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EVALUATION OF DETECTION METHODS OF BIOFILM FORMATION BY *BACILLUS CEREUS* AND *STAPHYLOCOCCUS AUREUS* ISOLATES FROM FOODS

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ABSTRACT: Biofilm formation is a common feature of food processing surfaces. Biofilm producing bacteria are different from their planktonic counterparts because of their altered gene expression. Biofilm production is an important property of pathogenic bacteria being extraordinarily resistant to different control measures. The objective of the study was to detect biofilm-producing ability of food isolates by three different methods and to evaluate these methods for suitability. Previously isolated cultures of *Bacillus cereus* and *Staphylococcus aureus* from road-side foods sold in and around Kolkata were used in the study. Three different methods viz., congo red agar (CRA) method and tube method (TM) (both qualitative) and tissue culture plate (TCP) method (quantitative) were employed. The two qualitative methods viz., CRA and TM could detect 60.71 and 17.86% of isolates, respectively, as biofilm-producer. Most of the isolates were non or weak biofilm-producer in TCP method. Only 3.57% of isolates were capable of producing moderate biofilm by TCP method. In conclusion, TCP is a quantitative, accurate and reliable method to detect biofilm forming microorganisms and thus recommended as general screening method for detecting biofilm-producing isolates from food.

KEYWORDS: Bacterial community, food pathogens, biofilm, congo red agar, tube method, tissue culture plate method

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1.INTRODUCTION

Biofilm is a community of microbes embedded in an organic polymer matrix. Many bacteria spend the most part of their life cycle within surface-attached sessile communities encased in a polymer matrix [1]. Biofilm formation is an age old process and integral component of the prokaryotic life cycle for survival in diverse environments [2]. Formation of biofilm starts when bacterial cells come in contact with surfaces in aqueous environments and excrete a slimy, glue-like substance. Biofilm formation takes place in various phases [3]. In the first phase planktonic cells come in contact with suitable surfaces and bind reversibly by weak van der Waals forces. However, because of weak interaction cells may wash away quickly. If the cells can overcome the prevailing mechanical forces, various surface appendages including fimbriae, pili, flagella, lipopolysaccharide and membrane proteins may aid in permanent attachment of the bacterial cells to the surface. Both abiotic and biotic surfaces including metals, plastics, soil particles, medical implant materials and most significantly, human or animal tissue are important surfaces where biofilm formation takes place. Conditioning of surfaces by deposition of organic and inorganic matter is an important pre-requisite of biofilm formation over abiotic surface. Complex polysaccharides, glycoproteins and humic compounds are important conditioning materials altering the physicochemical properties of the surfaces including free energy, hydrophobicity and electrostatic charges. Production of exopolymeric substances (EPS) by the initial colonizers is the most important pre-requisite of biofilm formation. EPS help the primary colonizers to anchor the surface irreversibly as well as provide the structural matrix of biofilm. Now the bacterial cells start multiplication resulting in microcolony formation. Over this structural matrix newer cells are recruited and secondary colonizers may attach in succession. At the final stage of biofilm formation also called maturation, biofilm is established and may only change in shape and size. During maturation of biofilm, the pathogens can communicate with each other via a group of chemical messengers called auto-inducers. Biofilm mode of life cycle is continued by dispersion of surface attached cells into the surroundings where the dispersed cells attach to new sites to form another biofilm [4,5]. Besides bacteria, viruses, fungi and Archaea have been found to possess mechanisms of biofilm formation. Cells inside biofilm matrix become more resistant to antibiotics and disinfectants; some having thousand times more antibiotic resistance have also been reported. Biofilms are often compared to the tissues of higher organisms having differential gene expression pattern with channels for nutrient transport and waste removal. Biofilm formation in food processing environments is a public health concern. Food processing surfaces, conveyors, pipe lines, valves, pumps and tanks are frequently associated with biofilm producing microorganisms [6]. Spores of *Bacillus* spp. being hydrophobic can easily attach themselves to pipelines, joints and bakery equipments causing spoilage of dairy and bakery products. Research on clinical isolates of *Staphylococcus aureus* demonstrated biofilm production in both static and continuous flow

