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Comparative evaluation of methods to detect biofilm formation by bacterial isolates of poultry environments

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R. Manikandan Suresh, S.S. Raja and R. Vijayakumar*

Research Department of Microbiology, Bharathidasan University Constituent College, Kurumbalur - 621 107, Perambalur District, Tamilnadu, India

Abstract

The importance of biofilm study in the environment of the poultry production and processing industries and inhibition of their growth is inevitable to maintain the hygienic atmosphere and ultimately ensure overall quality of poultry. Biofilm forming microbes on the surface of materials can also caused biofouling smell. In the present study, a total of 6 different biofouling bacteria (BFB) were isolated from poultry samples like cage, feeding bottle, tape connections and floor surface of several poultries on nutrient agar medium. Of the 6 different biofouling bacteria, three isolates belonged to the genus *Bacillus* spp. (BFB1, BFB2 and BFB3), one each belonged to *Staphylococcus* sp. (BFB4), *Micrococcus* sp. (BFB5) and *Streptococcus* sp. (BFB6). The biofilm formation was detected by tube method (TM), Congo red agar (CRA) method and tissue culture plate (TCP) method. The biofilm forming ability of the biofouling bacteria was varied between the species and methods employed. Our study indicates that the tube method is an accurate and reproducible method for detecting biofilm and this technique can serve as a reliable quantitative tool for determining biofilm formation.

Keywords: Poultry, biofilm formation, screening methods.

INTRODUCTION

The formation of biofilm in food processing environments is a special concern in particular with pathogenic bacteria because of their survival rate and higher chance to contaminate food items. In food industry, biofilms have a high food residues and mineral contents that originate with product and processed water. These constituents also provide protection to microorganisms held within the biofilm. Biofilms in nature can have a high level of organization, as they may exist in single or multiple species communities, form a single layer or 3 dimensional structures, or take the form of aggregates such as flocs or granules (Bryers, 1987; Allison and Sutherland, 1987; Bagge *et al.*, 2001). Biofilms exist on various types of surfaces of food plants, such as metal, concrete floors, walls, pipes, nylon materials, glass, rubber, steel, wood, plastic, etc (Costerton *et al*., 1995; Chmielewski and Frank, 2003;). Biofilm formation can cause mechanical blockage in fluid handling systems, the impedance of heat transfer (Sandu and Singh, 1991) and corrosion to metal surfaces (Bryers, 1987), though these problems are not common in the food industry. In natural habitats, majority of microorganisms are capable to attach to the surfaces (Davey and O'Toole, 2000), indicating the extent of the selective advantage for biofilm growth. In particular, poultry is one of the fastest growing agricultural and food sector in India. This directs to noteworthy raise in the production of broilers at 8 to 10% per annum with an annual turnover of 30,000 crores (Mehta and Nambiar, 2007). Because of lowest investment and requirement of small area the numbers of poultry shops have increased creating vast employment opportunities (Agblevor *et al*., 2010). Unfortunately, these poultry industries leads to the generation of huge wastes usually composed of broiler and layers, feathers, bones, blood, hatchery debris and dead birds. These wastes pose serious environmental pollution issues through pathogenic microbial infection, offensive odours, promotion of flies and rodent breeding (Adeoye *et al.,* 1994). There are no proper waste managing units for these wastages in developing countries like India but attempts are in progress to reuse these materials as beneficiary ones such as fertilizers and animal feed supplements (Kelleher *et al.,* 2002). As these wastes are composed of tissues and blood, they may serve as a reservoir for the multiplication of several pathogenic microorganisms that can cause severe disease outbreaks. Both the gram positive and gram negative bacteria have the capability to form biofilms, it is very important to screen all the microorganisms isolated from contaminated area for the production of biofilm by various techniques at *in vitro* level so as to achieve the real picture of biofilm producers. Hence, the present study was carried out to screen the biofilm producing ability of bacteria isolated from biofouling samples of poultry by various

*Corresponding Author :
email: *vijayakumarkmng@gmail.com*

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techniques and identify those bacteria using conventional techniques.

