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RESEARCH ARTICLE

DIVERSITY CHARACTERIZATION OF BIOFILM FORMING MICROORGANISMS IN FOOD SAMPLED FROM LOCAL MARKETS IN KOCHI, KERALA,INDIA

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ABSTRACT

Biofilms are of considerable interest in food hygiene given that it leads to serious health problems and economic loss due to food spoilage. Food samples including beef, cheese, raw milk, pasteurized milk, curd, chilly powder, turmeric powder, coriander powder, soft drinks, fresh fish, dried fish and dried prawn were tested for the presence of food borne pathogens using standard plate count assay. Quantification of biofilm formation was by microtitre plate assay. Thirty six isolates which are indicative of great threat in the food industry were characterized. Twenty isolates were strong biofilm producers, ten moderate biofilm formers, while only one was a weak producer. Five did not produce any biofilm. The antibiogram of the twenty strong producers showed multiple resistance to antibiotics. The enzyme profiling of the strong producers showed that most produced more than one enzyme, which is indicative of their competence in reduction the nutritional value of food and causing spoilage.

INTRODUCTION

Biofilms are microbially derived sessile communities characterized by many cells attached to an abiotic or living surface, and embedded in a matrix of extracellular polymeric substances produced by the cells. Biofilm formation has been noted in fossil records (~3.25 billion years ago) (Maric and Vranes, 2007). Antony Von Leeuwenhoek is credited with the discovery of biofilm on his own tooth surface. The first published report on biofilms by Zobell in 1943, used buried slide culture method, where the slides buried in the soil had an attachment of microorganisms (Kokare*et al*., 2009). Angst (1923) observed that the marine bacterial load on hulls of ships was higher than the free floating cells, and also proposed that bacterial biofilms led to serious corrosion of these hulls. By 1980s, bacteria were observed on the solid surfaces of many ecological environments including waste water treatment systems, equipments used in the manufacture of vinegar, industrial water systems, tooth decay, urinary tract and also on other implanted medical devices (Zottola and Sasahara, 1994). These observations led to the development of new electronic techniques including scanning electron microscopy, transmission electron microscopy and laser scanning confocal microscopy.

The formation of biofilm is a complex and dynamic process involving different steps (Costerton*et al*., 1994(b) and Melo*et al*., 1992), such as conditioning of the surface, adhesion of cells, formation of microcolony, biofilm formation, detachment and dispersal of biofilms.

In the food industry, biofouling causes grave problems, impeding heat flow across surfaces, increase in fluid frictional resistance and corrosion rate at the surface, all leading to energy and product losses. Biofilms due to spoilage and pathogenic microflora on surfaces of food like poultry, meat and in processing environments also pose considerable problems of cross contamination and post-processing contamination. Therefore in the context of food hygiene biofilms have been of considerable interest, as they may contain spoilage and pathogenic bacteria increasing contamination and risk to public health.

The microbes involved in biofilm formation and health risks include bacteria belonging to the genera *Vibrio, Salmonella, Pseudomonas, Listeria, Bacillus, Escherichia, Clostridium,* to name a few. With the emergence of resistance in pathogenic bacteria to traditional antibiotics, development of alternative control measures gained momentum. In addition, microorganisms produce saccharolytic, proteolytic, pectinolytic and lipolytic enzymes, whose metabolic end products are associated with food spoilage and poisoning. Thus the food industry faces multitude of challenges to keep products safe and free of pathogenic microorganisms for the consumers and also to augment product shelf life

The present study focuses on the bacterial biofilm formers in food available in the markets and their characterizations, since food poisonings and food pathogen related health risks and deaths are increasing by the day.

MATERIALS AND METHODS

Quantification of biofilm forming pathogens by microtitre plate assay

The food samples including beef, cheese, raw milk, pasteurized milk, curd, chilly powder, turmeric powder, coriander powder, soft drink, fresh fish, dried fish and dried prawn collected from the local stores and markets in Kochi, Kerala were analyzed using standard plate assay. 1 g of sample was serially diluted in10 mL of sterile distilled water. 0.1 mL of each dilution was plated on nutrient agar (HiMedia,

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Mumbai, India) plates using spread plate technique. The isolated bacterial colonies were picked and preserved in nutrient slants at 4^0C. These bacterial isolates were tested for their biofilm forming ability using microtitre plate assay (Rode *et al*., 2007).

