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Evaluation of the Biofilm Forming Capacities of Bacterial Isolates Recovered in Raw and Treated Effluent from Wastewater Treatment Plant of Ahmadu Bello University Zaria, Nigeria

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ABSTRACT: Biofilm producing bacteria are associated with many recalcitrant infections and are highly resistant to antimicrobial agents, hence notoriously difficult to eradicate. This study aimed at determining the biofilm forming capacities of bacterial isolates recovered in the raw wastewater and treated effluent from Wastewater Treatment Plants of Ahmadu Bello University Zaria using Tube Method (TM) and Congo Red Agar (CRA) method; and from the results, among the isolates recovered from the raw wastewater, TM detected 62.5% isolates as positive and 37.5% as negative for biofilm production, CRA detected 37.5% isolates as positive and 62.5% as negative for biofilm production. TM also demonstrated to be more suitable in detecting biofilm producing bacterial isolates from the treated effluent were it detected 50% isolates as positive and 50% as negative. However, CRA detected only 12.5% isolates as positive and 87.5% as negative for biofilm production. We therefore, conclude that the TM is more efficient and reliable for detection of biofilm producing bacteria in the laboratory when compared to CRA method and can be recommended as one of the suitable standard screening method for the detection of biofilm producing bacteria in laboratories.

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Biofilm is as an assemblage of microbial cells that is surrounded by a matrix of Extra-Polymeric Substance (EPS) secreted by those cells. Biofilms can be composed of a pure culture, but more commonly comprise a community of mixed microbial species. The formation of a biofilm is a developmental process in which a quorum sensing signal molecule, an auto-inducer, functions to induce the secretion of EPS and leads to the formation of a characteristic three-dimensional biofilm architecture (Nadell *et al*., 2008) and also exhibit an altered phenotype with respect to growth rate and gene transcription (Donlan *et al*., 2007). Within a biofilm, bacteria communicate with each other by production of chemotactic particles or pheromones, a phenomenon called quorum sensing (Thomas *et al*., 2007). Availability of key nutrients, chemotaxis towards surface, motility of bacteria, surface adhesins and presence of surfactants are some factors which influence biofilm formation (Thomas *et al*., 2007). Microorganisms growing in a biofilm are intrinsically more resistant to antimicrobial agents than planktonic cells. High antimicrobial concentrations are required to inactivate organisms growing in a biofilm, as antibiotic resistance can increase 1,000 fold (Stewart *et al*., 2001). One mechanism of biofilm resistance to antimicrobial agents is the failure of an agent to penetrate the full depth of the biofilm (Crossley *et al.,* 2009). Polymeric substances like those that make up the matrix of a biofilm are known to retard the diffusion of antibiotics, and solutes in general diffuse at slower rate within biofilms than they do in water. Antibiotics have been shown to penetrate biofilms readily in some cases and poorly in others, depending on the particular agent and biofilm (Stewart *et al*., 2001). According to a publication by the National Institutes of Health, more than 80% of all infections involve biofilms (NIH, 2002). Biofilms are associated with many medical conditions including indwelling medical devices, dental plaque, upper respiratory tract infections, peritonitis, and urogenital infections (Reid, 1999). Both Gram-positive and Gram-negative bacteria have the capability to form biofilms. Bacteria commonly involved include *Enterococcus faecalis*, *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus viridans, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis* and *Pseudomonas aeruginosa* (Aparma *et al.,* 2008) There are various methods to detect biofilm production. However, for the purpose of this study the choice for Congo Red Agar (CRA) and Tube method (TM) was adopted because of their specificity, low operational cost and availability of materials. We screened 16 bacterial isolates by these two different methods, which could

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be used in a routine clinical laboratory, for determining their ability to form biofilm.

MATERIALS AND METHODS

Sampling Site: All the samples were collected from the Wastewater Treatment Plants of Ahmadu Bello University Zaria Kaduna State Nigeria and further analyzed at the Microbiology department laboratory from February 2018 to April 2018.

Samples collection: Batches of 100ml samples of raw wastewater and treated effluent were collected in sterile sample bottles at the intake tanks were the screened raw wastewater is emptied and at the point of discharged into the recipient water way respectively and transported in ice packs to the laboratory for physicochemical and microbiological analysis.

