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# Prevalence of Biofilm associated Pathogenic *Escherichia coli* in Seafood Pre-processing factories - A potential threat to seafood safety

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## Abstract

Microbial adhesion and biofilm formation on food contact surfaces in the seafood industry pose major problems and risks to human health. *Escherichia coli* is a common inhabitant of the intestinal tract of humans and animals and can be easily disseminated in different ecosystems through the food chain and water causing serious infection in human beings. The present study attempted to analyse the biofilm forming capacity of 90 *E. coli* strains isolated from food contact surfaces in seafood pre-processing plants. Quantification of biofilm formation was done by microtiter plate assay. Among them, 20% of the isolates were strong biofilm producers, 26.67% and 13.33% were moderate and weak producers respectively. Biofilm production was not observed in 40% of the isolated *E. coli.* All the strong biofilm producers were exo-enzyme producers, which is indicative of their capability in reducing the nutritional value of food and causing spoilage. In addition, the distribution of the genes encoding virulence and biofilm functions in strong biofilm forming *E. coli* isolates were examined. Multiplex PCR analysis revealed the presence of shiga toxin genes *stx1* (44.44%), and *stx2* (77.78%), intimin (*eae*, 38.89%) and enterohemolysin genes (*hlyA*, 66.67%). Biofilm-associated genes, *sdiA* and *rpoS* (100%), were most prevalent, while *rcsA* (55.56%) was least prevalent in strong biofilm producing *E. coli* strains. Field emission scanning electron microscopy (FESEM) showed that strain with strongest biofilm producing capability by *in vitro* methods established biofilm on stainless steel, plastic and rubber. The findings of this study revealed that food contact surfaces in seafood pre-processing plant harbors biofilm forming *E. coli*, indicating chance of contamination of seafood with *E.coli* and high risk of seafood-related illnesses in humans.

**Keywords:** Biofilm, *E. coli*, food contact surface, seafood safety, pathogenicity

## Introduction

Seafood is of great importance for nutrition and has traditionally been part of the human diet in many countries. It is the fastest-growing food sector and a source of income for a large number of people across the globe (FAO, 2018). Although healthy, there is substantial evidence that seafoods and seafood products can cause a variety of foodborne diseases (Vogel, 2009) because they are susceptible to contamination in the marine environment and can harbor pathogenic microorganisms (Iwamoto et al., 2010). The situation is even more concerning with the possibility of the formation of densely populated sessile bacterial communities, which is known as biofilm. Biofilms are aggregates of microorganisms which adhere to each other as well as to surfaces and remain enclosed in a self-synthesized layer of complex polysaccharides, proteins, lipids and extracellular DNA, collectively called the extracellular polymeric substance or EPS (Phillips, 2016). In seafood processing plants, microorganisms could attach themselves on to solid surfaces and form biofilms, in the presence of required nutrients, minerals, and organic matter. It is important to note that sanitizers do not easily penetrate the matrix of the biofilms formed after an inefficient cleaning process (Srey et al., 2013).

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Biofilms being a predominant and successful existence of microbial life can implicitly develop on natural as well as man-made substances (Nandita et al., 2019). The equipment and materials used for fish processing are similar to those used in other food processing plants and include buna-N rubber, polyvinyl chloride (PVC), chlorinated PVC, glass, fiberglass and stainless steel (Joseph et al., 2001). Biofilms were shown to form on these materials in food processing environment (Mizan et al., 2016). Biofilm formation on equipment surfaces increases the bio transfer potential that can be described as the ability of any microorganisms present on equipment surfaces both before and after the cleaning procedures to contaminate a product during processing and cause hygiene/health issues as well as economic losses in the seafood industry (Coughlan et al., 2016). Most seafood-borne pathogens can adhere itself to the food matrixes and the food processing surfaces for a long period of time and form biofilms, in the presence of required nutrients, minerals and organic matter (Vogel et al., 2001). One of the most important sources of contamination of the seafood is enterobacterial contamination (Guerin et al., 2004). Although the presence of *E. coli* in shrimp is uncommon, it is considered emergent in aquaculture, is considered as a gold standard for assessing the fecal contamination of seafood in India and other countries (Ahmed et al., 2015) and it is one of the most important public health concerns worldwide. Contamination of seafood with *E. coli* from landing centers in Gujarat (Sivaraman et al., 2017) and Tamil Nadu and Cochin (Murugadas et al., 2016) as well as hypervirulent strains from Cochin estuary (Divya & Hatha, 2018) have been reported earlier.

