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Research Article

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Screening and Characterization of Biofilm Forming Food Pathogens and their Biocontrol

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Abstract: Microorganisms grow as a complex aggregation on a solid substrate to form a biofilm. This film serves as a defensive tool for various pathogenic microorganisms at different stress conditions. A well developed biofilm forms a complex diverse structure of dormant and active bacterial colonies with their enzymes and its excretory products. The main features of biofilm forming bacteria are their ability for surface attachment, high population density and extracellular polymeric substances. Biofilm has positive effects on biotechnology but it is extremely harmful in industry and medicine. Numerous chronic infections are caused by bacterial biofilm. Besides, biofilm also causes various infections of biomaterial used in medicine such as intravascular urethral catheters, orthopedic devices, contact lenses, heart valves and vocal cord prosthesis. Biomaterials used in medicine such as intravascular urethral catheters, orthopedic devices, contact lenses, heart valves and vocal cord prosthesis also gets infected by biofilm. The proposed work was carried out to study the biofilm-forming ability of bacteria from different food items. These bacteria were isolated and tested for biofilm-forming ability using Congo red and microtiter plate assay. All strong biofilm producers were tested for antibiotic sensitivity. Enzyme profiling and molecular characterization were done. Morphological and biochemical characterization of isolates was also carried out.

Keywords: Biofilm, congo red, microtiter plate assay

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1. INTRODUCTION

Food borne pathogens cause a number of diseases and food poisoning. It is stated that biofilms have become a critical problem in the current food industry.[1] A complete removal is a big challenge in food processing industries. The formation of biofilm provides safer mode of growth keeping cells to survive in unsuitable environments to disperse and colonize new niches.[2,8] Anton van Leeuwenhoek credited the discovery of biofilm on his own tooth surface.[3] Biofilm primarily consists of viable as well as nonviable microorganisms embedded in polyanionic extracellular polymeric substances attached to a surface.[4] Food industry provided with mineral content and high food residue from process water and food products which provide protection to microorganisms held within the biofilm.[5,,31,32] The four basic steps in biofilm formation are deposition of the conditioning film, attachment of microbes to the conditioning film, growth and microbial colonization and finally form biofilm followed by dispersion. [6]Several studies showed that the pathogens inside communicate with one another by a quorum sensing but this mechanism is not fully understood. A bacterium can be able to sense other pathogens growing around them and are more inclined to join and contribute to the formation of a biofilm.[7] Quorum sensing bacteria communicate through chemical messages for their presence either within a single bacterial species or between diverse species. The bacteria produce responses with stronger messages. It regulates a host of different processes and many different molecules can act as signals .[2]Biofilmed bacteria move either by rippling or rolling across a surface or detaching in clumps. It disperses through swarming and seeding that differentiates to form an outer stationary bacterial wall and inner region of the biofilm liquefies. This allows planktonic cells to swim out of the biofilm and leavea hollow mound [2,8]Medicinal plants used in primary health care gained importance in developing countries in the past few decades.[9] Herbal extracts or essential oils of medicinal plants constitute different compounds with various biological activities confirmed by *in vitro* and *in vivo* studies such as antibacterial, antifungal, antiviral, antiprotozoal, antihelminthic, antiseptic, antioxidative, anti-inflammatory, antitumor, contraceptive, antiallergic, anticonvulsant, antidepressant, antimutagenic, analgesic and diuretic properties. [26]

2. MATERIALS AND METHODS

2.1 Screening of Bacterial Food Borne Pathogens

Food samples such as raw milk (Sample 1), curd (Sample 2), cheese (Sample 3), soft drink (Sample 4), chilly powder (Sample 5), turmeric powder (Sample 6), coriander powder (Sample 7) and jam (Sample 8) were collected from the local market in Palakkad, Kerala and were analyzed using standard, or viable, plate count method.[10] The samples were serially diluted; plated and bacterial colonies were picked, preserved in nutrient slants at 4 °C.