The wells of a sterile 96 well polystyrene microtitre plates were filled with 230 µL of tryptone soy broth (TSB) (HiMedia, Mumbai, India). 20 µL bacterial cultures (OD_{600} =1) were added into each well separately, with triplicates for each bacterial culture and incubated aerobically for 24 h at 37^0C. Negative control included only TSB. The contents of the plates were poured off, wells washed 3 times with phosphate buffer (0.01 M, pH 7.2) and the attached bacteria were fixed with methanol. After 15 minutes, the plates were decanted, air dried and stained with of 1% crystal violet for 5 minutes. The excess stain was rinsed under running tap water. After air drying, the dye bound to adherent cells was extracted with 33% (V/V) glacial acetic acid per well and the absorbance was measured at 570 nm using a UV-VIS spectrophotometer (Schimadzu, Japan). Based on the absorbance (A_{570}) they were graded A= A_c= No biofilm producers; A_c< A= Weak biofilm producers; $2A_c$<A= Moderate biofilm producers; $4A_c$<A= Strong biofilm producers; where cutoff absorbance A_c was the mean absorbance of the negative control. All tests were interpreted thrice independently and statistically analysed (Christensen *et al*., 1988; Stepanovic*et al*., 2000);

Molecular characterization of biofilm producers

Genomic DNA was isolated and purified (Ausubel*et al*., 1987). A portion of the 16S rDNA was amplified using a primer pair for 16S rDNA (Reddy *et al*., 2002). The identity of the sequences was determined by comparing the 16S rDNA sequence with the sequences available in the NCBI nucleotide databases using BLAST (Basic Local Alignment Search Tool) algorithm (Altschul*et al*., 1990). A phylogenetic tree was also constructed for the biofilm producers by neighbor joining method (Saitou and Nei, 1987) using the MEGA 4 software (Tamura *et al*., 2007)

Antibiotic sensitivity tests

All strong biofilm producers were tested for antibiotic sensitivity in accordance with the Kirby- Bauer method (Bauer *et al*., 1966), with 12 antibiotics (HiMedia, Mumbai, India) belonging to different classes, namely ampicillin (5 µg/disc), azithromycin (15 µg/disc), cefixime (5 µg/disc), cefuroxime (30 µg/disc), ceftriazone (15 µg/disc), chloramphenicol (30µg/disc), ciprofloxacin (5 µg/disc), gentamicin (10 µg/disc), nalidixic acid (30µg/disc), norfloxacin (5 µg/disc), tetracycline (30 µg/disc), and trimethoprim (5 µg/disc). The results were interpreted as per the manufacturers' instructions.

Enzyme profiling of the biofilm producers

The qualitative assessment of enzyme activities including amylases, proteases, cellulases and lipases was using starch agar, skimmed milk agar, carboxymethyl cellulose agar and tributyrin agar respectively, as a part of characterization of the strong biofilm producers and consequently for the determination of their ability to degrade the nutritional substances in the food samples.

Amylases activity

For detecting amylase activity, organisms were patched onto 0.5% starch agar plate and incubated for 24 hours at room temperature. Gram's Iodine solution was flooded onto the inoculated plate. A clear zone around the colony indicates that amylase has hydrolysed the starch thereby giving no blue colour on reaction with iodine (Murray *et al*., 2007).

Proteases activity

The test organisms were patched onto 10% skimmed milk agar plate and incubated overnight at room temperature. Clear zones produced around the colony indicate that the casein in the mediumhas been hydrolysed. . No clearance of the medium is seen as the negative test (Mahendran*et al*., 2010).

Lipases activity

The test organisms were patched onto 1% tributyrin agar plate and incubated the plates for 48 - 72 hours at room temperature. Clear zones around the colony indicate the presence of lipases (Karnetova*et al*., 1984).

Cellulases activity

For cellulolytic activity, the test organisms were patched onto 0.5% carboxy methyl cellulose agar and incubated for 48 hours at room temperature. The plates were flooded with 0.1% Congo red solution and kept for 20-30 minutes with intermittent shaking, drained flooded with 1N NaCl solution and kept for 15 minutes. A yellow colour around the colony leaving the other portion of the plate red indicates positive reaction (Eggins and Pughg, 1962).