conditions under the influence of *ica* operon [7]. Contamination of food processing surfaces and utensils by biofilm producing strains of *B. cereus* and *S. aureus* is a serious public health concern especially for those foods which do not undergo heat treatment before consumption. Biofilm production by clinical isolates is well reported but from food isolates especially of ready-to-eat type is still under-reported. This paper deals with the evaluation of three different biofilm detection methods employing previously isolated *B. cereus* and *S. aureus* from foods sold in road-side eateries in and around Kolkata.

2. MATERIALS AND METHODS

Formation of biofilm was tested by following three methods.

Congo red agar (CRA) method

Sterile Brain heart infusion (BHI) broth supplemented with 8% glucose ($wv^{-1)}$ and 1% (wv^{-1}) agar was mixed with sterile solution of congo red at a final concentration of 0.08% (vv^{-1}) in molten condition to prepare CRA plate [8]. Black colonies with a dry crystalline consistency indicated biofilm production (Fig 1.). Biofilm negative strains produced white or very light pink coloured colonies.

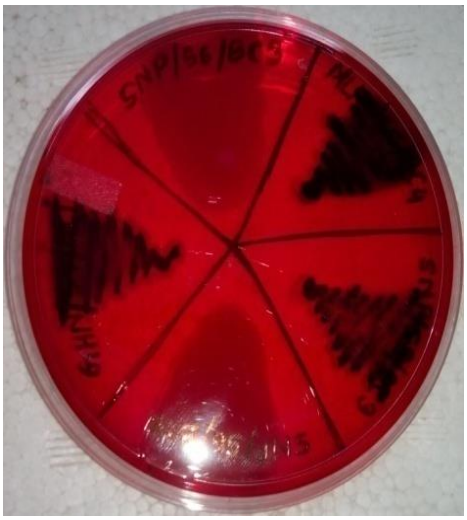

Fig 1. CRA method showing positive and negative results

Tube method (TM)

Isolates were grown in tryptone soya broth (TSB), washed with phosphate buffer saline (PBS) of pH 7.4 and dried. Dried tubes were stained with 0.1% (wv^{-1}) crystal violet solutions, washed with distilled water and again dried. Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube (Fig 2.). Ring formation at the liquid interface was not indicative of biofilm formation [9].

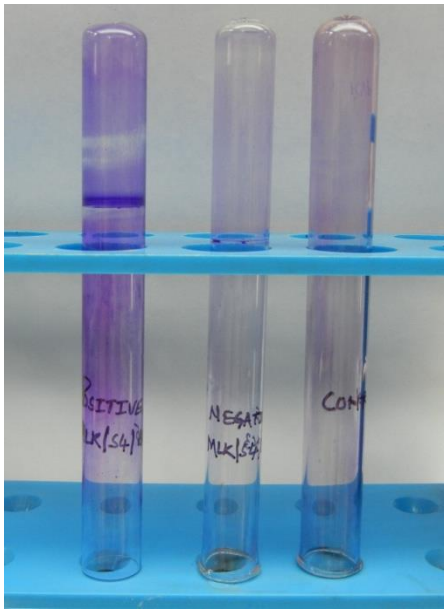

Fig 2. Tube method showing positive and negative results with control

Tissue culture plate (TCP) method

Isolates were grown in TSB at 37 °C for 18 h and then diluted 100 times with fresh TSB. An aliquot of 200 µl of diluted culture was dispensed in each well of polystyrene tissue culture plate and incubated at 37 °C for 18 h. The negative control wells contained TSB only. After incubation content of each well was gently removed by tapping and washed three times with sterile PBS (pH 7.4). The remaining attached bacteria were fixed with 2% sodium acetate for 15 min, dried and stained with 0.1% crystal violet solution for 5 min. Excess stain was rinsed off by distilled water, dried and optical density of the content (A) in each well was then recorded at wavelength of 560 nm (OD_{560}) [9]. The cutoff absorbance (Ac) was the mean absorbance of the negative control. Biofilm formation was interpreted as in Table 1 [10].

