**COMPARISON OF DISAGGREGATION PROCEDURES FOR ENUMERATION AND CHARACTERIZATION OF MUNICIPAL SEWER BIOFILM BACTERIA**

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Running Title: municipal sewer biofilms.

**ABSTRACT:** *Biofilms are complex communities where bacteria stick firmly to any moist surface. The main role in biofilm formation is played by the tangled mass of exopolyssacharides. Different technologies are needed and exist to prevent biofilm formation. In the present study biofilm dispersal techniques are compared prior to enumeration and characterization of biofilm bacteria. Biofilm samples were collected from municipal sewerage. Vortex mixing, sonication and blender mixing procedures were used to disperse biofilms. Mixing and dispersion by blending gave the best results. Isolated bacteria were cultivated on rich/selective media using spread plate technique. After 24 hours incubation colony forming units (CFU) were calculated. The viable counts were recorded more on NA and PA medium as compared to other growth medium. EPS producing strains were identified based on slimy colony morphology on different culture media (E medium, SBS medium and EPS medium). Another screening was performed based on the Alcian blue 8GX staining of cells from 48 hour cultures on media supporting mucoid mode of growth.*

**Key words;** Biofilms, EPS, gycocalyx, alcian blue, mucoid.

**INTRODUCTION**

Biofilms are ubiquitous and found in all environments (natural, medical, and industrial) where bacteria can reach, grow and exist (1) Due to slimy nature of EPS the bacterial cells held either tightly, loosely or free floating in biofilms, Some are younger in outer layers and some are older in deeper layers. Hence they differ in density, porosity and spatial arrangement with many phenotypic variants (2,3,4). Extracellular polymeric substances (EPS) make the biofilm communities unique and robust. Different chemical, physical and mechanical technologies are available to disintegrate the biofilms (5-11).Wastewater biofilms may be more complex, they may possess a thick, overlying, less firmly bound, filamentous bacterial component, glycocalyx (12). Without disintegration it difficult to grow all types of bacteria and quantify the biofilm viability from waste water biofilms .The study of wastewater biofilm formation is limited to studies on biofilm-forming activated sludge bacteria grown in laboratory reactors (13). In this study bacterial strains were isolated from mixed domestic and industrial wastewater biofilms growing on the concrete lined walls of a municipal disposal unit’s wastewater pool at Satukatla Drainage, Lahore, Pakistan. In order to disaggregate biofilm bacteria different dispersal techniques are compared prior to enumeration and characterization of biofilm bacteria. Vortex mixing, sonication and blender mixing procedures were used to disperse biofilms. Isolated bacteria were cultivated on rich/selective media using spread plate technique. After 24 hours incubation colony forming units (CFU) were calculated. The selected isolates were characterized following Gerhardt *et al.,* (14). EPS producibility of the selected isolates was investigated by using different media supporting mucoid mode of growth.

**MATERIALS AND METHODS**

**Sampling:** Biofilm samples were scratched with the help of sterile steel devices from concrete lined walls of a municipal disposal unit’s wastewater pool at Satukatla Drainage (SD), Lahore, Pakistan. Different physical characteristics such as colour, temperature and pH of wastewater were noted on site.

**Homogenization of biofilm sample to disperse bacterial cells**

One gram biofilm sample in PBS was subjected to following disaggregation procedures to disperse bacterial cells embedded in biofilm slime matrix:

1. Sonication; biofilm sample in PBS was taken in a thick walled pyrex glass tube in a beaker containing ice and sonicated for 30 seconds at 6 amplitude (Sanyo sonictor).
2. Vortex mixing; Sample was vortexed for 6 minutes on Sanyo vortexer.
3. Blender mixing; slight blending of the sample for about 30 seconds was repeated 5-6 times in a blender mixer

**Isolation, enumeration and purification of bacteria:** The homogenized sample after serial dilution in PBS was cultivated on rich (Nutrient agar) as well as on selective media, (EMB= Eosin Methylene Blue agar; SX = starch and beef extract; (SX) agar medium based on Starr’s medium; TSA = Trypticase soy agar; M9 = Kahn’s minimal medium; PA = Pseudomonas P agar base), using spread plate technique to isolate maximum bacterial diversity.

