# Role of sugars in formation and maintenance of biofilm in Coagulase negative staphylococcal (CoNS) strains

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## Abstract

Two different Coagulase negative staphylococcal environmental isolates BP/SU1(Staphylococcus epidermidis; MTCC accession number:9538) and BP/SU2 (Staphylococcus haemolyticus; MTCC accession number:5731) show a tendency to propagate in the biofilm mode specially on low surface energy mediums. In this study we have co-related the biofilm formation of the two species on polystyrene tissue culture plates with the quantity of utilizable sugar present in the culture medium. The dispersal of the same biofilms with common dispersing agents like $NaIO_4$, DNase, and proteolytic enzymes has also been studied. Results indicate that though polysaccharides undoubtedly enhance the biofilm formation for both the species, their role as biofilm - material adhesive agents are minimal for these two strains.

**Keywords**: CoNS, biofilm, polysaccharides, $NaIO_4$, DNase, proteolytic enzymes

## INTRODUCTION

In natural environments, instead of living as free cells, bacteria grow as biofilms, i.e. "surface-attached microbial communities embedded in a self-produced extracellular matrix" [Costerton et al., 1995] which primarily constitutes of polysaccharides, proteins and DNA [Sutherland, 2001]. This conglomeration of biopolymers is known as extracellular polymeric substances (EPS) and it functions and it is responsible for the three-dimensional architecture of the biofilm. In modern medicine, coagulase negative staphylococci (CoNS) is one of the major causes of foreign material associated infections [Mack et al., 2006] and their habit of propagating as a biofilm remains their most important virulence factor. Due to their opportunistic tendency, the ecological importance of these organisms is often overlooked. However, coagulase negative staphylococci are frequently encountered in agroindustrial waste environment. In this study, two coagulase negative staphylococcal strains have been used *S. epidermidis*, BP/SU1 previously isolated from activated sludge [Roy et al.,2009] and a strain of *S.haemolyticus*, BP/SU2 isolated from the spent flow of a sugar industry[Sarkar et al.,2015].

Biofilm development relies upon availability of nutrients. Bacterial cells, though not actually starved but surviving under limited nutrient conditions tend to grow as biofilm. At either extreme, when there is too much food or none of it, higher numbers of cells are in the planktonic phase where they easily disperse through the fluid medium in order to access other nutrient rich environment.A substantial body of research has shed light on polymeric intracellular adhesion(PIA) mediated biofilm formation by CoNS with special emphasis on *S.epidermidis* strain RP62A (model *ica* positive strain). However, role of sugar and other environmental factors in *ica* independent biofilms is still quite unclear. Genes encoded on mobile genetic elements carrying virulence factors and antibiotic

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resistance genes confers large variations between different isolates and staphylococcal strains"*S. epidermidis* strains show a high level of diversity, with 74 identified sequence types" [Miragaia et al., 2007]. "Among CoNS,*S. haemolyticus*is second only to*S. epidermidis*inits frequency of isolation from human blood cultures" [Falcone et al., 2006]. In a study, biofilm forming capacity of 72 clinical isolates of *S. haemolyticus* was analysed and only three isolates were positive for *ica* D gene. "Such a low prevalence of the *ica* operon in this strain collection indicates that *S. haemolyticus* mainly forms PIA independent biofilms" [Fredheim et al., 2009]. Our study proposes to do a comparison between biofilm development of two CoNS strains namely BP/SU1(staphylococcal epiderrmid is (MTCC: 9854) and BP/SU2(staphylococcal haemolyticus (MTCC: 5731) in presence of various sugars. Chemicals targeting specific EPS components is an effective means to detach biofilms from a surface. The main components of staphylococcal biofilm is poly-β-1,6-*N*-acetylglucosamine (PNAG) which is a polysaccharide composed of repeating units of β-1,6-*N*-acetylglucosamines with partly deacetylated residues. It maintains the biofilm integrity by ensuring cell to cell adhesion. Beyond (PNAG) intercellular adhesion has been reported to be mediated by proteins, nucleic acids and other polysaccharides. Periodate ($IO_4^{-1}$) modifies the polymeric chainof PNAG ,poly-β-1,6-*N*-acetylglucosamine,by oxidizing the carbons bearing vicinal hydroxyl groups and cleaving the C3–C4 bonds of the *N*- Acetylglucosamine residue [Mack et al., 1996; Maira-Litran et al.,2004].DNase I catalyses the hydrolytic cleavage of phosphodiester linkages in the DNA backbone, thus degrading the DNA to release 5'-phosphorylated di-, tri-, and oligonucleotide products [Vanecko & Laskowski, 1961]. DNase I can disrupt biofilms in which DNA play a major role in binding the biofilm together. Proteinase K and trypsin are broad spectrum serine proteases with different substrate specificity: Proteinase K endolytically cleaves the peptide bonds of aliphatic, aromatic or hydrophobic amino acids [Morihara& Tsuzuki, 1975], whereas trypsin is specific for cleaving the peptide bonds of lysine and arginine [Rawlings & Barrett, 1994]. They can be used to disperse biofilms where proteins molecules are a major contributing factor in biofilm formation.

