Himedia make) as the inducer at 200, 400, 600, 800 and 1000µg concentrations in LB media. DNase production was monitored during these experiments and the obtained results were presented in graph 4.

From the graph it was observed that maximum DNase production was at 200µg of DNA in *LB* medium with enzyme yield of 12 units/ml by 72hrs of incubation. At all higher concentration the enzyme yield was comparatively very low (Graph 4). From these results it was clear that the DNase production could be induced by the addition of DNA .There was a dramatic increase in DNase production from 2units/ml to 12 units /ml at the end of media optimization. Similar studies were carried out to identify the optimum pH and temperature for the DNase production by the isolate (data not shown). There was profound enhancement in the DNase production from 2units/ml to nearly 21units /ml at optimum pH (7-8) & temperature ($30^0$C). Approximately an 10 fold enhancement in the yield was observed.

After achieving an 10 fold improvement in the enzyme production, *Serratia* culture supernatants were used to check for the biofilm dispersal activity. Many researchers had demonstrated that DNA is present in biofilms and plays an important role in biofilm architecture. Biofilms are supposed to be the protective barrier for microorganisms in general and specifically for clinical pathogens. Inorder to inhibit or control the biofilm formation by these clinical pathogens like *Pseudomonas, Staphylococci*, novel approaches are being investigated. The use of human recombinant deoxyribonuclease in the management of cystic fibrosis stands as an example of this type of approach. Reindert Nijland in 2010 demonstrated that secreted bacterial nucleases can be employed to control the development and dispersal of bacterial biofilms.

An attempt was made to investigate the biofilm dispersal activity of the extracellular DNase produced by the *Serratia sps YAJS* isolate. Dispersal of biofilms of both gram positive and gram negative bacteria like *Staphylococcus aureus, Enterococcus feacalis, Klebsiella pneumonia, Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa, Bacillus sps.* were carried in a standard biofilm dispersal assay using a 96 well plate format in triplicates.

All bacterial cultures were grown in microtitre plate for 96hrs for biofilm formation. Later dispersal assay was carried out by adding crude enzyme i.e *Serratia* culture supernatants. Microtitre plate was incubated for 1hr at $37^o$C for testing biofilm dispersal activity. At the end of incubation the plate was washed and stained with CV (0.5%). This was followed by destaining and measurement of absorbance at 595nm using microtitre plate reader. Crude enzyme or culture supernatants could show biofilm dispersal activity. However, to identify the component responsible for Dnase activity or Dnase protein, isolation and bioassay guided fractionation of proteins from the supernantant was performed.

| Strain | Source |
|---|---|
| *Serratia sps.YAJS* | Novel isolate obtained from rhizosphere soil samples of BVC campus, identified and characterized by microbiological, biochemical and molecular characterization by 16s rRNA analysis. |
| *Escherichia coli* | Clinical isolate obtained from diagnostics |
| *Pseudomonas aeruginosa* | A lab isolate |
| *Enterococcus faecalis* | Obtained from Chromogenic |
| *Staphylococcus aureus* | Clinical isolate obtained from diagnostics |
| *Bacillus sps* | A lab isolate |
| *Proteus vulgaris* | Obtained from Chromogenic |
| *Klebsiella pneumonia* | Obtained from Chromogenic |