The waste water samples were allowed to stand on the laboratory bench for 1 hour to sediment. The supernatant were decanted to 20ml volume and the sediment re-suspended by shaking.

Congo Red Agar Method (CRA): The medium was composed of Brain Heart Infusion agar (37 g/L), sucrose (50 g/L), agar no.1 (10 g/L) and Congo red stain (0.8 g/L). Congo red stain was made ready as a strong aqueous solution and sterilized (121°C for 15 minutes) separate from the rest of the medium components and supplemented to the agar when the temperature reached 55°C.

Agar plates were prepared and inoculated and kept in the incubator at 37°C for 24 hours. The production of black colonies with a dry crystalline consistency by the organisms was taken to indicate biofilm production against red colonies produced by non-biofilm forming strains (Freeman *et al*., 1989).

Tube Method (TM): A qualitative assessment of biofilm formation was determined as previously described by Christensen *et al*. (1982). A 5ml of tryptone soy broth was inoculated with a loopful of microorganism from overnight culture plates and incubated at 37°C for 48 hours. T

he tubes were decanted and washed with Phosphate buffer saline (pH 7.3) and dried. The tubes were stained with crystal violet (0.1%), and excess stain removed and tubes washed with deionized water. Tubes were then dried in inverted position and observed for biofilm formation. Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube.

RESULTS AND DISCUSSION

In this present study, both pathogenic and potentially pathogenic bacteria were recovered in the raw wastewater (RWW) and treated effluent (TE); *Escherichia coli, P. mirabilis, S. hominis K.oxytoca* and *Klebseilla pneumonia* was detected in all samples examined as shown in Table 1 and 3. *Vibrio vulnificus*, *S. ureus* and *Pantoea agglomerans* were found only in the raw wastewater while, *Acinetobacter baumannii*, *Burkholderia cepacia* and *Aeromonas hydrophila* were found only in the treated effluent. In this study, Congo red agar method and Tube method were used as screening methods to demonstrate their varying capacity to detect biofilm formation in the raw wastewater and treated effluent isolates; among the isolates recovered from the raw wastewater, it was observed that *Escherichia coli* displayed a strong tendency to form biofilm by the two screening methods. On the other hand, Congo red agar method demonstrated highly suitable to detect biofilm formation in *Klebsiella pneumoniae* and *Klebsiella oxytoca,* but they were unable to form biofilm by the tube method. *Proteus mirabilis, Vibrio vulnificus, Staphylococcus hominis and Staphylococcus aureus* demonstrated weak tendency to produce biofilm by Congo red agar method but were able to form biofilm by tube method, while *Pantoea agglomerans* was clearly unable to form biofilm by Congo red agar and tube method because it lack the tendency to form biofilm by these two screening methods in this study as shown in Table 1. However, in the results shown in Table 2, among the isolates recovered from the raw wastewater; tube method detected 5(62.5%) isolates as positive and 3(37.5%) as negative for biofilm production, whereas Congo red agar method detected 3(37.5%) bacterial isolates as positive by which only three isolates showed black colonies with consistence crystalline appearance and 5(62.5%) were negative for biofilm production without black colonies with crystalline appearance.

Furthermore, in the isolates from the Treated effluent, Congo red agar detected only *Klebsiella oxytoca* to be positive for biofilm formation in all the isolates that exited with the effluent which could be the reason they went away with the final effluent because they could not form biofilm in the wastewater treatment plant while the tube method demonstrated a high tendency to detect biofilm formation in *E.coli, Klebsiella pneumoniae, Proteus mirabilis and Acinetobacter buamannii,* whereas *B.cepacia*, *A.hydrophilla* and *S.hominis* was clearly unable to form biofilm by the two screening methods maybe because the methods and composition of the media used in this study were not favourable for them to produce slimy layers or biofilms as shown in Table 3.

Table 1: Biofilm Production Capacities of Isolates from the Raw Wastewater

Isolates code	Isolates	Screening Methods Congo Red Agar	Tube Method
RW1	*E.coli*	+	+
RW2	*K.pneumoniae*	+	_
RW3	*K.oxytoca*	+	_
RW6	*P.mirabilis*	_	+
RW7	*V.vulnificus*	_	+
RW11	*S.hominis*	_	+
RW12	*S.aureus*	_	+
RW13	*P.agglomerans*	_	_

Key: (RW) =Raw wastewater; Number (1-13)=Isolates identification code; (+)=Positive;(−)=Negative.