MATERIALS AND METHODS

Study area: The biofouling samples were collected from the interior area like cage, feeding bottle, and tape connections of poultry farms in and around Namakkal district, Tamil Nadu, India. Samples were collected from six different locations namely P. Velur, Mohanur, Vasanthapuram, Pachudayam Patty, Nallipalayam and Thummankurichi.

Collection and processing of biofouling samples: The samples were collected according to the standard microbiological procedures of Cappuccino and Sherman (2005). Two grams of samples were collected in sterile containers using sterile spatula and transported to the laboratory for further analysis.

Isolation of biofouling bacteria: About one gram of fouling sample was serially diluted using sterile distilled water blanks. One hundred microlitre of aliquot from 10^{-3} to 10^{-5} was transferred and spread over nutrient agar plates using sterile L-rod. Plating was done in duplicate and all the plates were incubated at 37ÚC for 24 h. Morphologically different bacterial colonies were selected, purified and sub-cultured on nutrient agar slants (Bavya *et al*., 2011).

Characterization and identification of biofouling bacteria: Characterization and identification of biofouling bacteria were carried out based on their growth pattern and colony characteristics on selective and differential media. In addition, Gram staining, motility determination, IMViC tests, and production of catalase, oxidase and urease were carried out according to the standard procedures (Cappuccino and Sherman, 2005). Based on the phenotypic characteristics, the biofouling bacteria were identified at genus level.

Detection of biofilm formation biofouling bacteria

1. Plate Method: Mucoid nature of the bacterial colonies was studied by cultivation of all the strains on Congo red agar (CRA) plates (Mariana *et al*., 2009). Eighteen hours old bacterial cultures were streaked on the CRA plates. Plates were incubated at 28°C for 24-48 h and observed for the characteristic colony morphology.

2. Tube method (TM): The capabilities of biofilm formation by the bacterial isolates were observed by the way of adherence to the walls of culture tubes (Mathur *et al*., 2006). Inoculum was prepared using 2 ml of nutrient broth. After 24 h of incubation at 28°C, turbidity was adjusted to 0.5 McFarland standards. For biofilm experiment, 100 ìl of inoculum was transferred into 3 ml of nutrient broth in 10 ml test tubes. All the test tubes were kept in shaker at 95 rpm speed for 24-48 h. After incubation, culture broth containing the cells was discarded. The tubes were washed with 3 ml of 1X phosphate buffer saline (PBS). About 3 ml of 2% crystal violet solution was added and allowed for 5 min. All the tubes were washed with sterile water and allowed to dry, and the tubes were visually observed for the presence of biofilms on the inner walls of the test tubes. All the tubes were added with 1.5 ml of 33 % glacial acetic acid and mixed gently. Optical density (OD) value was measured in colorimeter at 570 nm. The OD values of the test samples were compared with the PBS as control. The biofilm formation experiment in tubes was performed using both glass and plastic (PVC) tubes.

3. Tissue culture plate method (TCP): The TCP assay for the detection of biofilm formation was carried out by the standard method as described by Christensen *et al.* (1985). All the biofouling bacterial isolates were screened for their ability to form biofilm on three different media, tryticase soy broth (TSB: Difco), TSB with 1% glucose (TSB: glu) and brain heart infusion (BHI: Difco) with 2% sucrose (BHISuc). Fresh cultures of biofouling bacteria were inoculated with 0.2 ml aliquots in to all the three different media and transferred to sterile, polystyrene, 96 well-flat bottom tissue culture plate (TCP) (Tarson, Kolkata, India) wells. All the TCP were incubated for 18 h at 37°C, un-inoculated broth was maintained as control. After incubation, content of each well was gently removed by tapping the plates. The wells were washed four times with 0.2 mL of PBS (pH 7.2) to remove free-floating 'planktonic' bacteria. Biofilms formed by adherent 'sessile' bacteria in plates were fixed with sodium acetate (2%) and stained with crystal violet (0.1% w/v). Excess stain was rinsed off by thorough washing with deionized water and plates were allowed for drying. Optical density (OD) of stained adherent bacteria was determined with a micro ELISA auto reader (model 680, Bio rad) at wavelength of 570 nm. These OD values were considered as an index of bacteria adhering to surface and forming biofilms. All the experiment was performed in triplicate and standard deviation calculation was made for the average data. Based on OD values obtained for individual strains of biofouling bacteria, their biofilm formation ability was categorized to 3 types as mentioned below (Table 1).