## Materials and Method

### Materials

Bacterial growth media components and staining solutions have been purchased from HiMedia (India). Rest of the chemicals including solvents and inorganic salts are of analytical grade and are obtained from E-Merck (India).Polystyrene tissue culture and petri plates are from Tarson.Bacterial strains BP/SU1 and BP/SU2 are earlier isolates from the same laboratory from where this study has been done.

### Methods

Growth Kinetics

To plot the growth curve of the isolate, aliquots are withdrawn every hour from a flask culture of 20 ml LB broth with 1% inoculum incubated at 37°C on an orbital shaker and growth is monitored as a function of absorbance at 600nm against LB broth for 10 hours from the point of inoculation.

**Sugar utilization**

5ml LB broth, supplemented with sugar (1% w/v) is taken in test tubes and 0.018 g/L phenol red is added to it. Durham's tubes are placed into the test tubes and the media is autoclaved. After addition of 1% inoculum, the tubes are incubated at 37℃ for 48 hours.

At the end of incubation, all the tubes are observed for changes in color (red to yellow due to acid production) or bubble in Durham's tube due to gas production.

**Biofilm cultivation and quantification**

Overnight culture of bacteria in LB broth supplemented with the appropriate sugar ,or Trypticase Soy broth is diluted 100-fold and 100 µl aliquots of the diluted culture is placed in wells of multi-well polystyrene tissue culture plates which was then incubated for 24 hours at 37°C. Post incubation, the spent media with non-adhering cells was aspirated off followed by washing of the wells with saline. The plate is then left to dry. 125 µl crystal violet stain was added to the wells, stained for 15 minutes and washed off. After drying, 200 µl 33% glacial acetic acid was placed in the wells to destain them. Biofilm formed in each well was quantified by measuring the absorbance at 570 nm by VERSA MAX Microplate reader.

**Biofilm detachment assay**

CoNS biofilms are cultivated in 96 well plates as described in the previous section. To ensure substantial biomass accumulation, glucose (1% w/v) fortified LB broth is used as growthmedium. The biofilms are washed with 200 µl of saline (0.9% NaCl) and then treated for 2hoursat 37°C with 100 µl of –

- 10 mM potassium periodate [in 50 mM sodium acetate buffer (pH 4.5)]
- Proteinase K at 1.0 mg/ ml [in 20 mMTris (pH 7.5), 100 mMNaCl]
- Trypsin at 1.0 mg/m1 [in 20 mMTris (pH 7.5), 100 mMNaCl, 10 mM EDTA]
- DNAase at 1U per well [in 10 mMTris (pH 7.6), 2.5 mM MgCl2, 0.5 mM CaCl2]

Control wells were filled with appropriate buffers. After the treatment, the fluid from each wellis aspirated off, biofilms are washed with Milli Q water, dried at room temperature and stainedwith crystal violet. Quantification of biofilm is done as described above. Percent detachment is calculated from the A570 value