**Fig.1** Gram reaction of *Serratia* isolate

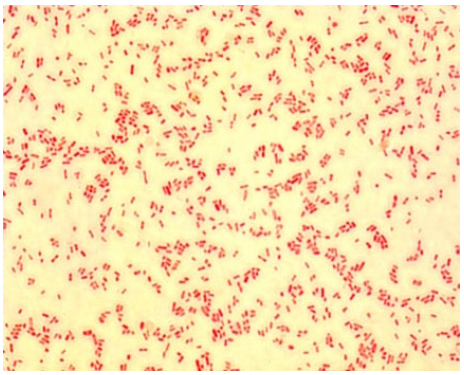


**Fig.2** Pigmented colonies of isolate on Nutrient agar plate

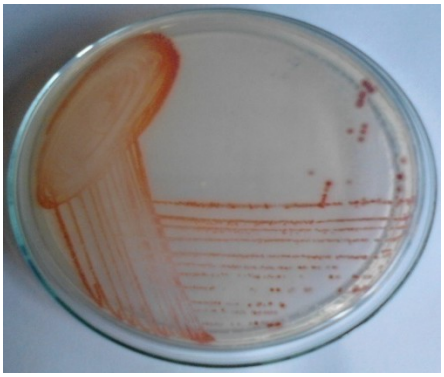


**Fig.3** DNase Production

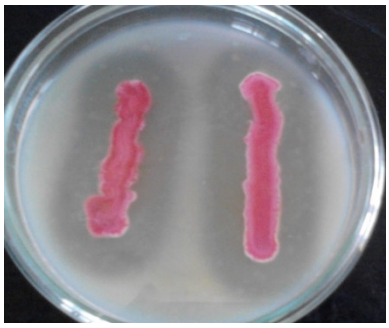
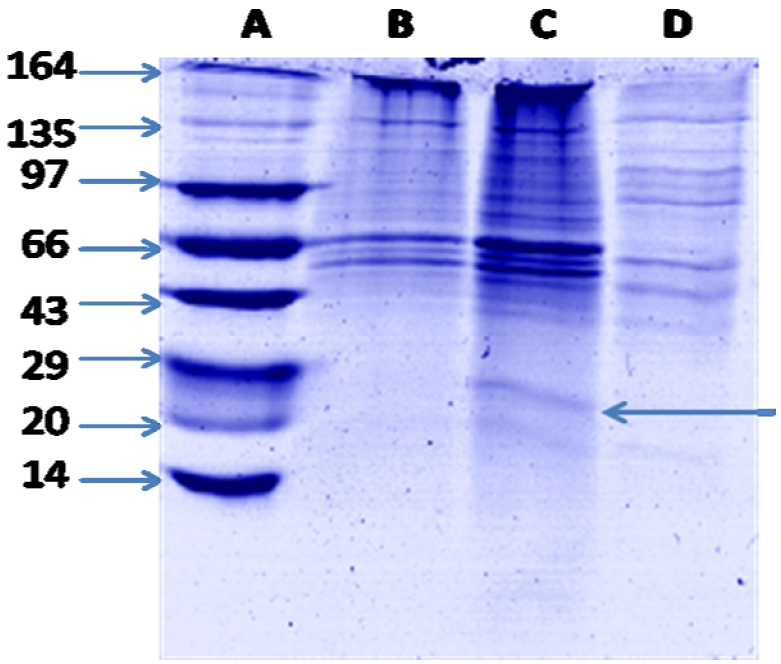
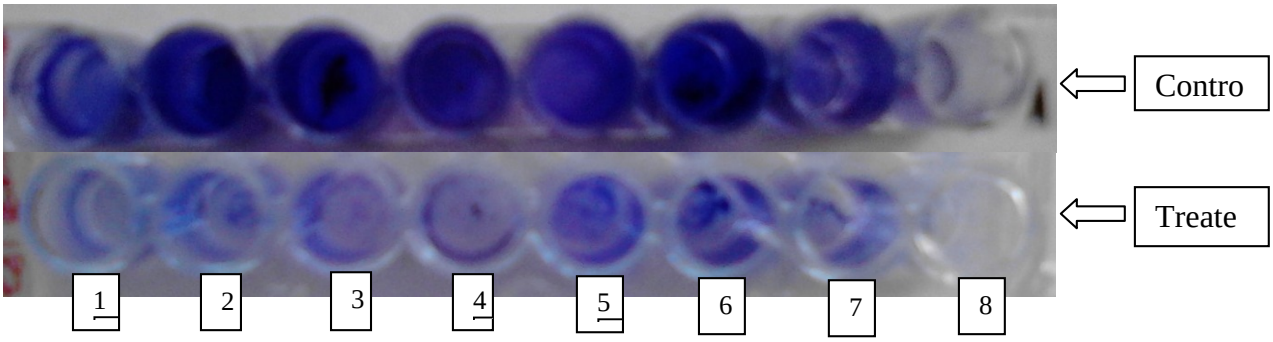