Table 1. Classification of bacterial adherence by TCP method

Mean OD values (at 570 nm)	Adherence	Biofilm formation
<0.120	Non	Non / weak
0.120-0.240	Moderately	Moderate
>0.240	Strong	High

RESULTS AND DISCUSSION

Generally, in most locations including natural, industrial, or clinical, biofouling bacteria are found in biofilms rather than in the planktonic state. Biofilms also provide important environmental reservoirs and protection for pathogenic bacteria (Parsek and Singh, 2003). Investigations to understand the pathogenesis of these infections have focused upon the process of adherence of these microorganisms on these devices. Investigators have used various methods to quantify number of microorganisms adhering to surfaces (Freeman *et al*., 1989; Donlan *et al*., 2001; Johannes *et al*., 2002). In the present study, 6 different biofouling bacterial isolates were isolated from the biofouling samples of poultry industry of in and around Namakkal district, Tamilnadu. Among the 6 isolates, three bacterial isolates belonged to the genus *Bacillus* spp. (BFB1, BFB2 and BFB3), one isolate each belonged to *Staphylococcus* sp. (BFB4), *Micrococcus* sp. (BFB5) and *Streptococcus* sp. (BFB6) (Fig. 1). Characteristic features of all the biofouling bacteria were studied already and reported by Manikandan and Vijayakumar (2016).

Biofilm detection from biofouling bacteria was done by TM method, CRA method and TCA method. These methods require the use of especially solid media-brain heart infusion broth supplemented with 5% sucrose and Congo red stain (Rewatkar and Wadher, 2013). It was observed that, most of the strains produced white coloured colonies on CRA medium, while *Staphylococcus* sp. BFB4 isolate produced black coloured colonies, which was typical indication for the production of biofilm. The isolate BFB1, produced red coloured colonies with dry crystalline morphology after 24-48 h (Fig. 2). Correspondingly, Mathur *et al.* (2006) reported that screening of biofilm formation by CRA method provided very different results, most of the bacterial strains displayed red (pink to orange) colonies on CRA medium. The present study has been reported that the nutrient composition of the CRA medium in particular supplementation of various sugars will greatly influenced the colony morphology as well as biofilm formation efficiency of the biofouling bacteria.

Biofilm forming capacity of all the isolates was also evaluated by adopting plate as well as tube method (Gopikrishnan *et al*., 2013). By TM, the biofilm formation was confirmed by formation of visible thick film inside the wall of tube and bottom of the tube. The isolates BFB1, BFB2 and BFB4 were showed thick film inside the bottom of the glass tube indicating strong biofilm production, while other isolates were not showed the formation of biofilm in the glass tubes (Fig. 3a).