2.2 Morphological and Biochemical Identification

Morphological features were identified by growing the isolated cultures on nutrient medium and Gram staining[11,12] was performed for each isolate. An isolate exhibiting maximum biofilm potential was subjected to biochemical tests such as IMViC, Indole test, Methyl Red test, Voges Proskauer test and Citrate utilization test (IMViC), catalase, oxidase, urease, nitrogen reduction, H_2S production and Triple Sugar Iron (TSI) [12].

2.3 Qualitative and Quantitative Analysis of Biofilm producers

The qualitative analysis of biofilm producers were done by Congo red assay method .Quantification of biofilm formation was carried out by microtiter plate assay[13]The wells of a sterile 96 well polystyrene microtiter plates were filled with 230 µl of Tryptone Soy Broth (TSB) (HiMedia, Mumbai, India). 20Twenty µl of bacterial suspension in TSB with turbidity or concentration equivalent to 1 in OD600bacterial cultures (OD 600 =1) were added into each well separately, with triplicates for each bacterial culture; and incubated aerobically for 24 h at 37 °C. 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 min, the plates were decanted, air dried and stained with 1% crystal violet for 5 min. 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 (A570) they were graded A=Ac= No biofilm producers; Ac< A= Weak biofilm producers; 2Ac<A= Moderate biofilm producers; 4Ac<A= Strong biofilm producers; where cutoff absorbance Ac was the mean absorbance of the negative control. All tests were conducted and interpreted thrice independently and statistically analyzed.[14]All data from biofilm quantitative assays were expressed as mean± SD with each assay conducted in triplicates. The statistical significance of associations between variables in different categories of isolates (Strong, moderate and weak) was calculated using Kruskal- Wallis test one way analysis of variance test, which is an extension of Mann Whitney U test, for more than two groups using Stats Direct statistical software (version 3.0, Cheshire, UK) computer program. Finally, the percentage of reduction in biofilm formation was calculated as:

% in biofilm reduction = (OD of Control - OD of Test/ OD of Control) X 100

2.4 Characterization of Strong Biofilm Producers

2.4.1 Antibiotic Sensitivity Tests

All strong biofilm producers were tested for antibiotic sensitivity in accordance with the Kirby- Bauer method[15], with 8 antibiotics (HiMedia, Mumbai) belonging to different classes, namely Ampicillin, Chloramphenicol, Erythromycin, Kanamycin,Nalidixicacid,Rifampicin, Streptomycin andTetracycline.[27,28] The results were interpreted as per the manufacturers' instructions shown in table 4.1.

2.4.2 Enzyme Profiling and Molecular Characterization of Biofilm Producers

The qualitative assessment of enzyme activities including amylase [16] protease [17] cellulase[18] and lipase[19] were performed using starch agar, skimmed milk agar, carboxymethyl cellulose

agar and tributyrin agar respectively.[30] Genomic DNA was isolated and purified (Ausubel*et al*., 1987). A portion of the 16S rRNA was amplified using a primer pair for 16S rRNA .[20] The sequences for the primer pair is shown below:

Forward primer - 5'AGAGTTTGATCCTGGCTCAG 3'
Reverse primer - 5'ACGGCTACCTTGTTACGACTT 3'

2.4.3 *Bio control of Biofilm Producers - Antimicrobial Screening of Plant Extracts*

Leaves of Indian Borage and Rose were obtained, air-dried, grinded into fine powders and stored (Fig: 5). Powdered plant material (1g) (Fig: 6) was weighed into a conical flask and 10 ml of distilled water was added to cover the powder. The flask was sealed, incubated at 45 °C in a water bath for 3 h with intermittent shaking. Following incubation, the extract was filtered and the extraction process was repeated twice on the residue using 5 ml of water at the second extraction. The filtrates obtained from the filtration process were pooled, dried and stored at 4 °C. [2-,23]