Table2: Percentage Biofilm Formation Capacities of Isolates from the Raw Wastewater.

Screening method	Positive (%)	Negative (%)
Congo Red Agar	3(37.5)	5(62.5)
Tube Method	5(62.5)	3(37.5)

Table 3: Biofilm Formation Capacities of Isolates from the Treated Effluent

Isolates code	Isolates	Screening Methods Congo Red Agar	Tube Method
TE14	*E.coli*	_	+
TE15	*K.oxytoca*	+	_
TE16	*K.pneumoniae*	_	+
TE17	*P.mirabilis*	_	+
TE18	*A.baumannii*	_	+
TE19	*B.cepacia*	_	_
TE20	*A.hydrophilla*	_	_
TE21	*S.hominis*	_	_

Key: (TE) =Treated Effluents; Number (14-21) = Isolates identification code; (+) = Positive; (−) = Negative

Table 4: Percentage Biofilm Formation Capacities of Isolates from the Treated Effluent

Screening methods	Positive (%)	Negative (%)
Congo Red Agar	1(12.5)	7(87.5)
Tube Method	4(50)	4(50)

Additionally, the results also depicted that in the treated effluents isolates, TM detected 4(50%) isolates as strong positive and 4(50%) to be negative, while CRA detected 1(12.5%) isolate as positive with which only one isolates showed black colonies with crystalline appearance and 7(87.5%) as negative for biofilm production as shown in Table 4.The occurrence of these bacterial isolates in these samples from the WWTP Ahmadu Bello university Zaria Samaru campus, may be attributed to the isolates ability to develop various mechanisms which help and supported their growth within the environment and for the facts that the raw wastewater is heavily loaded with microbial contaminants both organic and inorganic substances. Bacteria isolates obtained in this finding were similar to those obtained in other study by Chikere and Azubuike (2014) where they isolated bacteria from different micro-habitant in Rivers state. The occurrence of *E. coli* in the raw wastewater and treated effluent explain widely its used as a fecal contamination indicator in aquatic environment, its survival in a water environment requires the ability to withstand environmental stresses, such as nutrient deprivation, low temperature, salinity, exposure to solar radiation, protozoan grazing and competition with autochthonous microbial communities (Pachepsky and Shelton,2011). Similar occurrence of *Staphylococcus aureus, Proteus* spp *and E. coli* in the raw wastewater samples has been also reported by (Nusa, *et al.,* 2016), in the isolation of bacterial community from the wastewater treatment plant in Zaria Nigeria. This result was in line with the study done by Khalid and Afaf (2014) who reported poor biofilm production by bacterial isolates using Congo red agar method in a similar study. In another study, Ruzicka *et al.* (2004) noted that out of 147 isolates of *S. epidermidis*, Tube method detected biofilm formation in 79 (53.7%) and Congo Red Agar method detected 64 (43.5%) isolates. They showed that Tube method is better for biofilm detection than Congo Red Agar (Ruzicka *et al*., 2004). Baqai *et al (*2008*)* tested TM to detect biofilm formation among uropathogens. According to their results, 75% of the isolates exhibited biofilm formation (Baqui *et al*., 2008) With the Congo Red Agar method, 11 were found to be biofilm producing bacteria and 99 as non-biofilm producers. Knobloch *et al* (2002) did not recommend the Congo Red Agar method for biofilm detection in their study, Out of 128 isolates of *S. aureus*, Congo Red Agar method detected only 3.8% as biofilm producers as compared to other screening methods such as Tube method and Tissue Culture Plate method

which detected higher number of biofilm producers from *S.aureus*. All the bacterial isolates detected to be positive for biofilm production in this study could be attributed to their acquisition of new genetic trait which gave them chances to transcribe the necessary genes and became active members of the biofilm community.

Conclusion: We therefore, conclude from our study that Tube method (TM) is a more efficient and reliable method to detect biofilm forming bacteria when compared to Congo red agar (CRA) methods and can be recommended as a general screening method for detection of biofilm producing bacteria in laboratories.

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