**Figure.4** SDS-PAGE depicting Dnase bands



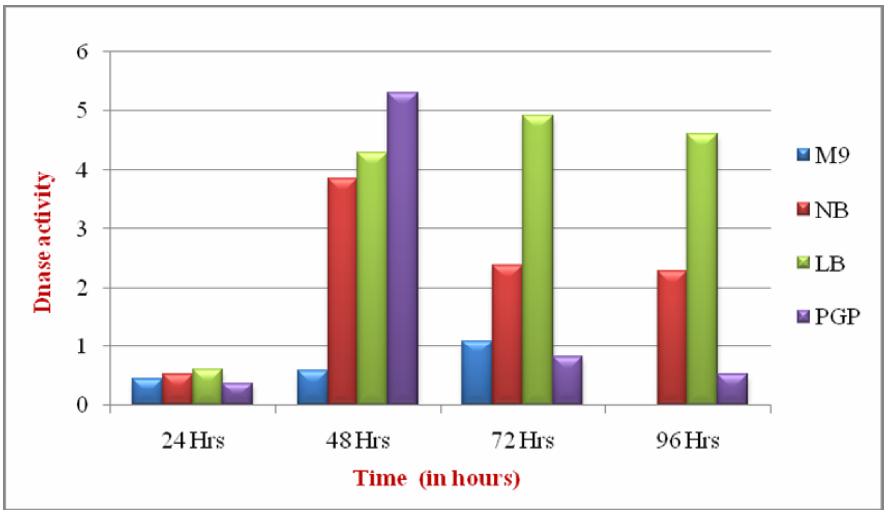

A. Protein marker B. *Serratia* 24hrs of culture supernantent C. 48h hrs of culture supernatant D. *Serratia* cell pellet

**Figure.5** Biofilm dispersal activity

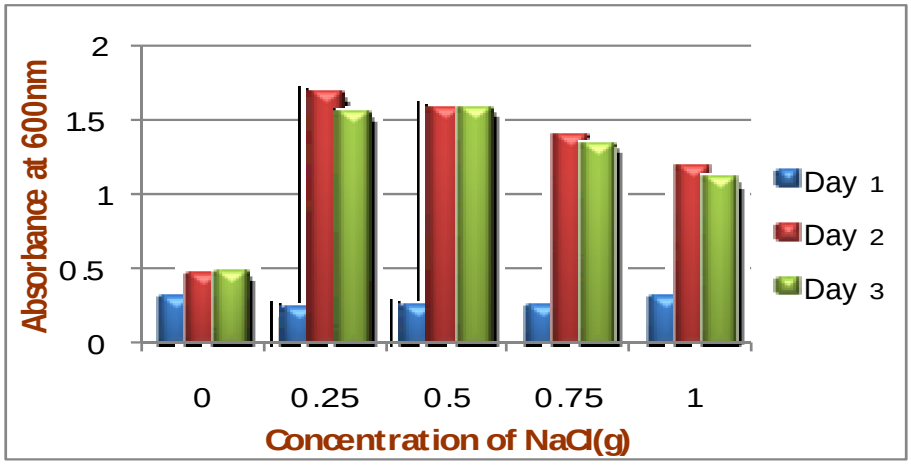



1. *Staphylococcus aureus* 2. *Enterococcus feacalis* 3. *Klebsiella pneumonia* 4. *Escherichia coli* 5. *Proteus vulgaris* 6. *Pseudomonas aeruginosa* 7. *Bacillus sps* 8. Control (Water)

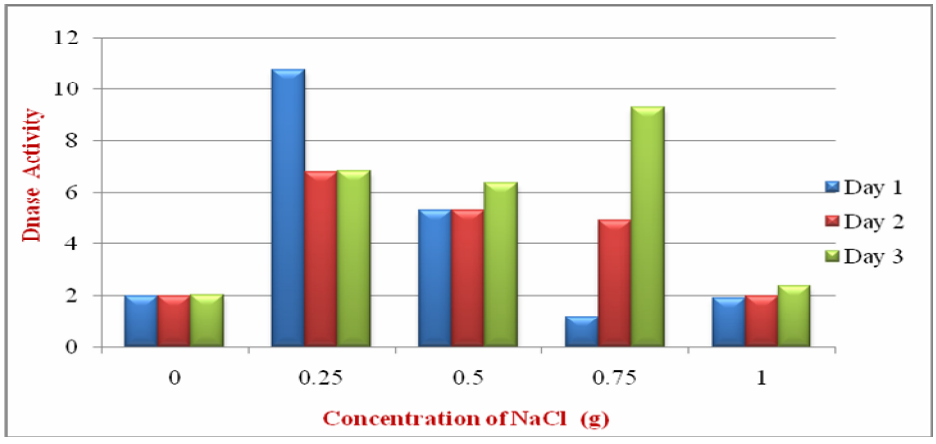
**Graph.1** Effect of different media on DNase production