Statistical analysis

The TCP method was considered the gold standard for biofilm production tests. Parameters like sensitivity, specificity, positive predictive value, negative predictive value and accuracy were calculated for CRA and TM method comparing with data obtained from TCP method [11].

Table 1. Interpretation of biofilm production by TCP mehod

Average OD_{560}	**Biofilm production**
A=Ac	No
Ac<A=2Ac	Weak (+)
2Ac<A=4Ac	Moderate (++)
4Ac<A	Strong (+++)

3. RESULTS AND DISCUSSION

Two types of bacteria viz., *B. cereus* and *S. aureus* used in this study were previously isolated from four different types of foods collected from road-side shops in and around Kolkata, West Bengal [12]. Of these, ghugni is a popular snack while laddu and soan papdi are sweets. A total of thirty-five *B. cereus* and forty-nine *S. aureus* isolates from seventeen number of food samples were tested for possible biofilm production (Table 2). The bacteria were isolated using selective differential culture media and confirmed by morphological, biochemical and physiolological methods.

Table 2. Sources of bacteria used to study biofilm production

Bacteria	Source			
	Ghugni (n=4)	Laddu (n=6)	Milk powder (n=3)	Soan papdi (n=4)
B. cereus	9	10	6	10
S. aureus	9	19	10	11

Result of biofilm production by *B. cereus* and *S. aureus* isolates is presented in Table 3. Of the 35 *B. cereus* isolates, 20 i.e., 57% showed positive biofilm production by CRA method. Food-wise analysis showed that 70, 56 and 50% of the isolates from laddu, ghugni and both milk powder and soan papdi, respectively, produced biofilm by CRA method. Similarly 6 i.e., 17% of the *B. cereus* showed biofilm production by TM. Food-wise percentages were 30, 20 and 11 for soan papdi, laddu and ghugni isolates, respectively. Nineteen i.e., 54% percent of the *B. cereus* isolates were biofilm-producer by TCP method. Food-wise positive percentages were 67, 60, 50 and 33, respectively, for ghugni, soan papdi, laddu and milk powder isolates.

Table 3. Bacteria producing biofilm by different methods

Bacteria	Source	Total isolates	Biofilm positive		
			CRA method	TM	TCP method
B. cereus	Ghugni	9	5	1	6
	Laddu	10	7	2	5
	Milk powder	6	3	0	2
	Soan papdi	10	5	3	6
S. aureus	Ghugni	9	6	3	6
	Laddu	19	12	2	17
	Milk powder	10	7	3	10
	Soan papdi	11	6	1	7