2.4.4 *Minimum Inhibitory Concentration*

To examine the inhibitory effect of Indian Borage and Rose Leaves on the biofilm growth, modified crystal violet assay was carried out. The positive control for bacterial cultures was ciprofloxacin. Controls were prepared at a stock concentration of 0.01 mg. 100 µL Hundred µl of sterile distilled water was aliquoted into all the wells of the microtiter plate. The prepared extracts were then pipetted into the wells A1-A12 of the plate. Doubling dilutions were performed in the direction A to H resulting in decreasing concentrations, following doubling dilutions, 100 µL of the bacterial suspension in TSB bacterial cultures (0.5 McFarland) was added to all the wells. The plates were incubated at 37 °C for 24 h for all bacterial strains. Water was used as negative control. Following incubation, the crystal violet assay was performed to assess biomass of the attached cells. The percentage of biofilm inhibition was calculated using the following equation.[24]

$$1 - \frac{OD\ of\ cells\ treated\ with\ test\ agent}{OD\ of\ non-treated\ control} \times 100$$

2.4.5 *Inhibition of the Preformed Biofilm*

Inhibition of a preformed biofilm was investigated using plant extracts that exhibited >50% inhibition of cell attachment. Biofilm formation was achieved by aliquoting 100 µL of culture into a 96 well microtiterplate. The plates were then incubated at 37 °C for 4 h to allow cell attachment. Following the 4 h incubation, 100 µL of each plant extract was added to a final concentration of 200mg/ml in the wells and ciprofloxacin was used as the positive control. The plates were further incubated at 37 °C for 24 h. Following incubation the crystal violet assay was performed[25].

3. STATISTICAL ANALYSIS

Statistical analysis was performed using spss version software (IBM Corporation, Ny, USA). Differences between means for the variables were evaluated using repeated measures ANOVA. p test was used to compare the biofilm OD_{590} mean values. The level of significance was set at $p< 0.05$To ensure the reliability and reproducibility of data the assays were done in triplicates. Statistical analysis indicated that there was a significant difference in the inhibition of preformed biofilm ($p<0.05$).