Safety of seafoods is one of the most important public health issues directly linked to farming and food production steps. As with other food types, seafoods are also not free of food-borne pathogens and several risk factors are associated with its consumption. Regarding seafoods, there are regulatory hygienic alerts in importing countries (Novoslavskij et al., 2016). Feeding habits of the consumers vary due to cultures of the countries across the world. Ready-To-Eat (RTE) and raw foods are now an increasing global trend, including seafood, and this situation may cause serious risks for the consumers' health if the seafoods are produced under poor hygienic conditions (Mizan et al., 2015). The foodborne diseases associated with contaminated seafoods have increased tremendously in last decade (Kim et al., 2017). Numerous studies have reported Shiga toxigenic *E. coli* in fresh fish and shellfish, and their RTE products in retail markets (Prakasan et al., 2018; Kim et al., 2020), suggesting that human activities, such as handling, processing and ingestion of the products might be a major source of Shiga toxigenic *E. coli* contamination.

The bacterial contamination of food-contact surfaces is a major factor in pathogen persistence in food processing environments and is believed to have a significant public health impact. About 60% of foodborne infections result from the microbial transfer from equipment surfaces to processed foods (Bridier et al., 2015). Foodborne illnesses due to biofilm formers are difficult to abolish as they may be 100 times more resistant to antimicrobials compared to planktonic cells (Nandita et al., 2019). Several previous studies have reported the presence of pathogenic and multidrug-resistant hypervirulent *E. coli* from Cochin estuary (Sukumaran & Hatha, 2015; Divya & Hatha, 2018). Antony et al. (2021) also reported the higher prevalence of pathogenic *E. coli* serotypes associated with shellfish harvested from Cochin estuary indicates potential food safety concerns. However, reports on the presence of pathogenic biofilm forming *E. coli* in seafood pre-processing plant have been very few (Frozi et al., 2017; V´azquez-S´anchez et al., 2018). The biofilm formation by *E. coli* contributes to the occurrence of various infections and makes their eradication difficult. Hence, it is increasingly important to monitor and verify the seafood safety risks along the entire seafood production chain. The present study, therefore, is aimed at determining the biofilm formation ability of *E. coli* isolated from food contact surfaces in seafood pre-processing plant and its characterizations, since the disease burden associated with contaminated seafoods has increased tremendously in last decade (Kim et al., 2017; Ali et al., 2020); however, with the increasing threat, the general awareness of seafoodborne diseases has also been increased worldwide.

## Materials and Methods

Food contact surfaces such as processing tables made up of stainless steel, where peeling and grading are done, plastic crates used to store the peeled and graded shrimp and the rubber gloves on the worker's hands were selected for screening biofilm forming *E. coli*. In the present study, a

qualitative analysis of 144 swab samples collected from biofilm formed on food contact surfaces in seafood pre-processing plant were analyzed as per ISO protocols for *Enterobacteriaceae*, *coliforms* and *E. coli* respectively (ISO 4832, 2006). Typical *E. coli* like colonies with a green metallic sheen from Eosin Methylene Blue (EMB) Agar (Hi-Media, India) were further confirmed on Hicrome E. coli Agar (Hi Media, India) and biochemical characterization of the presumptive isolates was done by Indole, Methyl Red, Voges-Proskauer, and Citrate (IMViC) tests. Isolates showing ++— reaction in IMViC tests were further confirmed by PCR-based molecular detection of the specific *uidA* gene (Antony et al., 2016). The presence of 147 bp amplicons confirmed the presence of *E. coli*. The biofilm-forming capacity of these isolates was analyzed by qualitative and quantitative methods.