**Extracellular DNA extraction**

Extracellular DNA has been extracted from spent culture using CTAB [Corinaldesi et al.,2005]. CoNS biofilm is cultivated on PE(1) and 500µl sample (planktonic cells in spent media)is collected after 72 hours incubation at 37ºC. To this, equal volume of CTAB in buffer 1 (1% CTAB (w/v), 50 mMTris, 10 mM EDTA, pH 8.0) is added and incubated at 65ºC water bath,for 30 minutes. Samples are then centrifuged at 10,000 rpm for 15 minutes. The supernatant is discarded, pellet resuspended in 100µl buffer 2 (10 mMTris, 0.1 mM EDTA, 1M NaCl, pH 8.0), 60µl isopropanol added followed by incubation at -20ºC for 60 minutes. Samples are then centrifuged at 10,000 rpm for 30 minutes at 4ºC. The supernatant is discarded and the pellet is air dried and finally re-suspended in 20-100µl buffer 3 (10 mMTris, 0.1 mM EDTA, pH 8.0). DNA samples thus isolated were analysed by agarose gel electrophoresis (1% agarose gel stained with ethidium bromide).

## Results and Discussion

### *Glucose in growth medium promotes biofilm formation*

Trypticase Soy Broth (TSB) is the preferred choice of growth medium for cultivating bacterial biofilms in the laboratory. It is nutritionally richer than Luria Broth(LB) (Table 1) due to the additional presence soy bean digest and glucose. Biofilm formation by BP/SU1 & BP/SU2 in three types of growth media – TSB, LB & glucose supplemented LB was compared (Figure1A) by quantifying the biofilm produced in polystyrene wells by crystal violet staining. In LB, the amount of biofilm formed by BP/SU2 on polystyrene is greater than BP/SU1, thus underlining a major difference between the two CoNS strains ($p = 3.2\times10^{-8}$). There is almost a 3-fold increase in biofilm for BP/SU1 cultivated in TSB and LB supplemented with 0.5%

(w/v) glucose over LB. The increase in biofilm for BP/SU2 is 1.9-fold and 3.5-fold for LB supplemented with glucose and TSB respectively. BP/SU2 prefers TSB as a growth medium to proliferate as biofilm over

Table1. Comparison of LB & TSB growth medium (concentration is w/v %)

| **Luria Broth (LB)** | |
|---|---|
| Pancreatic digest of Caesin | 1.0 % |
| Yeast extract | 0.5 % |
| Sodium chloride | 0.5 % |
| pH | 7.0 |
| **Tryptone Soy Broth (TSB)** | |
| Pancreatic digest of Caesin | 1.7 % |
| Papaic digest of soyabean meal | 0.3 % |
| Sodium chloride | 0.5 % |
| Glucose | 0.25 % |
| Disodium hydrogen phosphate | 0.25 % |
| pH | 7.3 |

glucose supplemented LB ($p = 1.3\times10^{-6}$). Also, dependency on glucose in the growth medium to form biofilm on polystyrene is more pronounced in the case of BP/SU1 when compared to BP/SU2 (Figure1B**)**.

***Growth kinetics and generation time of CoNS is differentially controlled by sugars in growth medium***

Growth kinetics of BP/SU1 and BP/SU2 is LB is compared and generation time (G) is calculated for each strain. After a lag period of about 2 hours, both the organisms start growing exponentially. In the log/exponential phase, generation time of BP/SU1 is significantly greater than that of BP/SU2 ($G_{BP/SU1}$ = 125.4 minutes & G BP/SU2 = 99.7 minutes). From Figure.2 it is evident that by the 10th hour, BP/SU1 approaches the stationary phase whereas BP/SU2 continues growing exponentially. Thus, in LB, BP/SU1 grows at a slower rate than BP/SU2 glucose supplemented LB ($p = 1.3\times10^{-6}$). Also, dependency on glucose in the growth medium to form biofilm on polystyrene is more pronounced in the case of BP/SU1 when compared to BP/SU2 (Figure1B**)**.