Thirty-one of the 49 i.e., 63% of the *S. aureus* isolates produced biofilm by CRA method. Food-wise percentages were 70, 67, 63 and 54 for milk powder, ghugni, laddu and soan papdi, respectively. TM could confirm 9 i.e., 18% of *S. aureus* isolates as positive biofilm producer. Food-wise percentages were 33, 13, 11 and 9, respectively, for ghugni, milk powder, laddu and soan papdi isolates. By TCP method, 40 i.e., 82% of *S. aureus* isolates were found to be biofilm-producer. Food-wise positive percentages were 100, 89, 67 and 64 for milk powder, laddu, ghugni and soan papdi isolates, respectively. *B. cereus* is a notorious food pathogenic as well as spoilage causing bacteria present predominantly in milk and dairy products, cereals, pulses, spices etc. Besides stress resistant endospores having high hydrophobicity, formation of biofilm by *B. cereus* in different substrata is a major problem [13]. *S. aureus* on the other hand is a frequent contaminant of food processing plants and has been implicated in severe outbreaks [14]. Adherence to surfaces and formation of biofilm enhance growth and survival of the cells as the common sanitizers hardly penetrate thick exopolysaccharide matrix surrounding the cells. The presence of undesirable biofilms on food processing contact surfaces may lead to: (1) transmission of diseases; (2) food spoilage; (3) shortened time between cleaning events; (4) contamination of product by nonstarter bacteria; (5) metal corrosion in pipelines and tanks; (6) reduced heat transfer efficacy or even obstruction of the heat equipment. Despite the significant problems caused by biofilms in the food industry, biofilm formation in these environments is still poorly understood and effective control of biofilms remains challenging [15]. Food processing surfaces are favorable for biofilm production being laden with moisture, nutrients and constant supply of inoculums from raw materials [16]. Air-liquid interface has been found to be the most preferred environment for *B. cereus* biofilm formation [17]. However detection rate varies by test procedures because of differences in medium composition, hydrophobicity of test surfaces, inoculum size etc. The three methods viz., CRA plate, TM and TCP could detect 60.71, 17.86 and 70.24% of isolates, respectively, as biofilm-producer. Of these three methods, TCP, which is a quantitative method, detected most of the isolates as non or weak biofilm-producer. Only three i.e., 3.57% of isolates, one each from ghugni, laddu and soan papdi samples were capable of producing moderate biofilm by TCP method (Table 4.). All these three positive isolates were *S. aureus* as none of the *B. cereus* isolates could be detected as even moderate biofilm-producer by this method. Two isolates one each of *B. cereus* from soan papdi and *S. aureus* from laddu produced biofilm all over the bottom, wall and interface of test tube in TM to be designated as strong biofilm-producer. These two isolates produced biofilm by CRA method too. But the *B. cereus* isolate was detected as weak biofilm-producer by TCP method.

Table 4. Quantitative biofilm production by TCP method

Food	Biofilm negative	Biofilm positive			Total
		Weak	Moderate	Strong	
Ghugni	6	11	1	0	18
Laddu	7	21	1	0	29
Milk powder	4	11	1	0	16
Soan papdi	8	13	0	0	21

Among the three different methods of testing, CRA plate method is simple, economical and sensitive. CRA plate method detected highest number of isolates as biofilm-producer. Supplementation of congo red agar with high concentration of glucose stimulates slime production which may combine with Congo red and yield a black colour. Addition of sugar to TSB has profound effect on quantitative biofilm production by TCP method as sugar helps in biofilm formation [11]. No supplementation of sugar to TSB in our study may be the reason behind un-detection of any strong biofilm-producing bacteria by TCP method. Five of the isolates were found to be false positive by CRA method while sixteen were false negative. CRA method is 100% sensitive, 41% specific and 43% accurate for biofilm detection. In TM none was false positive but forty-six were false negative when compared with TCP method considering weak and moderate biofilm producers. TM is 100% sensitive, 85% specific and 86% accurate for biofilm detection (Table 5). Thus TM correlates better than the CRA method with TCP method. But none of the methods correlates well with TCP method as TSB was not supplemented with sugar and no strong biofilm producing bacteria was detected by TCP method. However, detection of biofilm producing bacteria from the road-side eateries in and around Kolkata poses serious public health concern.

Table 5. Diagnostic parameters of CRA method and TM for biofilm production

Screening method	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Accuracy (%)
TM	100	85.19	20	100	85.71
CRA	100	40.74	5.88	100	42.86

4. CONCLUSION

B. cereus and *S. aureus* isolates from foods are potential biofilm producers. However detection rate varies by different methods. Presence of sugar stimulates biofilm production. Of the three detection methods TCP is a quantitative, accurate and reliable method to detect biofilm forming microorganisms and thus recommended as general screening method for detecting biofilm-producing isolates from food.

5.ACKNOWLEDGEMENT

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6. CONFLICT OF INTEREST

The authors declare that there is no financial interest or any conflict of interest.

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