RESULTS

Quantification of biofilm forming pathogens by microtitre plate assay

Screening of the various food sample types for bacterial food

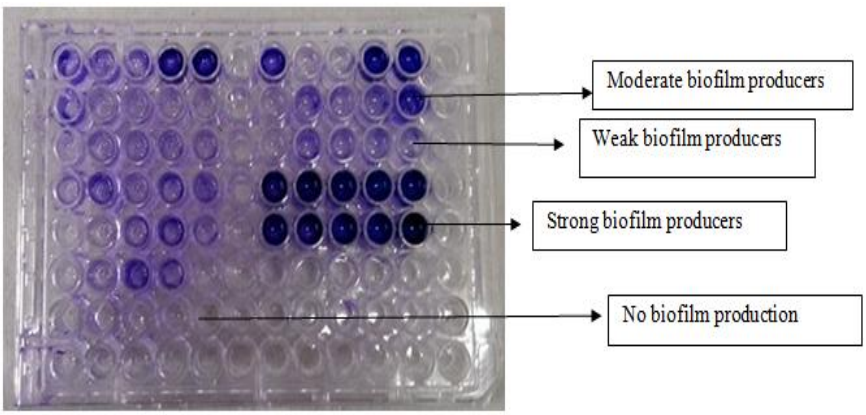

Figure 1 Microtitre plate for quantification of biofilm producers after the crystal violet staining

borne pathogens using standard plate count assay yielded thirty six isolates. Thirty one (86.11%)were biofilm producers. Figure 1 shows the quantification of biofilm production by microtitre plate assay. They were classified as strong, moderate and weak biofilm producers.

Figure 2 shows the strength of the biofilm production in all the 36 isolates obtained. Figure 3 shows biofilm production in the twenty strong producers in accordance to the A_C value.

The fig (1) shows varying intensities of crystal violet, which is indicative of the strength of the biofilm formed. The more intense the color, stronger the biofilm formed. From the figure, the levels of biofilm formed by different food pathogens is evident and can be clearly utilized to differentiate the strong, moderate and weak biofilm producers.

Molecular characterization of biofilm producers using 16S rDNA sequence analysis

Genomic DNA was isolated from the twenty isolates. Polymerase chain reaction based 16S rDNA amplification and sequence analysis thereafter was used for molecularcharacterization of the biofilm formers.. Following BLAST the identity of the biofilm formers was determined and the sequence data was submitted to the NCBI database and accession numbers obtained. Table 1 shows the identity of the twenty biofilm producers based on NCBI BLAST analysis.

The 16S rDNA analysis revealed that 14 of the biofim formers were *Bacillus* species, 4 were lactic acid bacteria and one each *Brevibacterium* and *Pseudomonas* species.

Table 1 The identity of the isolates with biofilm forming ability

Isolate	Organism	Genbank Accession number	Isolate	Organism	Genbank accession number
BTMW1	*Bacillus altitudinis*	KF460551	BTTP1	*Bacillus altitudinis*	KF460561
BTMY2	*Bacillus pumilus*	KF460552	BTDF1	*Brevibacteriumcasei*	KF573739
BTMG1	*Bacillus altitudinis*	KF460553	BTDF2	*Staphylococcus warneri*	KF573740
BTMW2	*Bacillus pumilus*	KF460554	BTDF3	*Micrococcus luteus*	KF573741
BTCW2	*Bacillus altitudinis*	KF460555	BTDP2	*Micrococcus sp*	KF573742
BTMW3	*Bacillus altitudinis*	KF460556	BTDP3	*Bacillus niacini*	KF573743
BTMY4	*Bacillus pumilus*	KF460557	BTSD1	*Bacillus sp*	KF573744
BTRY1	*Pseudomonas aeruginosa*	KF460558	BTSD2	*Bacillus licheniformis*	KF573745
BTPW1	*Bacillus altitudinis*	KF460559	BTFF1	*Micrococcus luteus*	KF573746
BTCP1	*Bacillus pumilus*	KF460560	BTFF2	*Geobacilluss tearothermophilus*	KF573747

Out of thirty six isolates obtained, 56% (n=20) were strong biofilm producers, 28% (n=10) were moderate producers while 3 % were weak producers. 14% (n=5) did not form biofilm. The biofilm producers were classified and this is depicted in figure (2).