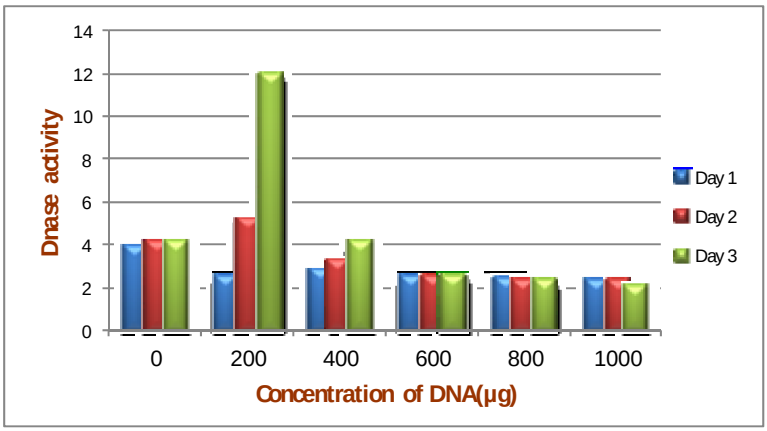

**Graph 2:** Growth of Serratia isolate in Luria Broth supplemented with NaCl




**Graph.3** DNase activity in Luria Broth supplemented with NaCl



**Graph.4** DNase activity in Luria Broth supplemented with DNA




**Graph.5** Biofilm dispersal activity of *Serratia* DNase

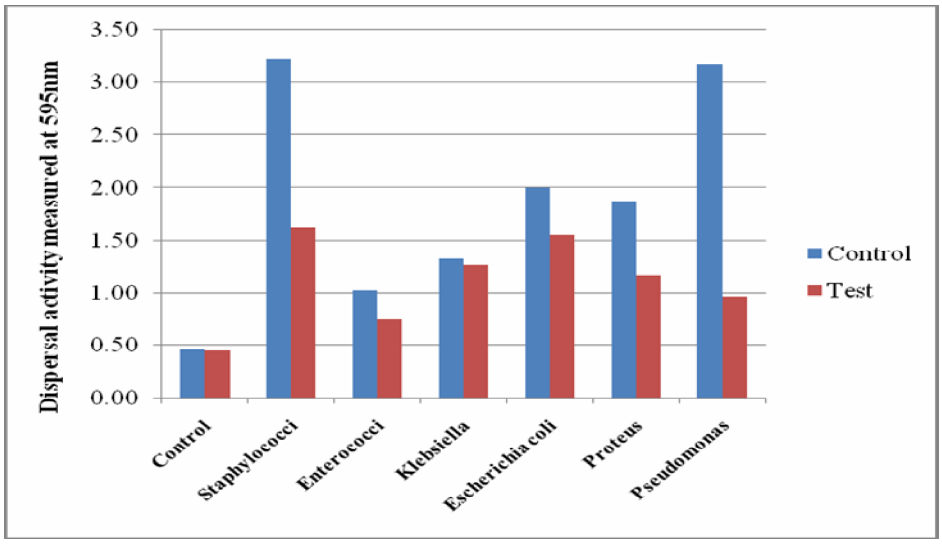



**Table.1** Effect of DNase treatment on Antibiotic resistance and sensitivity of clinical pathogens

| Organism | Tetracycline | | Chloramphenicol | | Trimethoprim | | Penicillin | |
|---|---|---|---|---|---|---|---|---|
| | Before treatment | After treatment | Before treatment | After treatment | Before treatment | After treatment | Before treatment | After treatment |
| *E.coli* | 22 | 23 | 21 | 24 | 21 | 28 | **R** | **10** |
| *Psuedomonas* | **R** | **25** | **R** | **19** | R | R | R | R |
| *Enterococci* | 11 | 25 | **R** | **26** | **R** | **27** | **R** | **22** |
| *Staphyloccoci* | 18 | 24 | 20 | 25 | **R** | **28** | **R** | **19** |
| *Proteus* | 9 | 10 | **16** | **26** | 26 | 26 | 10 | 11 |
| *Klebsiella* | **R** | **19** | **12** | **24** | **R** | **23** | R | R |

*Values represent Zone of inhibition measured in millimeter (mm)
R stands for Resistance

Extracellular DNase was concentrated by giving TCA treatment as per the protocol of Reindert Nijland et al 2010. This treatment had helped to concentrate the Dnase protein in culture supernatant and analysed by SDS PAGE analysis.

SDS- PAGE analysis was performed as per the Hanna et al, 2000 method for the proteins present in the supernatant and cell extracts. From the fig 4, the DNase protein band was clearly observed in 48hrs culture supernatant (Lane 3) whose molecular weight was approximately 29kd when compared with the standard protein molecular weight marker. The DNase protein band was observed only in supernatant but not in cell pellet, proving that it is an extracellular enzyme.

The TCA concentrated DNase enzyme was used for biofilm dispersal activity testing. Figure 5 depicts the results of the biofilm dispersal activity of *Serratia* DNase on the seven clinical pathogens tested against control in a microtiter plate. The absorbance values were represented in graph 5. The effect of DNase on the biofilms is calculated as percent of reduction in biofilm formation with control values i.e. untreated bacterial cultures.