Congo Red Agar method (Freeman et al., 1989) and Tube adherence method (Wolfe et al., 2004) were used for qualitative assessment of biofilms formation. The bacterial strains were streaked on CRA plates and after overnight incubation, observed for the presence of black crystalline colonies which are indicative of biofilm production whereas, pinkish-red colonies were evaluated as non-biofilm formers. For tube adherence test, strains were grown in Tryptone Soy Broth (TSB) under shaking conditions to allow biofilm formation and then transferred to glass test tubes, incubated without shaking for 24 h at 30°C; the culture broth was discarded and the biofilm at the interface between the air and medium was visualized using 0.5% Crystal Violet. Un-inoculated TSB was used as a control for the biofilm assays.

The quantification of biofilm was done by microtiter-plate technique (Stepanovic et al., 2007). A cell suspension was prepared in Tryptone Soy Broth (TSB- Himedia) with turbidity adjusted according to 0.5 McFarland standard (equivalent to $1.5 \times 10^8$ cfu $ml^{-1}$). For the microtiter-plate test, as a negative control, sterile medium (without bacterial suspension) was used to determine the cut off value. The reading of the optical density (OD) was performed using a microplate reader (Varioskan® LUX Microplate Readers, Thermo Scientific) at λ of 570 nm. The mean and standard deviation of the negative control was also calculated, and the cutoff (ODc) of negative control was established. The isolates with OD≤ODc were considered non-biofilm forming; isolates with ODc < OD ≤ (ODc x 2) were considered weak formers; isolates with (ODc x 2) < OD ≤ (ODc x 4) were considered moderate formers; isolates with (ODc x 4) < OD were considered as strong biofilm formers. All the isolates were analyzed in three independent replicates and OD was given as mean±S.D.

The Genomic DNA of the strong biofilm forming isolates was extracted by the boiling method (Antony et al., 2016). The presence of shiga toxin genes such as *stx1*, *stx2* and virulence genes such as intimin gene (*eae*) and enterohemolysin gene (*hlyA*) were detected using multiplex PCR as described by Paton & Paton (1998). The biofilm genes such as *sdiA*, *rcsA* and *rpoS* were detected using multiplex PCR as described by Noie Oskouie et al. (2019). The primers used in the detection of toxigenic/virulence genes and biofilm genes in strong biofilm forming *E. coli* are listed in Table 1. PCR products were analyzed by electrophoresis on a 1.5% agarose gel (Sigma-Aldrich, United States) stained with ethidium bromide (Sigma-Aldrich, United States) and visualized by Gel Documentation System (BioRad Gel Doc™ EZ Imager, United States).

Strong biofilm forming isolates namely, EC-45 and EC-73 were subjected to *16S rRNA* gene amplification. The PCR product was sent for sequencing at Agrigenome, Kochi. The sequence obtained was submitted to the basic local alignment search tool (BLAST; http://www.ncbi.nlm.nih.gov/BLAST) at NCBI (National Center for Biotechnology Information) to determine the percentage similarity with already identified 16S rRNA sequences in the Genbank database. The sequence was deposited in the Genbank and an accession number was allotted to it. The qualitative assessment of enzyme activities *viz.,* amylases, proteases and lipases were determined using starch agar, skimmed milk 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 nutrients in the food items (Laxmi & Sarita, 2014).

Biofilm formation of strongest biofilm forming isolate (EC-45) among *E. coli* strains was monitored on SS, plastic and rubber surfaces using FESEM. Stainless steel (SS), plastic and rubber surfaces collected from seafood industry were processed as described by Shen et al. (2012). A single colony from each plate was inoculated into 5 ml of Tryptone Soy

Broth (TSB) (HiMedia, India) and incubated overnight at 30°C in a shaking incubator at 120 rpm. Subsequently, the *E. coli* cultures were centrifuged at 11,000 × g for 10 min, washed and resuspended in fresh TSB broth to a final OD600 of 1.0. These cultures are referred to as 'standardized cultures'. The standardized culture was diluted 1:50 and added to 50 ml Falcon tubes containing stainless steel (SS), plastic and rubber coupon (2 × 2 × 0.1 cm) completely submerged in TSB broth. The tubes were incubated without shaking at 30°C for 24 h to allow for biofilm formation on the SS, plastic and rubber coupon (Mizan et al., 2016). FESEM sample processing was performed as previously described by Jahid et al. (2014).