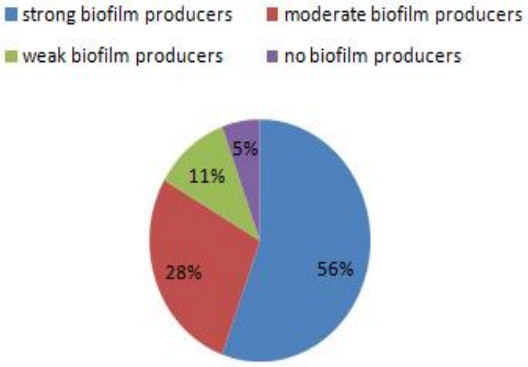

Figure 2 Classification of biofilm producers

Out of the twenty strong producers, the maximum biofilm production was shown by the strain BTSD2.. These biofilm formers were further identified using 16S rDNA sequence analysis.

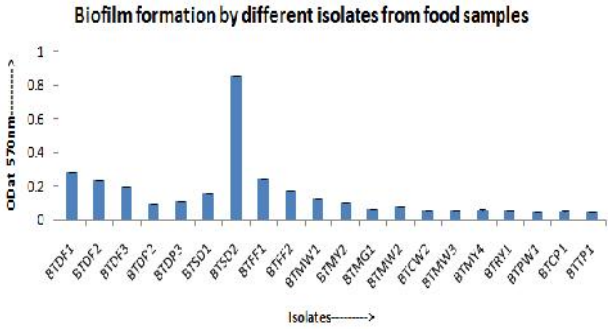

Figure 3 Biofilm production by 20 strong biofilm producers

This furthermore revealed that most of these strong biofilm producers are also food pathogens.

Phylogenetic analysis of the biofilm strains obtained in the study was done to understand their interrelatedness and is depicted as in figure (4).

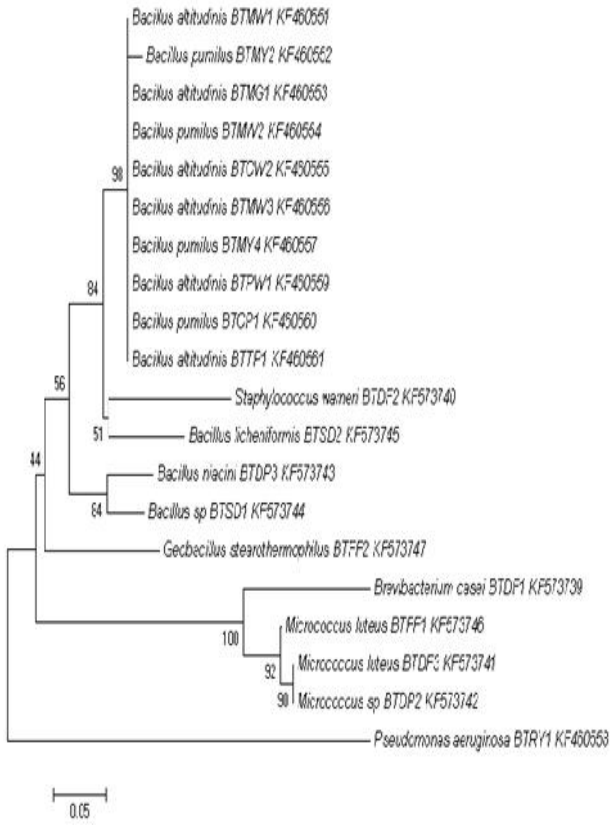

Figure 4 showing phylogenetic analysis of the obtained biofilm strains in the study

The number at the nodes of the phylogenetic tree are percentages indicating the levels of bootstrap support based on the Neighbour-Joining analysis of 1000 resampled data sets using MEGA 4 software.

It was observed that the *B.altitudinis* and *B. pumilus*strains grouped together in a single clade as did the three *Micrococcus* sp.