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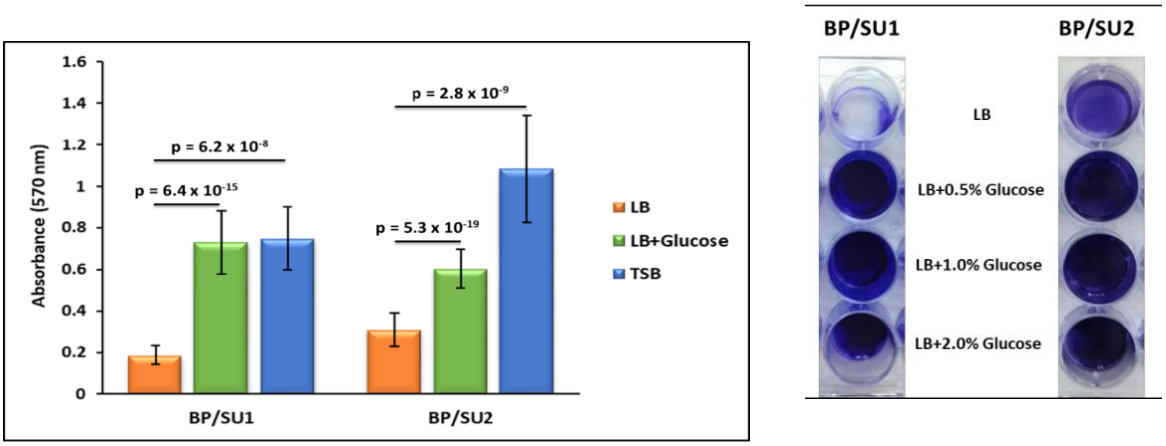
LB without any additional carbon source. The two strains utilize sugars differentially. BP/SU2 can use trehalose as a carbon source but not BP/SU1 (Figure 3**)**. There isn't any gas production in Durham's tube after 72 hours of incubation.Based on the capacity of CoNS to utilize them, the tested sugars have been classified as C+ or C-. Glucose, sucrose and lactose are C+ sugars for BP/SU1 whereas mannitol and trehalose are C-.




(A) (B)

**Figure 1** Biofilm formation on polystyrene in TSB, LB & glucose supplemented LB. Cells from 16 hours old overnight cultures of BP/SU1 & BP/SU2 in LB were harvested by centrifuging equal aliquots of culture. Cell pellets were washed with Milli-Q water and resuspended in equal volumes of TSB, LB & glucose supplemented LB. The cell suspensions were diluted to 1:100, dispensed into 96-well tissue culture plate and incubated at 37°C for 24 hours. Biofilm formation was determined as a function of A570 (described in Materials and Methods). Mean values of three independent experiments are shown (p value is of Student's t test and error bars are standard deviation).

In addition to glucose, sucrose and lactose, BP/SU2 ferments trehalose. Thus, except for mannitol, all the other sugars are C+ for BP/SU2.

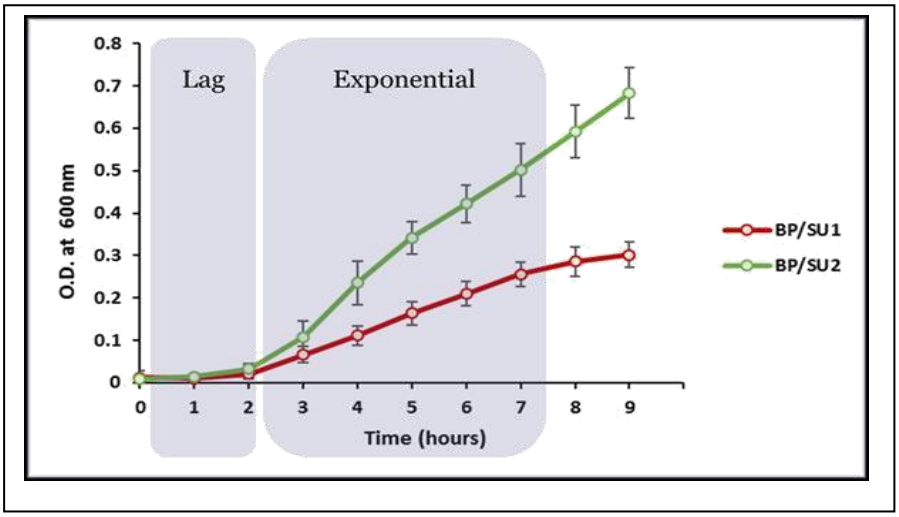
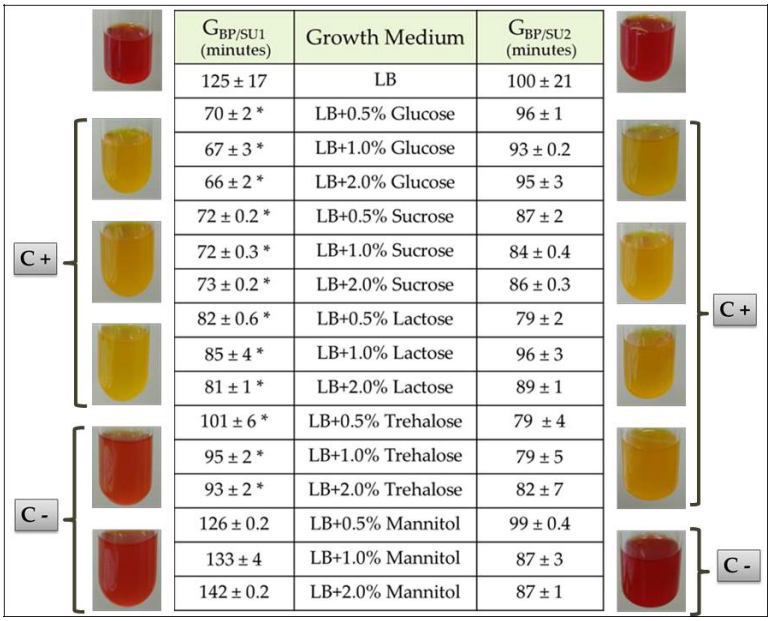