## Results and Discussion

In the present study, we analysed the biofilm forming ability of 90 *E. coli* strains isolated from various food contact surfaces in seafood preprocessing plants. These isolates were biochemically characterized and confirmed by molecular based PCR assay using *uidA* gene which codes for *E. coli* β-D-glucuronidase enzyme. Cardozo et al. (2018) reported that the presence of *E. coli* may be due to the use of poor-quality water or ice during the processing of seafood. The lack of good hygiene practices among fish handlers and the direct contact of raw fish with contaminated work surfaces may be responsible for *E. coli* contamination (Silva et al., 2019). Montville et al. (2002) have reported that, during handling and preparation, bacteria may be transferred from contaminated hands of food workers to food and subsequently to other surfaces including food contact surfaces. Sudheesh et al. (2013) revealed a high level of contamination of food contact surfaces by indicator organisms such as *E. coli* as well as by pathogenic bacteria in seafood processing plants in Oman.

Congo red agar method is used for the qualitative assessment of biofilm producers. Biofilm producing strains produced black crystalline colonies on CRA plate and non-biofilm producers produce red coloured colonies (Kaiser et al., 2013) (Fig. 1A). Out of ninety isolates, 54 isolates showed black coloured colonies with variations in colour intensity, while the remaining of them did not produce any black colour. In tube adherence method, eighteen isolates produced a thick visible film lined the wall and bottom of the tube indicative of strong adherence while thirty six isolates had less visible film formation, suggestive of their weakly adherence to glass materials (Fig. 1B). The results were confirmed by the quantitative method.

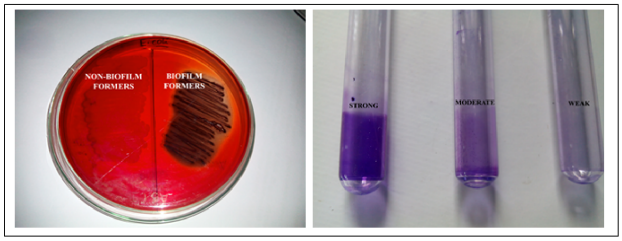


Fig. 1. Qualitative biofilm formation assay: (1A) Congo red agar plate assay (1B) Tube test

The biofilm-forming ability of *E. coli* isolates was determined quantitatively by microtiter plate assay. The average absorbance produced by negative controls at 570 nm was 0.095±0.002 (mean ± SD). The cut-off OD (ODc) which is the three standard deviations above the mean OD of the negative controls, obtained was 0.101. Among 90 *E. coli* strains, fifty-four (60%) were biofilm producers. They were classified as strong, moderate and weak biofilm producers based on the ODc (Fig. 2). Out of ninety isolates obtained, 20% (n=18) were strong biofilm producers, 26.67% (n=24) were moderate producers, while 13.33 % (n=12) were weak producers. 40% (n=36) of them did not produce biofilm. The values used for the categorization of the isolates into the four classes based on its OD in MTP assay are: Strong = 0.404< OD, Moderate = 0.202< OD≤ 0.404, Weak = 0.101< OD≤ 0.202, Non biofilm producers = OD ≤ 0.101

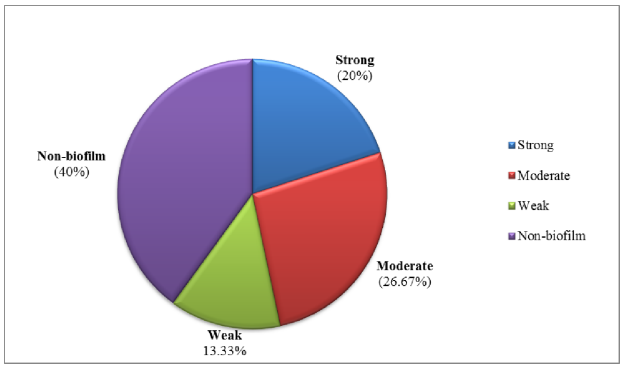


Fig. 2. Classification of biofilm formers

Fig. 3. shows biofilm production at OD570 nm by the 18 strong biofilm producers as per microtiter assay. These 18 strong biofilm producers were selected for further study. Out of the eighteen strong biofilm producers, the maximum biofilm production was shown by the strain EC 45 and EC 73.