Antibiogram of the strong biofilm producers

Antibiotic Sensitivity Test was done and the antibiogram of the strong biofilm producers is as given in the figure (5). It was observed that percentage of biofilm producers resistant or intermediately resistant to most of the antibiotics was greater. From the figure (5) it was observed that eventhough the percentage of sensitivity was more, the sum total of intermediate resistance and total resistance was higher. This indicated that increased risks of food poisonings and food related deaths.

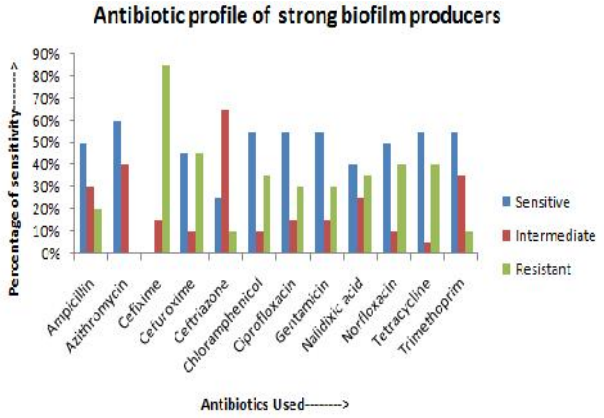

Figure 5 The antibiotic profile (%) of the twenty strong producers

Enzyme profile of biofilm producers

The enzyme profile showed that most of the strong biofilm producers were capable of producing more than one enzymes and thus were able to diminish the nutrient content of the food samples. The enzyme profile of is as listed in the table (4). The figures [6] – [9] depicts the different qualitative enzymatic assays using special media mentioned in the section 2.4.

The results of the qualitative enzymatic assays showed the ability of most of the isolates to produce more than one enzyme. This characteristic feature pointed out that these isolates, in addition to the biofilm formation, can reduce the nutritional value of the food.

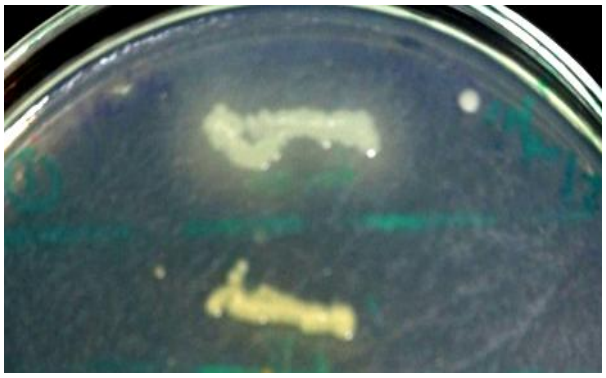

Fig 6 Lipase detection on tributyrin agar

Figure (6) – (9) shows the results of four qualitative enzymatic assays that include the lipase assay on tributyrin agar, cellulase assay on carboxy methyl cellulose agar, protease assay on skimmed milk agar and amylase assay on starch agar respectively

DISCUSSION

Several reports have been published on screening of food borne pathogens from different foods. The incidence of *Pseudomonas* sp in food items like beef, milk, anchovy and chicken was reported by Keskin and Ekmekci (2007). Agarwal*et al.*, 2011 evaluated the biofilm forming ability of different *Salmonella* serotypes using the microtitre plate assay

Table 2 showing the enzyme profile of strong biofilm producers

Strain	Amylase	Protease	Cellulase	Lipase
Bacillus altitudinis(BTMW1)	-	+	+	+
Bacillus pumilus (BTMY2)	-	+	+	+
Bacillus altitudinis (BTMG1)	-	+	+	+
Bacillus pumilus (BTMW2)	-	+	+	+
Bacillus altitudinis (BTCW2)	-	+	+	+
Bacillus altitudinis (BTMW3)	-	+	+	+
Bacillus pumilus (BTMY4)	-	+	+	+
Pseudomonas aeruginosa (BTRY1)	-	+	-	-
Bacillus altitudinis (BTPW1)	-	+	+	+
Bacillus pumilus (BTCP1)	-	+	+	+
Brevibacteriumcasei (BTDF1)	-	-	-	-
Staphylococcus warneri (BTDF2)	-	+	-	+
Micrococcus luteus (BTDF3)	-	+	-	-
Micrococcus sp (BTDP2)	-	-	-	-
Bacillus niacini (BTDP3)	-	-	-	+
Bacillus sp (BTSD1)	+	+	-	-
Bacillus licheniformis (BTSD2)	-	-	+	+
Micrococcus luteus (BTFF1)	-	+	-	+
Geobacillusstearothermophilus (BTFF2)	+	+	-	-