**Figure 2** Growth kinetics of BP/SU1 & BP/SU2 in LB medium. Aliquots are withdrawn every hourfrom a flask culture of 20 ml LB broth with 1% inoculum incubated at 37°C on an orbital shaker and growth is monitored as a function of A600 for 10 hours from the point of inoculation. Error bars are standard deviation from triplicate data sets).

### *Proteins and extracellular DNA are responsible for maintaining thearchitecture of CoNS biofilm*

BP/SU1 & BP/SU2 are cultivated in LB supplemented with 1.0% glucose, in polystyrene wells. Figure 4 highlights the result of the detachment assays on the preformed biofilms. Proteinase K (1mg/ml) detaches biofilm of BP/SU1 & BP/SU2 by 71% & 60% respectively

(Figure 4A). At the same concentration, trypsin results in 42% and 30% detachment of BP/SU1 and BP/SU2 respectively (Figure 4B). Thus, sensitivity of CoNS biofilm is greater towards proteinase K and proteins have a predominate presence in the Extra cellular polymeric substance of Co NS biofilm.(1 unit acting upon preformed biofilm from 100ul culture) results in 70% and 80% biofilm detachment of BP/SU1 and BP/SU2 (Figure 4C) DNase I 1 unit acting upon preformed biofilm from 100ul culture) results in 70% and 80% biofilm detachment of BP/SU1 and BP/SU2 (Figure 4C).In this study, $KIO_4$ failed to detach the CoNS biofilms from the polystyrene




| $G_{BP/SU1}$ (minutes) | Growth Medium | $G_{BP/SU2}$ (minutes) |
|---|---|---|
| 125 ± 17 | LB | 100 ± 21 |
| 70 ± 2 * | LB+0.5% Glucose | 96 ± 1 |
| 67 ± 3 * | LB+1.0% Glucose | 93 ± 0.2 |
| 66 ± 2 * | LB+2.0% Glucose | 95 ± 3 |
| 72 ± 0.2 * | LB+0.5% Sucrose | 87 ± 2 |
| 72 ± 0.3 * | LB+1.0% Sucrose | 84 ± 0.4 |
| 73 ± 0.2 * | LB+2.0% Sucrose | 86 ± 0.3 |
| 82 ± 0.6 * | LB+0.5% Lactose | 79 ± 2 |
| 85 ± 4 * | LB+1.0% Lactose | 96 ± 3 |
| 81 ± 1 * | LB+2.0% Lactose | 89 ± 1 |
| 101 ± 6 * | LB+0.5% Trehalose | 79 ± 4 |
| 95 ± 2 * | LB+1.0% Trehalose | 79 ± 5 |
| 93 ± 2 * | LB+2.0% Trehalose | 82 ± 7 |
| 126 ± 0.2 | LB+0.5% Mannitol | 99 ± 0.4 |
| 133 ± 4 | LB+1.0% Mannitol | 87 ± 3 |
| 142 ± 0.2 | LB+2.0% Mannitol | 87 ± 1 |