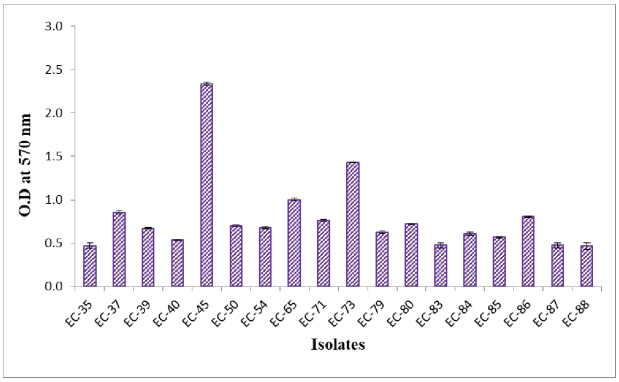


Fig. 3. Quantitative biofilm formation assay of 18 strong biofilm forming *E. coli* strains

It has been reported that various *E. coli* strains, either pathogenic or of environmental origin have the ability to colonize as a biofilm, which could result in longer persistence in the environment (Nesse et al., 2014). Biofilm forming bacterial pathogens commonly colonize certain types of seafood, such as shrimp, crabs and pacific oysters (Mizan et al., 2015). King et al. (2004) reported that *E. coli* isolated from seafood have been shown to be associated with biofilms.

The present study demonstrated the biofilm formation by *E. coli strain* (EC-45) on stainless steel, plastic and rubber. FESEM images were acquired to examine the cell and biofilm morphology of the *E. coli* isolates on SS, plastic and rubber (Fig. 4 A-C). The acquired images (representative images selected) showed that EC-45 formed extensive biofilms on SS, plastic and rubber.

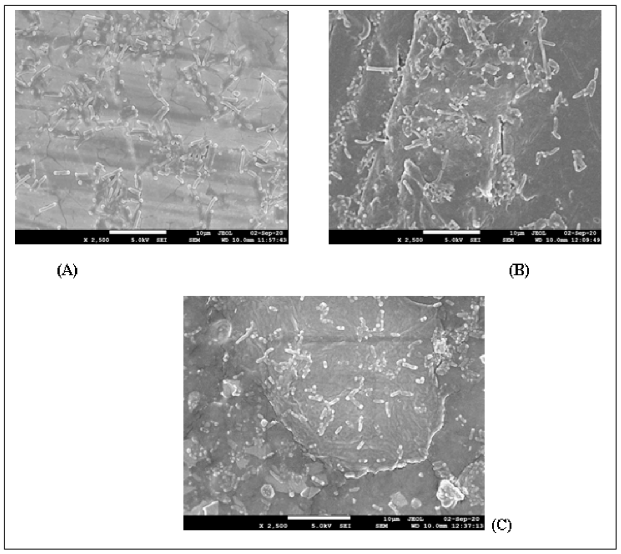


Fig. 4. FESEM images (2500 × magnification) of biofilms formed by *E. coli* isolates on: (A) Stainless steel (B) Plastic (C) Rubber

*E. coli* biofilm formation is an intricate process which involves a number of steps such as initial adhesion, early development, maturation and dispersion. At each step of biofilm development and dispersal, there is a specific genetic signal control (Grigore-Gurgu et al., 2019). Genes codify for cell surface structures and appendages (flagella, curli, fimbriae, and pili) that are facilitating biofilm formation by helping bacteria to move toward surfaces and to adhere to them, for extracellular polymeric substances that stabilize the biofilms and protect the cells and for quorum sensing communication (Álvarez-Ordóñez et al., 2013).