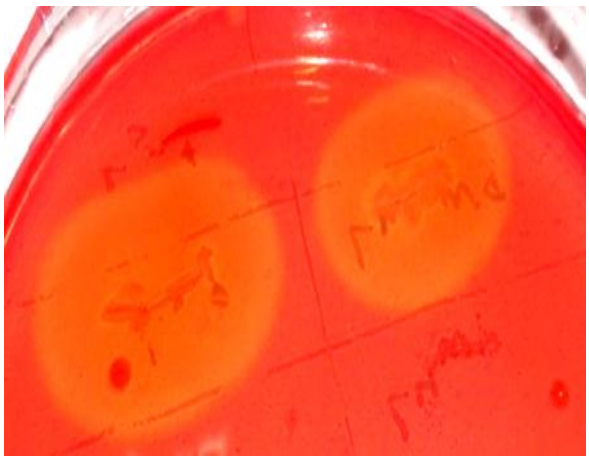

Fig 7 Cellulase detection on CMC agar

Fig 8 Protease detection on skimmed milk agar

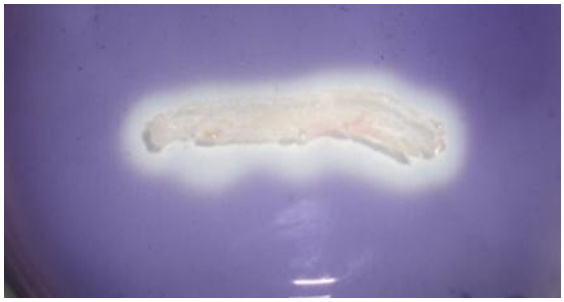

Fig 9 Amylase detection on Starch agar

with the crystal violet staining and their results showed that most of the strains in the study formed biofilm on plastic surfaces; t this study also categorized the isolated pathogens as strong, moderate and weak biofilm producers.

Murmann*et al*., 2008 collected food samples from outbreaks of Salmonellosis and molecular characterization was done. They also checked the antimicrobial susceptibility using antibiotics like amikacin (30 µg), ampicillin (10 µg), cefaclor (30 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), gentamicin (10 µg), nalidixic acid (30 µg), tetracycline (30 µg), tobramycin (10 µg), streptomycin (10 µg), sulphamethoxazole-trimethoprim (23.75/1.25 µg) and sulfonamide (300 µg) and a low frequency of antimicrobial resistance was observed in their study. Bacteria in biofilms are normally reported to have intrinsic mechanisms that protect them from most aggressive environmental conditions, including the exposure to antimicrobials (Davies, 2003). Teh*et al*., 2012 reported that enzymes secreted from biofilms into raw milk during transportation can potentially reduce the quality of different dairy products and could lead to severe economic losses in the food industry.

Thus biofilms production by food pathogens pose a immense threat to the food industry. In the present study, 20 strong biofilm producers were characterized by 16S rDNA sequencing and their identity revealed. The strains belonged to the genera *Bacillus, Pseudomonas, Micrococcus, Staphylococcus, Brevibacterium and Geobacillus.* The strongest biofilm producer was *Bacillus sp* (BTSD1). The enzyme profiling showed that the strongest biofilm producers produced most of the important starch, cellulose, proteins and lipids hydrolyzing enzymes and were thereby capable of easily diminishing the quality of the food samples. Multiple antibiotic resistance was observed among the strong biofilm producers, which were also food pathogens.

According to the present study, most of the biofilm forming food pathogens were multiple antibiotic resistant and produced more than one enzyme responsible for the food perishability. Several bioactive compounds find application against the biofilm formation of most of the strains and their safety needs to be confirmed prior to application in the food industry.
Since biofilm formation a serious issue, their control must be considered since it directly influences public health.

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