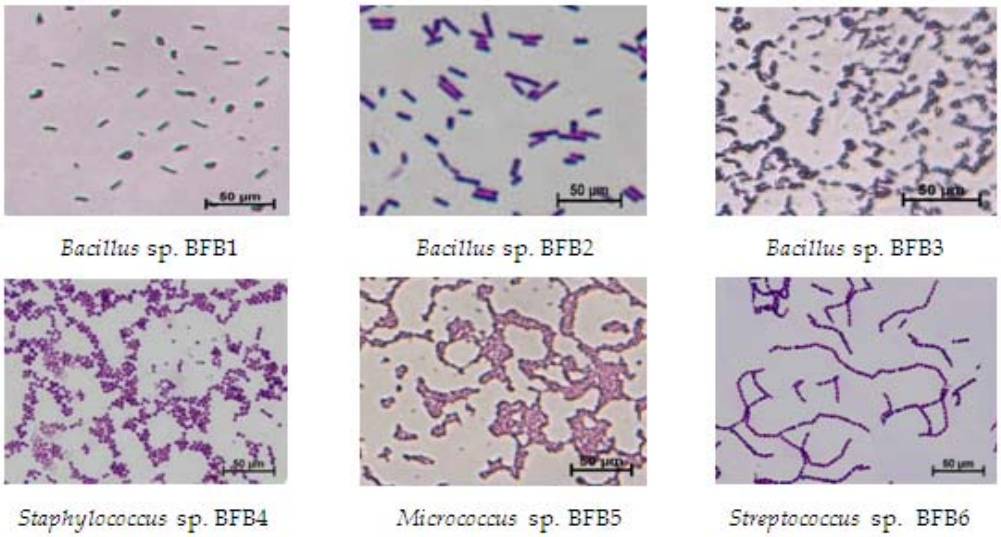

Fig. 1. Microphotograph of Gram's stained biofouling bacterial isolates

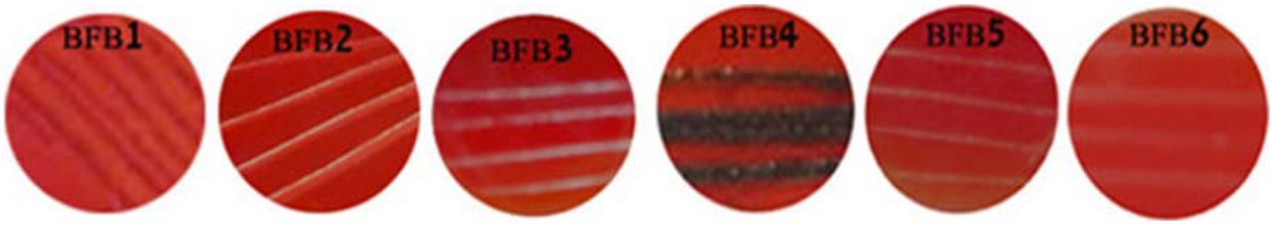

Fig. 2. Screening of biofilm production on Congo red agar (CRA) medium

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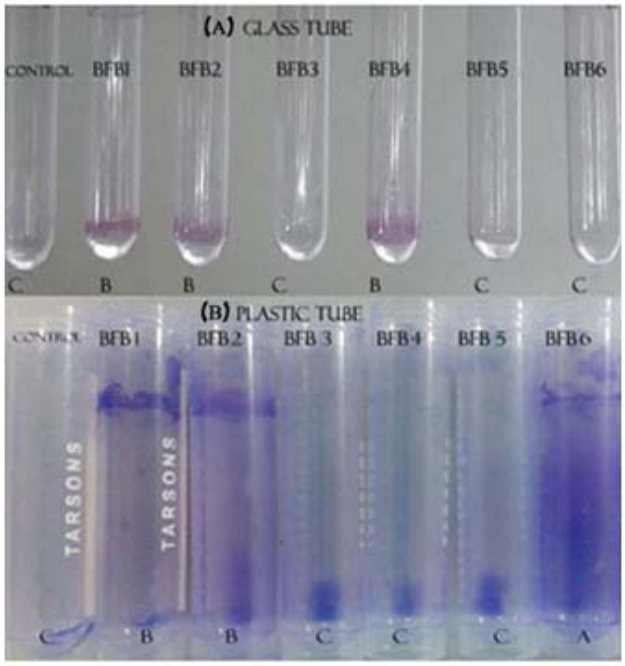

Fig. 3. Screening of biofilm production by tube methods

Whereas, the isolates BFB1, BFB2 and BFB6 were showed thick film inside the wall of the plastic tube indicating strong biofilm production, while other isolates showed weak biofilm formation (Fig. 3b). Based on the biofim formation of the isolates in TM, it has been reported that the ability of bacterial attachment and growth on the surface of the test tubes will be highly varied depends on the substrate nature like smooth and roughness.