4. RESULTS AND DISCUSSION

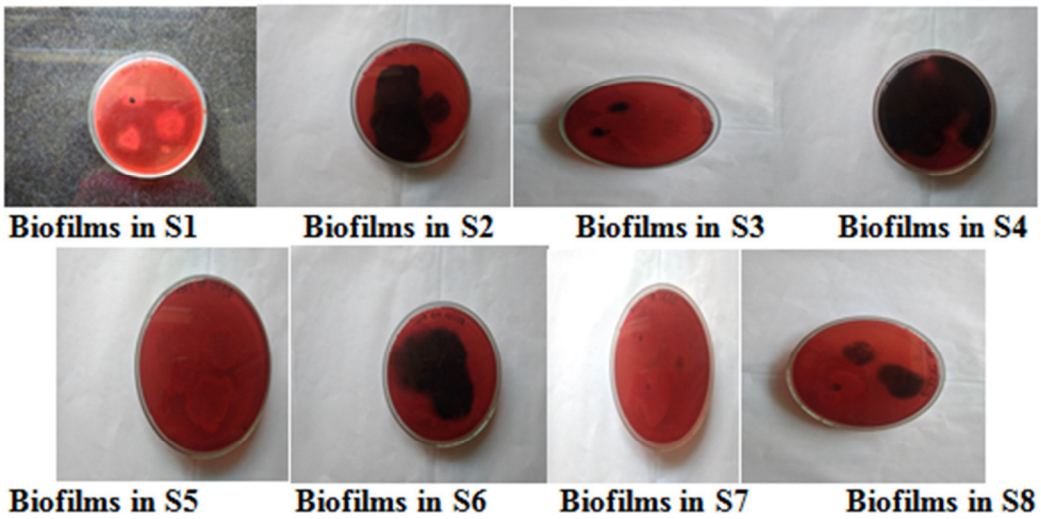


Fig 1. Biofilm Formation of Various Food Samples

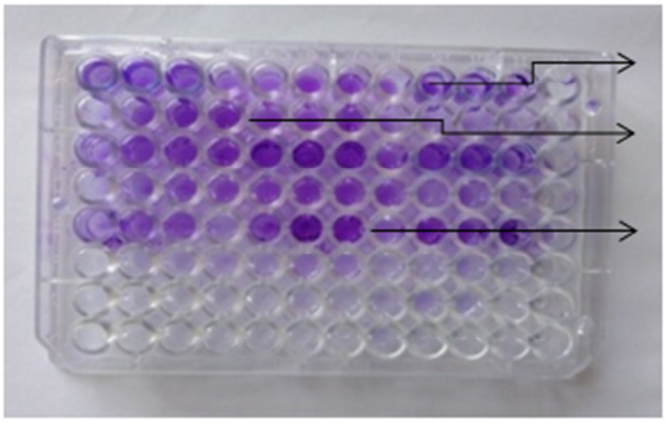
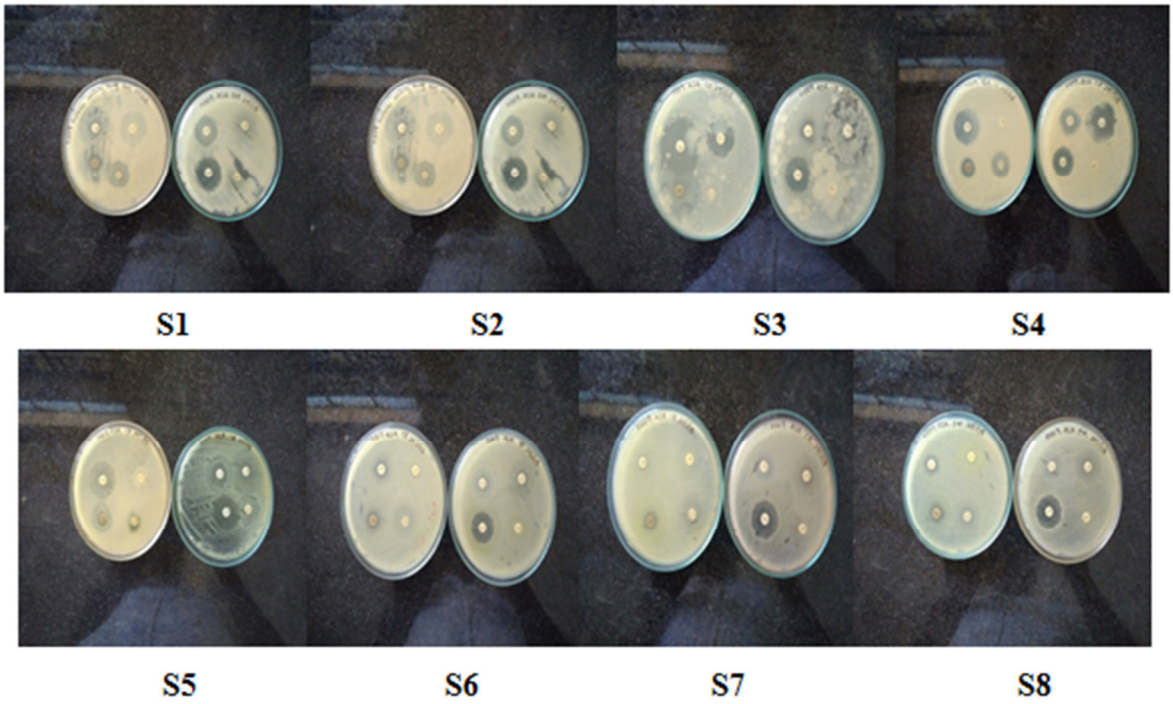


Fig 2 . Quantification of Biofilm Forming pathogen by Microtitre Plate Assay



Zone clearance around the disc represents no growth of the biofilm producers. Most strong biofilm producers (S2,S4,S6,S7,S8) are resistant to most of the antibiotics