**Morphological characterization of bacteria**:

Different morphologically different colonies were selected and characterized Gerhardt *et al.,* (14).

**Selection of exopolysaccharide producing bacteria:** Bacterialisolates with slimy colony morphology were selected as EPS producer (15). Different culture media, P medium (16), E medium (17), Soft Brown Sugar (SBS) medium (18), MSM medium (19) and EPS medium (20), were used that supported growth and EPS production to select bacteria exhibiting mucoid mode of growth. Alcian blue 8GX staining was used to verify the EPS presence (20).

**RESULTS AND DISCUSSION**

**Comparison of homogenization methods**

Among the three methods used for the dispersal of biofilm sample blender mixing gave the highest CFU values. Sonication gave much better CFU than vortex dispersal of

**Fig1:Comparison of homogenization methods to harvest bacteria from municipal sewer biofilms on different culture media**

the biofilm sample (Fig 1). These results clearly indicate that mechanical sheering is the most effective method to disperse bacterial cells are entangled in EPS matrix. Mechanical sheering resulted in better CFU results than sonication (22). Kaplan et al., (23) reported that *Actinobacillus* biofilms are resistant to chemical (detergents, proteases etc. ) and physical agents (like heat , sonication, vortexing). Fine *et al.,* (24) also reported that mechanical scraping in combination with periodic acid ca do so. Figure 1 showed the results of three independent trials as mean log10 CFU values per gram of biofilm sample for each dispersal method.

**Isolation, enumeration and purification of bacteria:** A total of 24 bacterial strains had been isolated from mixed domestic and industrial wastewater biofilms NA and PA media supported maximum growth, figure 1.

**Morphological characteristics of the selected strains**

On the basis of colony morphology four clusters were formed which were further sub-divided as follows:

In cell morphology gram staining, capsule staining and spore staining was performed. PAS11, PAS12, PAS2, PAS3, PAS9, NAS1, NAS2, NAS5, NAS9, TSS1, TSS3, TSS5, TSS7, XAS2, XAS4, XAS6 strains were gram +ve, spore +ve and capsule +ve except TSS5 that lacked capsule.

**Figure-2. Morphological characterization of Bacterial colonies isolated from municipal sewer Biofilms.**

wastewater biofilms. Biofilms are robust structures where

These strains did not show any growth on EMB or MacConkey agar.

PAS6, NAS6, NAS8, TSS6, EMS1, EMS2, AAS1, M9S1 strains showed gram negative behavior. Spores were also lacking in these strains. All these strains showed capsules. These strains show positive growth on EMB and MacConkey agar. EMS1 and AAS1 strain produced colonies with metallic green sheen on EMB agar. EMS1 and AAS1 produced red, TSS6 pink and EMS2and M9S1 light pink colonies on MacConkey agar. Motility test revealed TSS6 and M9S1 strain to be non-motile. These results suggest that the strains PAS11, PAS12, PAS2, PAS3, PAS9, NAS1, NAS2, NAS5, NAS9, TSS1, TSS3, TSS5, TSS7, XAS2, XAS4, and XAS6 may be related to family Bacillariaceaeand strains PAS6, NAS6, NAS8, TSS6, EMS1, EMS2, AAS1, M9S1 may belong to Enterobateriaceae**.** TSS6 and M9S1 being non-motile may belong to *Klebsiella* sp., Holt *et al.*(25). Table1.