**Figure 3** Effect of sugars; A) glucose, B) sucrose, C) lactose, D) trehalose & E) mannitol on growth kinetics & generation time of BP/SU1 & BP/SU2. To determine the generation time G of CoNS in LB as well as sugar supplemented LB, aliquots from the culture being monitored for growth kinetics was plated at two different time points and after 24 hours'incubation at 37°C, colony counts (X & x) were noted down $\boldsymbol{G = \frac{t}{3.3log\, X/x}}$. The sugars glucose, sucrose, lactose, trehalose & mannitol have been designated as C+ or C-based on the result of the sugar utilization test (x = number of cells at 0 minutes and X = number of cells after t minutes)

surface implying the lack of involvement of polysaccharides in maintaining the structural integrity of the biofilm (Figure 4D).

## *Cellular aggregation and extracellular DNA levels are nutrient dependent*

BP/SU1 and BP/SU2 cultivated under static condition in LB show aggregation after 24 and 72 hours of incubation respectively (Figure 5). The extent of aggregation is higher for BP/SU1. The cellular aggregate of BP/SU1 cannot be resuspended easily, even mechanical agitation also doesn't break the aggregate.

BP/SU2 aggregates can be dissolved to a certain extent much more compared to BP/SU1 but never completely. Addition of DNase completely solubilises the cellular aggregates of both strains (Figure 6) proving that extracellular DNA is playing the role of adhesin.

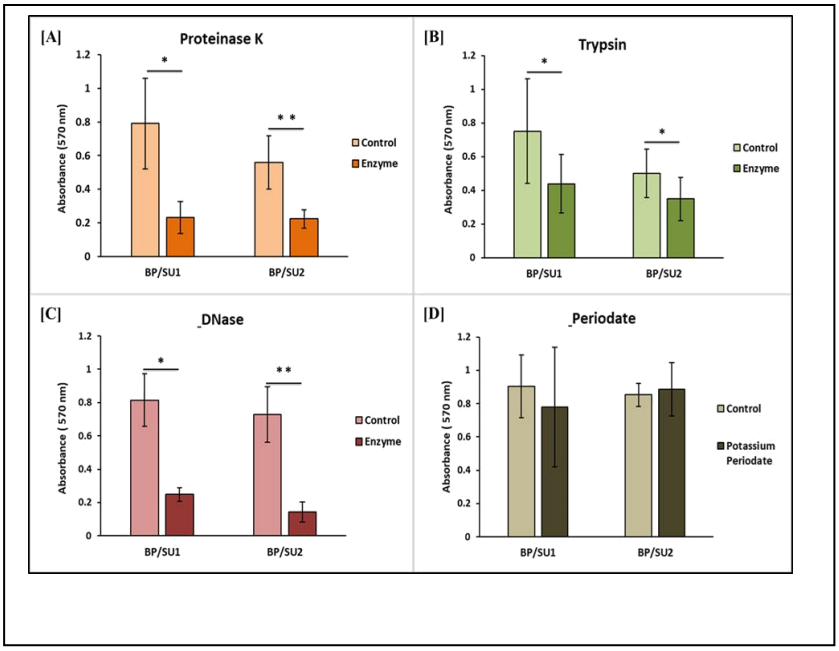



**Figure 4** Biofilm detachment assays of BP/SU1 & BP/SU2 using A) Proteinase K, B) Trypsin, C) DNase I & D) Potassium periodate. CoNS biofilms were cultivated in 96 well polystyrene plates. 16 hour old cultures were diluted to 1:100 in LB supplemented with glucose, dispensed into wells and incubated at 37°C for 24 hours. Planktonic cells were aspirated off and the plate was left to dry overnight. Enzymes and periodate was added in the wells with preformed biofilm. Buffer was added to control wells. Residual biofilm was determined as a function of A570 (described in Materials and Methods). Mean values of three independent experiments are shown (Error bars are standard deviation; * indicates significant biofilm detachment, $p<0.05$)