Fig 3. Kirby-Bauer Disc Diffusion Test

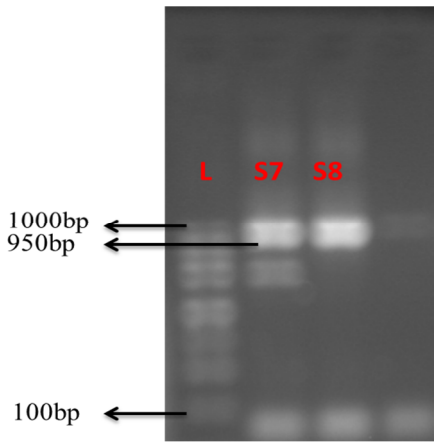


Fig 4. Gene Expression of 16S Rrna

Indian Borage Rose

Fig 5. Plant Samples

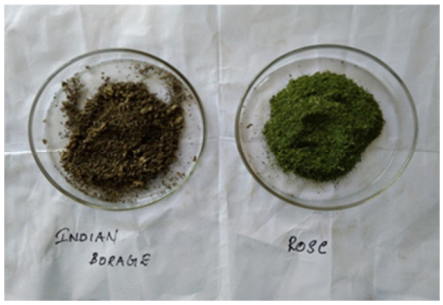
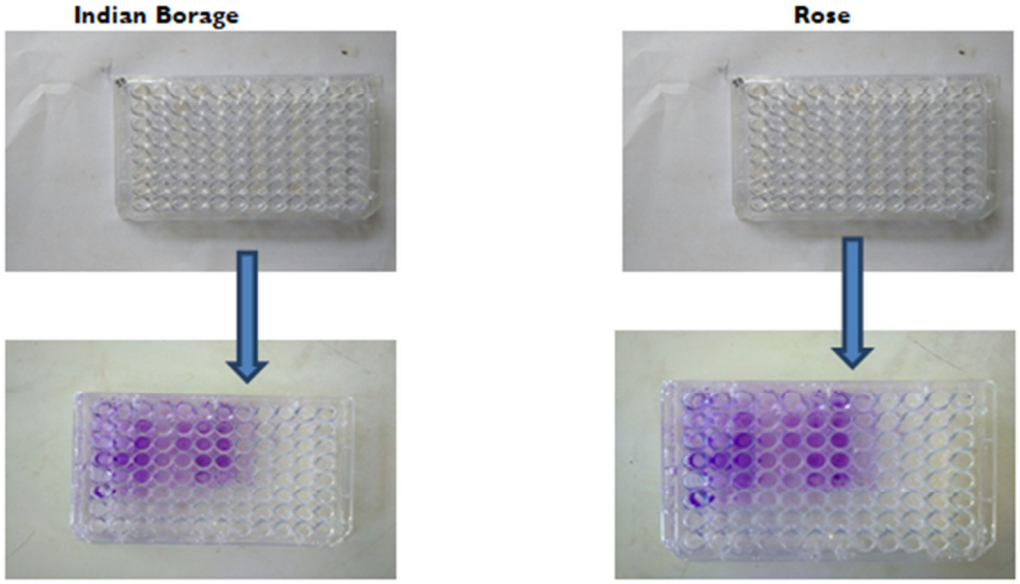


Fig 6. Powdered Form of plant Samples



Colourless - indicates Strong Biofilm Producers, Light Blue - indicates Moderate Biofilm Producers, Dark Blue - indicates week Biofilm Producers

Fig 7. Minimum Inhibitory Concentration of Biofilm Producers by Microtiter Plate Assay

Table 1. Absorbance of the Formed Biofilm by Microtitre Plate Assay

Sl.No	Isolate	Absorbance At 570nm	Criteria
1	S1	0.072	S
2	S2	0.117	S
3	S3	0.038	M
4	S4	0.071	S
5	S5	0.012	W
6	S6	0.083	S
7	S7	0.078	S
8	S8	0.112	S
9	Control	0.016	