From the results depicted in the graph, biofilm of *Psuedomonas* was effectively dispersed by 69%, while *Staphylococci* biofilm was about 50%. Biofilm of *Proteus* was dispersed to 38% while *Enterococci* & *E.coli* biofilms were cut down to 27% and 23% respectively. Present results supports that extracellular Dnase of *Serratia* could effectively disperse the biofilms of all clinical pathogens tested both gram positive and gram negative bacteria. Reduction in the bacterial biofilm matrix could be due to destruction of extracellular DNA by DNase, as the DNase generally does not penetrate bacteria and its effect is only outside the cell (Liao, 1973). Spectrophotometric analysis of biofilm dispersal assay helped to quantify the effect of Dnase on biofilms.

Dnase treatment of biofilms is known to weaken the matrix which makes bacteria more susceptible to antibiotics. This phenomena was studied by carrying out experiments on antibiotic resistance and sensitivity of bacterial strains before and after DNase treatment. Antibotics like tetracycline, chloramphenicol, trimethoprim and pencillin were used in the study as bacterial pathogens had resistance to one or the other antibiotic before treatment with DNase. To record the antibiotic sensitivity or resistance, Dnase treated bacterial pathogens were plated and standard antibiotic discs (Himedia) were placed on the petriplates and incubated for zone of inhibition. Table 1, depicts the results obtained during these experiments. A common and interesting observation in these results was that all pathogens were showing increased antibiotic sensitivity after the treatment of Dnase. Strikingly, *Psuedomonas* which was resistant to all four antibiotics before treatment was observed to record sensitivity to Tetracycline and Chloramphenicol after treatment. *Enterococci* was also noticed to be sensitive to Choramphenicol, Trimethoprim and Pencillin for which it was resistant before the treatment. In *Klebsiella*, antibiotic sensitivity after Dnase treatment was found in Tetracyline and Trimethoprim, while in *E.coli* sensitivity to Pencillin was observed. By and large there was an increase in the sensitivity of all pathogens tested.

Our results indicate that the destruction of biofilms eDNA by treatment with Dnase from *Serratia* leads to decrease in strength of biofilm matrix and as a result make them more sensitive to the antibiotic treatment**.**

## Acknowledgement

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## References

Aparna, Y and Sarada,J 2012. Molecular characterization and Phylogenetic analysis of *Serratia sp*-YAJS- An extracellular Dnase producer isolated from rhizosphere soil.

Blair EB, Emerson JS, Tull AH. 1967. A new medium, salt mannitol plasma agar, for the isolation of Staphylococcus aureus. Am J Clin Pathol. Jan; 47(1):30–39

Catlin BW (1956) Extracellular deoxyribonucleic acid of bacteria and a deoxyribonuclease inhibitor. Science 124: 441–442.

Hanna, S. L., Sherman, N. E., Kinter, M. T. & Goldberg, J. B. 2000. Comparison of proteins expressed by *Pseudomonas aeruginosa* strains representing initial and chronic isolates from a cystic fibrosis patient: an analysis by 2-D gel electrophoresis and capillary column liquid chromatography-tandem mass spectrometry. Microbiology 146, 2495–*2508.*

Kamekura, M., and H. Onishi. 1974. Halophilic nuclease from a moderately halophilic *Micrococcus varians*. J. Bacteriol 119:339-344.

Karatan E, Watnick P (2009) Signals, regulatory networks, and materials that build and break bacterial biofilms. Microbiol Mol Biol Rev 73: 310–347.

Lau PC, Dutcher JR, Beveridge TJ, Lam JS (2009) Absolute quantitation of bacterial biofilm adhesion and viscoelasticity by microbead force spectroscopy. Biophys J 96: 2935–2948.

Laemmli , UK 1970. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. N*ature* 227, 680 – 685.

Liao, T.H., and J. Salnikow. 1973. Bovine pancreatic deoxyribonuclease A. J. Biol. Chem. 248:1489-1495.

Reindert Nijland1¤, Michael J. Hall2, J. Grant Burgess(2010) Dispersal of Biofilms by Secreted, Matrix Degrading, Bacterial DNase PLoS ONE 5(12): e15668

Suri, R.(2005). The use of human deoxyribonuclease (rhDNase) in the management of cystic fibrosis. BioDrugs 19: 135–144.

Uhlenhopp EL (1975) Viscoelastic analysis of high molecular weight, alkalidenatured DNA from mouse 3T3 cells. Biophys J 15: 233–237.

Whitchurch CB, Tolker-Nielsen T, Ragas PC, Mattick JS (2002) Extracellular DNA Required for Bacterial Biofilm Formation. Science 295: 1487–1491.