Screening of biofilm producers by TCP method is highly effective method, in this method categorized the biofilm producers as high, moderate and non slime producers and easily differentiated with crystal violet staining in 96 well tissue culture plate (Mathur *et al*., 2006). The present study also screened the biofouling bacteria for their biofilm producing capacity by TCP method. The

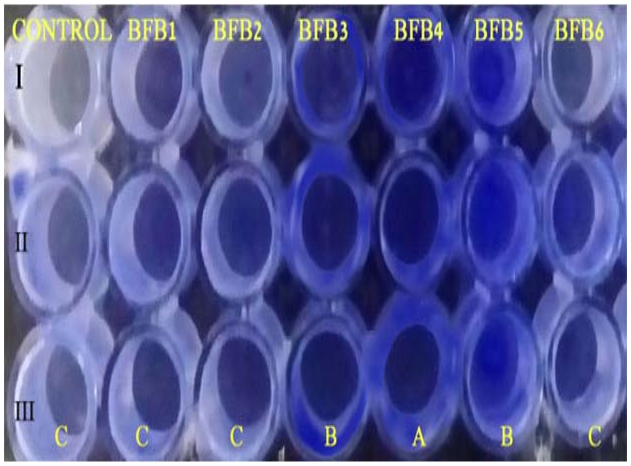

Fig. 4. Screening of biofilm production by TCP method (A: High; B: Moderate; C: Non/weak)

isolate *Staphylococcus* sp. BFB4 was produced strong biofilm, whereas *Bacillus* sp. BFB3 and *Micrococcus* sp. BFB5 were produced moderate level of biofilm. In contrast, the isolates BFB1, BFB2 and BFB6 were formed weak biofilm on the side wall of TCP (Fig.4-5). Similar type of work has also been reported by Gopikrishnan *et al*. (2013), they isolated a total of 55 biofouling bacteria belonged to the genus *Bacillus, Aeromonas, Micrococcus, Alcaligenes, Lactobacillus, Staphylococcus*, *Pseudomonas* and *Kurthia* from Muttom, Parangipettai, Nagapattinam and Ennore coastal areas. Out of 55 bacterial isolates, 25 isolates produced positive results for biofilm formation in which the species belongs to the genus *Staphylococcus, Micrococcus* and *Alcaligenes* were identified as strong biofilm. Likewise, in the present study, almost all the isolates produced biofilm at least any one method. Comparatively, the isolate BFB6 produced strong biofilm in TCP method (Fig. 4-5), but this isolate was not produced biofilm in CRA method and in glass tubes. This indicated strongly that the surface of the substratum will greatly influence the biofilm formation. Environmental stresses such as low nutrient availability trigger phenotypic changes of planktonic (free living) cells to the sessile (attached) form (Costerton *et al*., 1987; Carpentier and Cerf, 1993; Mittelman, 1998).

Conclusively, the biofilm forming ability of an organism would potentially enhance their survivability in any environment. The production of biofilm by an organism may influenced by several factors including substratum composition, surface chemistry and topography, moisture content and fluid flow. The present study

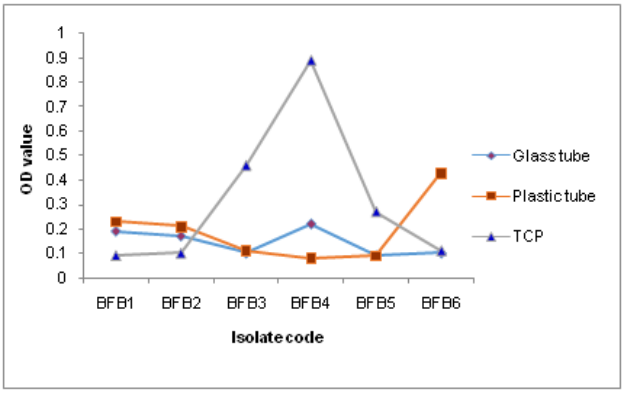

Fig. 5. Biofouling bacterial biofilm ability by TM & TCP methods

specifically reported that the methods adopted in *in vitro* condition also influence the detection of formation of biofilm. It is also suggested that the tube and TCP methods are accurate and reproducible method for screening of biofilm and these techniques can serve as a reliable quantitative tool for determining biofilm formation by poultry isolates of bacteria.

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