**Table 1 Morphological features of the municipal sewer biofilm isolates**

|  |  |
| --- | --- |
| **Characteristics** | **Strain** |
| **PAS11** | **PAS12** | **PAS2** | **PAS3** | **PAS6** | **PAS9** | **NAS1** | **NAS2** | **NAS5** | **NAS6** | **NAS8** | **NAS9** |
| **TSS1** | **TSS3** | **TSS5** | **TSS6** | **TSS7** | **EMS1** | **EMS2** | **XAS2** | **XAS4** | **XAS6** | **AAS1** | **M9S1** |
| **Cell Morphology** | **1** | + | + | + | + | - | + | + | + | + | - | - | + |
| + | + | + | - | + | - | - | + | + | + | - | - |
| **2** | + | + | + | + | + | + | + | + | + | + | + | + |
| + | + | - | + | + | + | + | + | + | + | + | + |
| **3** | + | + | + | + | - | + | + | + | + | - | - | + |
| + | + | + | - | + | - | - | + | + | + | - | - |
| **4** | + | + | + | + | + | + | + | + | + | + | + | + |
| + | + | + | - | + | + | + | + | + | - | - | + |
| **Growth on selective medium** | **5** | - | - | - | - | + | - | - | - | - | + | + | - |
| - | - | - | + | - | +(MG) | + | - | - | - | +(MG) | + |
| **6** | - | - | - | - | + | - | - | - | - | + | + | - |
| - | - | - | +(pink) | - | +(red) | +(LP) | - | - | - | +(red) | +(LP) |

1, gram +ve/–ve; 2, capsule; 3, spore; 4, motility; 5, EMB agar; 6, MacConkey agar; MG, colonies with metallic green sheen; LP, light pink colonies.

**Table 2: Detection of EPS Production mucoid colonies of isolated the bacterial isolates**

|  |  |  |
| --- | --- | --- |
| Strain | Mucoid colonies on respective media | EPS production on respective media |
| ***P***  | ***E***  | ***SBS***  | ***MSM***  | ***EPS***  | ***P***  | ***E***  | ***SBS***  | ***MSM***  | ***EPS*** |
| **PAS11** | + | - | + | - | - | **+** | - | **+** | - | - |
| **PAS12** | + | - | + | - | - | **+** | - | **+** | - | - |
| **PAS2** | + | - | + | - | - | **+** | - | **+** | - | - |
| **PAS3** | + | + | + | - | - | **+** | **+** | **+** | - | - |
| **PAS6** | + | + | + | - | - | **+** | **+** | **+** | - | - |
| **PAS9** | + | - | + | - | - | **+** | - | **+** | - | - |
| **+AS1** | + | + | + | - | - | **+** | **+** | **+** | - | - |
| **+AS2** | + | - | + | - | - | **+** | - | **+** | - | - |
| **+AS5** | +++ | +++ | +++++ | ++ | ++++ | **+** | **+** | **+** | **+** | **+** |
| **+AS6** | + | + | + | + | + | **+** | **+** | **+** | **+** | **+** |
| **+AS8** | + | + | + | + | + | **+** | **+** | **+** | **+** | **+** |
| **+AS9** | + | + | + | + | + | **+** | **+** | **+** | **+** | **+** |
| **TSS1** | + | - | + | - | - | **+** | - | **+** | - | - |
| **TSS3** | + | - | + | - | - | **+** | - | **+** | - | - |
| **TSS5** | ++ | +++ | +++++ | +++ | ++++ | **+** | **+** | **+** | **+** | **+** |
| **TSS6** | ++ | +++ | +++++ | ++++ | +++++ | **+** | **+** | **+** | **+** | **+** |
| **TSS7** | + | - | + | - | - | **+** | - | **+** | - | - |
| **EMS1** | ++++ | ++++ | ++++ | ++++ | ++++ | **+** | **+** | **+** | **+** | **+** |
| **EMS2** | + | - | + | - | - | **+** | **+** | **+** | **+** | **+** |
| **XAS2** | + | + | + | - | - | **+** | **+** | **+** | - | - |
| **XAS4** | + | - | + | - | - | **+** | - | **+** | - | - |
| **XAS6** |  | - | + | - | - | **+** | - | **+** | - | - |
| **AAS1** | ++++ | ++++ | ++++ | ++++ | ++++ | **+** | **+** | **+** | **+** | **+** |
| **M9S1** | + | - | + | - | - | **+** | - | **+** | - | - |

 For mucoid colonies + **= minimum; ++ =medium; +++= low ++++ = high +++++ = maximum**

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