Interestingly, this tendency to aggregate was lacking when the strains were cultivated in glucose supplemented LB (Figure 5) Aliquots were drawn from cultures of BP/SU1 & BP/SU2 growing under different conditions, namely- nutrient limited (LB) or nutrient rich (LB supplemented with C+ sugars). CoNS prefers to adhere to a surface like polystyrene with low surface energy of around 33mJ/m$^2$ over glass whose surface energy is almost 10 times greater. The adhesion of both strains to glass is extremely poor. During static incubation, in absence of Polystyrene micro titre wells the cells aggregate instead of adhering to glass which is the only surface available to them. Extracellular DNA was isolated from the aliquots by CTAB precipitation method and analysedby agarose gel electrophoresis. (Figure 7) shows that DNA release into the aqueous medium starts at 24$^{th}$ and 72$^{nd}$ hour of incubation for BP/SU1 & BP/SU2 respectively when grown in LB (Lane 2, Lane 11). Aliquots drawn from flask culture in LB show presence of extracellular DNA which implies that attachment to surface is not a prerequisite to DNA release. When LB is supplemented with sugars that the organism can use for growth, there is no detectable DNA in the cell free supernatant aliquoted from culture. Biofilm cultivated in presence of glucose is readily detached by DNase but interestingly, upon addition of any sugar that the organism could use for growth, there was no detectable DNA

This observation suggests that different levels of eDNA is available to the bacterial population trying to develop into a biofilm under different growth conditions. In *S. Epidermidis* biofilms, eDNA is generated through the lysis of a subpopulation of the bacteria, mediated by autolysin E [Qin et al.,2007]. This eDNA further promotes biofilm formation of the remaining population. In absence of sugar, both strains exhaust the nutrients at some point and cell lysis due to death begins generating DNA. Addition of carbon in form of sugar extends the exponential phase culminating in higher cell mass. DNA available for biofilm matrix is low due to delayed stationary phase and thus the available DNA is entirely sequestered in the biofilm. In such a scenario, there isn't any

detectable DNA in the cell free supernatant. Hence it is established that CoNS strains employ extracellular DNA as part of its biofilm matrix and biofilm development relies upon nutrient availability.

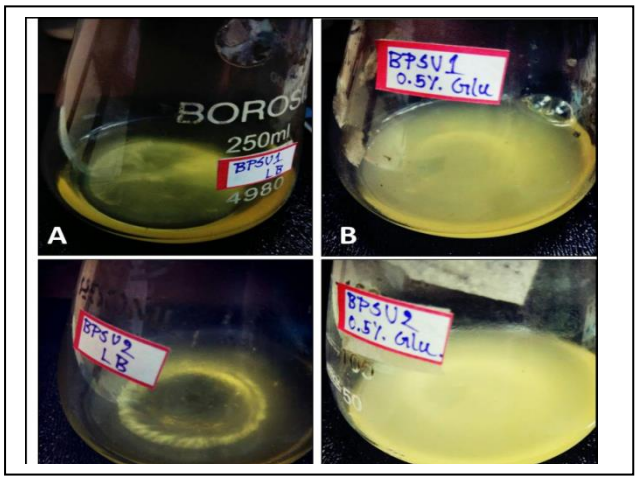



Figure 5 Cellular aggregation in static culture of A) BP/SU1 & C) BP/SU2 in LB. In presence of glucose, no such aggregates were observed (B & D). 50 ml growth medium (LB or LB supplemented with glucose) was inoculated with 1% overnight CoNS culture and incubated for 72 hours at 37°C without disturbing the flasks