Table 1. Primers used in the study

| Genes investigated | Primer sequence (5'-3') | | Annealing Temperature |
|---|---|---|---|
| | Forward | Reverse | |
| *16S rRNA* | agagtttgatcctggctcag | ggttaccttgttacgactt | 58ºC |
| Virulent/Toxigenic genes | | | |
| *stx1* (180 bp) | ataaatcgccattc | agaacgcccactg | 58ºC |
| *stx2* (255 bp) | ggcactgtctgaaa | tcgccagttatctg | 58ºC |
| *eae* (384 bp) | gacccggcacaag | ccacctgcagcaac | 58ºC |
| *hlyA* (534 bp) | gcatcatcaagcgt | aatgagccaagct | 58ºC |
| Biofilm genes | | | |
| *sdiA* (239 bp) | tcgctatctctgctgatgtc | tttaatgctgccaaatcggg | 52ºC |
| *rcsA* (306 bp) | gtgattcacagcgcccttca | tactcgattcggttcggctc | 54ºC |
| *rpoS* (120 bp) | gcagagcatcgtcaaatggctgtt | atcttccagtgttgccgcttcgta | 60ºC |

Table 2. Detection of genes involved in biofilm formation as well as pathogenicity of strong biofilm producing strains of *E. coli*

| Sl. No. | *E. coli* strains | Biofilm Genes | | | Virulent / Toxin Genes | | | |
|---|---|---|---|---|---|---|---|---|
| | | *sdiA* | *rcsA* | *rpoS* | *stx1* | *stx2* | *hlyA* | *eae* |
| 1. | EC-35 | + | + | + | + | + | + | - |
| 2. | EC-37 | + | - | + | + | + | + | + |
| 3. | EC-39 | + | + | + | - | + | + | - |
| 4. | EC-40 | + | - | + | + | + | + | + |
| 5. | EC-45 | + | + | + | + | + | + | + |
| 6. | EC-50 | + | - | + | + | + | + | - |
| 7. | EC-54 | + | + | + | + | + | + | + |
| 8. | EC-65 | + | + | + | - | - | - | + |
| 9. | EC-71 | + | - | + | + | + | + | - |
| 10. | EC-73 | + | + | + | + | + | + | + |
| 11. | EC-79 | + | + | + | - | + | - | + |
| 12. | EC-80 | + | - | + | - | + | + | - |
| 13. | EC-83 | + | + | + | - | + | + | - |
| 14. | EC-84 | + | - | + | - | + | - | - |
| 15. | EC-85 | + | - | + | - | - | - | - |
| 16. | EC-86 | + | - | + | - | - | - | - |
| 17. | EC-87 | + | + | + | - | - | - | - |
| 18. | EC-88 | + | + | + | - | + | + | - |

In the present study, we have analysed the genetic basis of biofilm (*sdiA*, *rcsA*, and, *rpoS* genes) in eighteen strong biofilm producers. Ten of them showed the presence of all these genes (Fig. 5), while 8 of them had only *sdiA and rpoS* genes (Table 2). Previous studies have reported the role of *rpoS*, *sdiA*, and *rcsA* genes in biofilm formation (Adamus-Bialek et al., 2015; Noie Oskouie et al., 2019).

The *sdiA* gene of *E. coli* is homologous to *luxR* in other bacteria and is the activator of quorum sensing in biofilm (Adamus-Bialek et al., 2015). The gene *rpoS* is an alternative sigma transcription factor that controls the expression of a large number of genes involved in the cellular response to stress is a key factor in the development of mature biofilms in *E. coli* (Álvarez-Ordóñez et al., 2013; Adamus-Bialek et al., 2015). Similarly, *rcsA* is a regulatory gene that belongs to the complex Rcs system for the regulation of cell wall integrity, cell division, stationary phase sigma factor activity, motility, and virulence. It has been shown that this gene has a role in adhesion to eukaryotic cells using curli (Adamus-Bialek et al., 2015).

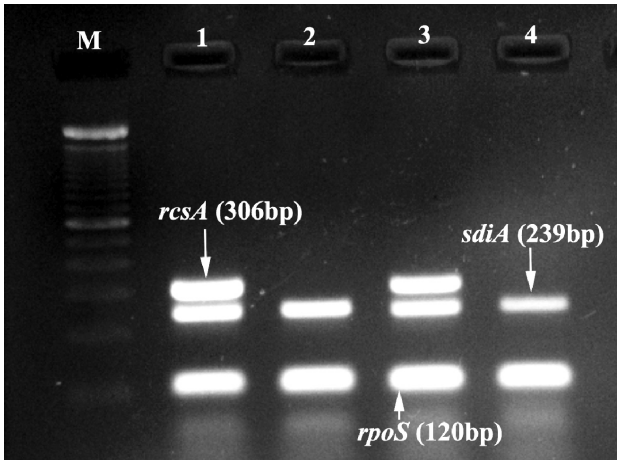


Fig. 5. Gel image of PCR products with representative isolates carrying biofilm genes: Lane M: 100 bp ladder; lanes 1-4: *sdiA* & *rpoS* gene; lane 1&3: *rcsA* gene of biofilm forming *E. coli* strains