S – Strong, M – Moderate, W – Weak

Table 2. Zone Size Interpretative Chart for Antibiotics

Antibiotics	Range (Indicates Zone Of Growth Inhibition In Mm)		
	R	I	S
Ampicillin	≤11	12-14	≥15
Chloramphenicol	≤12	13-17	≥18
Erythromycin	≤13	14-22	≥23
Kanamycin	≤13	14-17	≥18
Nalidixic acid	≤13	14-18	≥19
Rifampicin	≤10	11-15	≥16
Streptomycin	≤14	15-20	≥21
Tetracyclin	≤14	15-18	≥19

S – Sensitive, I – Intermediate, R – Resistant

Table 3. Zone Diameter of the Isolates

Antibiotics Used	Zone Diameter Of Isolates(Mm)							
	S1	S2	S3	S4	S5	S6	S7	S8
Ampicillin	25.03±0.06	23.2±0.25	0	0	13.1±0.31	10.2±0.2	22.9±0.12	25.1±0.23
Chlorampinecol	28.3±0.6	31.9±0.5	29	0	25.9±0.23	18.9±0.06	12.2±0.21	17±0.15
Erythromycin	22.8±0.25	14.3±0.52	31±0.2	9.93±0.12	13.9±0.12	10.2±0.26	11±0.06	10.3±0.55
Kanamycin	21.2±0.25	13.2±0.25	22.1±0.36	28±0.4	13.9±0.21	21.2±0.2	19	19
Nalidixic acid	19.2±0.21	0	18±0	12.2±0.21	16.1±0.1	14±0.06	0	14.1±0.15
Rifampicin	0	0	0	0	0	0	0	0
Streptomycin	25.3±0.26	22.23±0.25	22.1±0.26	19±0.1	25.03±0.35	22±0.15	23.9±0.32	23±0.12
Tetracyclin	17.1±0.1	22.3±0.31	15±0.2	11.2±0.25	17.2±0.26	11.1±0.36	12.2±0.29	13±0.1

Values are mean ± SD; (n=8); P<0.01 (Significant)

Table 4. Criteria of the Isolates Based on the Zone Diameter

Antibiotics Used	Isolates							
	S1	S2	S3	S4	S5	S6	S7	S8
Ampicillin	S	S	-	-	I	R	S	S
Chloramphenicol	S	S	S	-	S	S	R	I
Erythromycin	S	I	S	R	I	R	R	R
Kanamycin	S	R	S	S	I	S	S	S
Nalidixic acid	S	-	I	R	I	I	-	I
Rifampicin	-	-	-	-	-	-	-	-
Streptomycin	S	S	S	S	S	S	S	S
Tetracyclin	I	S	I	R	I	R	R	R

S:-Sensitive, I:-Intermediate, R:-Resistant

Table 5. Multiple Antibiotic Resistance (MAR) Index of the 5 Strong Biofilm Producers

Isolates	A	B	Mar Index (A/B)
S2	1	8	0.12
S4	3	8	0.37
S6	3	8	0.37
S7	3	8	0.37
S8	2	8	0.25

(A) - No. of antibiotics to which the isolate was resistant (B) - Total no. of antibiotics to which the isolate was subjected

Table 6. Exoenzyme Profile of the Strong Biofilm Producers

Isolates	Amylase	Cellulase	Lipase	Protease
S2	-	+	+	+
S4	+	+	-	+
S6	-	+	+	+
S7	-	+	-	+
S8	-	-	+	+

Table 7. Identification of the Strong Biofilm Producers -Morphological Identification

Morphological Features	S2	S4	S6	S7	S8
Gram Staining	Gram Positive	Gram Positive	Gram Positive	Gram Positive	Gram Negative
Shape	Rod	Slightly Curved Rod	Rod	Cocci	Rod
Colour of Colonies	White	White	Yellow	Yellow	Yellow

Table 8. Identification of the Strong Biofilm Producers - Biochemical Identification