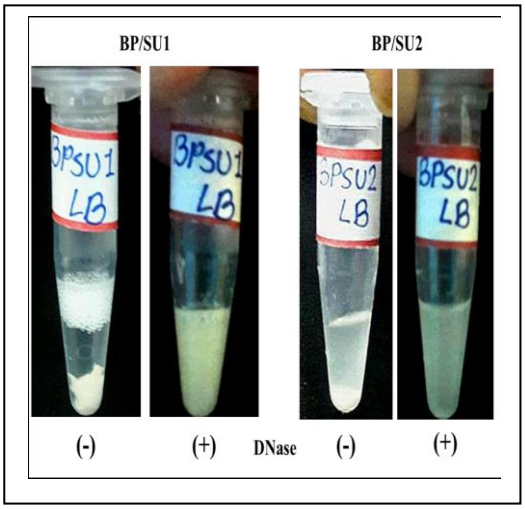



Figure 6 Disruption of cell aggregates of BP/SU1 & BP/SU2 by DNase. 72 hour old cultures of CoNS in LB & LB supplemented with glucose was centrifuged to obtain supernatant free cell pellet. DNase (40U/100 µl) was added to the cell pellet and incubated at 37°C for 3 hours.

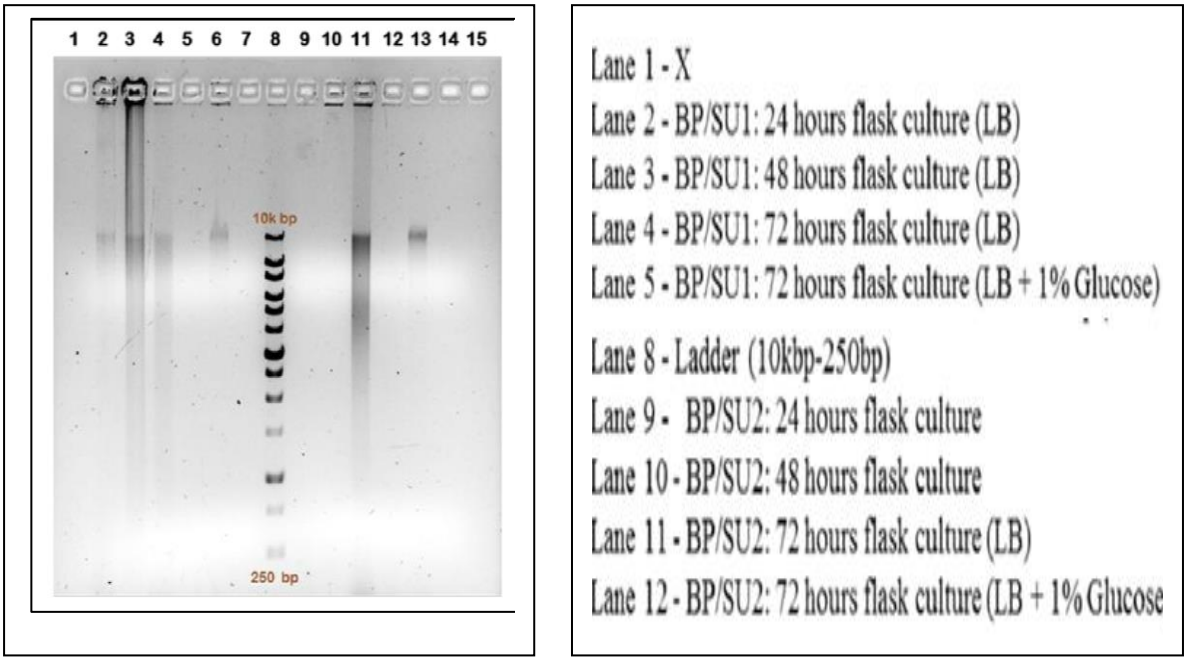



Figure 7 DNA-agarose gel

DNase but interestingly, upon addition of any sugar that the organism could use for growth, there was no detectable DNA in the culture.

## Conclusion

Biofilm formation for Bp/SU1 (S.epidermidis) is more dependent on the presence of utilizable sugar in the nutrient medium compared to BP/SU2 (S.haemolyticus ) which is a

natural biofilm former Without added sugars in the nutrient medium Bp/SU1 hardly propagates in the biofilm mode. The integrity of BP/SU1 aggregate is greater than that of BP/SU2 as it doesn't dissolve in aqueous medium in absence of DNase. DNase mediated detachment is greater for BP/SU2. Therefore, it will not be inaccurate to suggest that BP/SU1 employs eDNA to mediate intercellular adhesion and BP/SU2 uses it predominantlyfor surface attachment. Proteins have a greater role to play in the biofilm of BP/SU1 compared to BP/SU2.

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