Isolate	Indole Test	Methyl Red Test	Vogues Proskauererr Test	Citrate UtilizationTest	Catalase Test	Oxidase Test	Urease Test
S2	-	+	+	+	-	+	+
S4	-	+	+	+	+	+	+
S6	-	+	-	-	+	+	+
S7	-	-	+	+	+	+	+
S8	-	-	-	+	+	+	+

Table 9. Sugar Fermentation Test

Isolate	Glucose	Lactose	Sucrose
S2	-	-	-
S4	-	-	-
S6	-	-	-
S7	-	-	-
S8	-	-	-

Table 10. Five Strong Biofilm Producers

Sample	Organism
Curd (S2)	*Bacillus. pumilus***s.**
Soft Drink (S4)	*Bacillus. licheniformis*
Turmeric Powder (S6)	*Bacillus. altitudinis*
Coriander Powder (S7)	*Micrococcus. luteus*
Jam (S8)	*Pseudomonas. aeruginosa*

Table 10. Percentage Of Inhibition (%)

Isolates	Indian Borage	Rose	Indian Borage	Rose
S2	1.15	1.09	99.1%	93.9%
S4	1.13	1.08	97.4%	93.1%
S6	1.12	1.06	96.5%	91.3%
S7	1.11	1.04	95.6%	89.6%
S8	1.10	1.02	94.6%	87.9%
Control	1.16	1.16	-	-

Several food samples were screened for bacterial food borne pathogens using standard plate count assay. This yielded isolates were screened for their ability to produce biofilms. The isolates were subjected to qualitative and quantitative assays for biofilm production

4.1 *Qualitative Analysis for the Biofilm Producers by Congo red Plate Assay*

Qualitative analysis helps to segregate the strong, moderate and weak biofilm producers. Some of the isolates showed intense black coloured colonies, some produced lighter black coloured colonies, only few of them showed very light black precipitate, while others did not produce any black colour(Figure1) According to the intensity of the black colour, the isolates were categorized as strong, moderate and weak. The result was confirmed by the quantification assay by microtiter plate assay.

4.2 *Quantification of Biofilm Forming Pathogens by Microtiter Plate Assay*

After crystal violet staining, it showed biofilm formation on plastic surfaces by most strains, which also categorized the isolated pathogens as strong, moderate and weak biofilm producers based on the intensity of the colour formed. Dark blue colour indicates strong biofilm production, light blue colour indicates moderate biofilm production and very light blue colour indicates weak biofilm production.Absorbance of the Formed Biofilm by Microtiter Plate Assay was shown in the Fig 2 and Table 1.

4.3 *Antibiogram of the Strong Biofilm Producers*

All strong biofilm producers were tested for antibiotic sensitivity using Kirby-Bauer method, with 8 antibiotics shown in Fig 3 and the results were interpreted as shown in the following table 2. Food samples were molecularly characterized. Their antimicrobial susceptibility was tested to 8 different antibiotics, antimicrobial resistance was observed. Bacteria in biofilms are reported to have intrinsic mechanisms Antibiogram of the Strong Biofilm Producers were shown in the table 3. Zone diameter of the isolates and multiple antibiotic resistances index was calculated for strong biofilm food pathogens were shown in the table 4 and table 5 respectively.[29,33,34]

4.4 *Exoenzyme Profile of Biofilm Producers*

The enzyme profile showed the hydrolytic capabilities of the strong biofilm producers. It was observed that all were capable of producing more than one enzyme This characteristic feature pointed out that these isolates, in addition to biofilm formation, can also reduce the nutritional value of the food they contaminate (Table 6). The undesirable effects of the extracellular enzymes like protease, amylase, lipase, cellulase, etc., produced by the microbial biofilms were reported to degrade the food quality.[35] The amount of enzymes produced is also greater within biofilm community compared to the planktonic cell. [36-38]

4.5 *Molecular Identification of the Strong Biofilm Producers*

16S rRNA gene expression was assessed in this study for the evaluation of genotypic identification. Gene was highly expressed in coriander powder and jam samples. The gene 16S rRNA was expressed at 950bp which was run along with 1kb ladder shown in Fig : 4

4.6 *Identification of the Strong Biofilm Producers*

Strong Biofilm Producers were identified morphologically and biochemical tests were done. Results were shown in the table 7.0, 8.0 and 9.0. The strong biofilm producers were identified and tabulated (Table 10)

4.7 *Minimum Inhibitory Concentration*

Water extract of 2 plants showed potential in vitro activities against the biofilm producers. Comparing the average inhibition percentage of 2 extracts, Indian borage were found to be more effective (Fig: 7). Extracts Indian borage (97%) and Rose (91%) activities were comparable to ciprofloxacin (95%). These result shows that Indian borage was more active than the positive control shown in the table 11

4.8 *Inhibition of a Preformed Biofilm*

Extracts that showed at least 50% inhibitions were used in the preformed biofilm assay. Indian borage and Rose extracts exhibited good antibiofilm activity against the strong biofilm producers with percentage inhibition greater than 50%. These results show that inhibition of biofilm growth proved to be more difficult to achieve than cell attachment. An extensive multiplicity of microorganisms is equipped for shaping biofilm and subsequently biofilms exist in an assortment of situations. Some biofilms play a beneficial part in nature by serving as support for bigger living beings in the evolved way of life. However, those included as human and foodborne pathogens represent a huge danger to food security. Late flare-ups of foodborne ailment can be credited to biofilms.[39,40] The capacity of biofilm microorganisms to act on the whole to make a microbial province more grounded and more impervious to traditional sanitation and nourishment wellbeing strategies is overwhelming. Likewise, a refined system of cell-to-cell communication—majority detecting—upgrades biofilms' entrance to supplements and good ecological specialties, for example, new leafy foods. To decrease the dangers that biofilms stance to the nourishment business, further research is required not just to comprehend biofilm development in pathogenic life forms additionally to focus compelling systems for blocking majority detecting and inactivating biofilms on foods.[41,] Several reports have been published on screening of food borne pathogens from different food materials. Biofilm forming ability of different organisms were evaluated using the microtiter plate assay with the crystal violet staining, showed biofilm formation on plastic surfaces by most strains in the study, which also categorized the isolated pathogens as strong, moderate and weak biofilm producers. Biofilms formed on food-processing equipment and other food contact surfaces act as a persistent source of contamination threatening the microbiological quality and safety of food products, and resulting in food-borne disease and economic losses. Thus biofilm production by food pathogens poses immense threat to the food industry.

5. CONCLUSION

In the present study, the biofilm producers were subjected to antibiogram and the strongest biofilm producers were identified. Multiple antibiotic resistances were observed among the strong biofilm producers, which are also food pathogens. The enzyme profiling showed that the strongest biofilm producers produced most of the important starch, cellulose, protein and lipid hydrolyzing enzymes and were thereby capable of easily diminishing food quality. The 5 strong biofilm producers were characterized by 16S rRNA analysis and biochemical methods and their identity was revealed. According to the present study, most of the biofilm forming food pathogens were multiple antibiotic resistant and produced more than one enzyme responsible for food perishability. Several bioactive compounds which are present in various plant extracts find application against biofilm formation and their safety needs to be confirmed prior to application in the food industry. Since biofilm formation is a serious issue, their control must be considered since it directly affects public health.

6. AUTHORS CONTRIBUTION STATEMENT

M Flory Shobana and Roshna wilson contributed to the design and implementation of the research. M P Ayyappa Das, R Renugadevi and and V Subha Priya helped carry out the analysis of the result, K Vivekanandhan And V Manon Mani supervised the findings of this work. All authors discussesd the results and provided critical feedback and helped shape the research, analysis and manuscript.

7. CONFLICT OF INTEREST

Conflict of interest declared none.

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