*E. coli* is ubiquitous in the gut of humans and animals. It is often non-pathogenic, but some strains may cause gastrointestinal, urinary, or central nervous system infections (Nataro & Kaper, 1998).

Kumar et al. (2001) described seafood as a potential source of Shiga-toxigenic *E. coli* (STEC). This correlates well with our findings, with the identification of *stx1* and *stx2* genes in some of our biofilm forming *E. coli* strains. Among four toxin/virulent genes analyzed, *stx2* was most prevalent one (Table 2). Pradhan et al. (2016) reported that among shiga toxins, *stx2*, which is more potent than *stx1*, causes clinically severe weight loss and renal injury. The virulent genes such as *hlyA* and *eae* were present in 66.67% (12/18) and 38.89% (7/18) of the isolates. The presence of *eae* gene in *E. coli* strains allows them to adhere to the intestinal mucosa of the host by producing an outer membrane protein (intimin) and is considered as a potential factor required for the expression of virulence in STEC (Antony et al., 2021). STEC carrying both *stx* and *eae* genes are known as enterohemorrhagic *E. coli* (EHEC) and are responsible for serious systemic complications (Feng et al., 2011). These strains are able to produce a specific 60 MDa plasmid-mediated hemolysin (*hlyA*) (Kang et al., 2004). In the present study, 27.78% (5/18) *E. coli* strains among eighteen strong biofilm formers, harbour *stx1*, *stx2*, *eae* and *hlyA* together. Sheep, pigs, goats, dogs, cats and especially cattle are the principal reservoir of STEC strains (Paton and Paton, 1998). This, together with the fact that *E. coli* cannot survive in the marine environment for a long time (Kumar et al., 2005), reinforces the idea that the area where our seafood samples are harvested is likely to be contaminated with untreated effluent and sewage containing animal and/or human discharge. Presence of toxigenic/virulence genes revealed the pathogenic potential of the biofilm forming strains isolated from food contact surfaces in seafood pre-processing units.

16S rRNA sequence analysis of two strong biofilm formers (EC 45 and EC 73) was done and submitted in Genebank. When the sequence similarity search was done using the BLAST tool, our sequences was found to be 99% identical with the 16S rRNA gene sequences of the *Escherichia coli* strain in GenBank (KY367391 and MG198700). The sequence was submitted to GenBank and was assigned the accession numbers such as MN 368505 and MN 368506.

There are various types of foodborne micro-flora (pathogenic or non-pathogenic), that adheres itself to the food matrix and surfaces for a long period of time. These microorganisms play vital role in food degradation, intoxication and pathogenicity in consumers. This confers to affect the food quality and safety of food consumers (Bagge-Ravn et al., 2003; Ali et al., 2016). In the current study, the exoenzyme profiling showed that all the strong biofilm producers were capable of producing major enzymes such as amylase, protease, and lipase and thus were able to diminish the nutrient content of the food samples. This characteristic feature pointed out that these isolates, in addition to the biofilm formation, can reduce the nutritional quality of the seafood.

The results of the present study provide evidence of the hygienic status of the food processing surfaces and handlers, plays an essential part in microbial contamination of food and it has a direct influence on food quality and public health. In the food industry, biofilms are responsible for persistence of bacterial pathogens in food processing environments and recurrent cross-contamination of food products. There is an urgent need for periodic multistage monitoring programs. The inadequate cleaning and sanitation procedures could not remove adhered food residues on surfaces that can help in formation of microbial biofilms. So, the reformulation of hygienic policy must be implemented that effectively control biofilms to guarantee a safe quality of seafood and prevent its contamination. Good hygiene practices must be implemented in pre-processing industry to avoid contamination from both food handlers and food contact surfaces and to ensure the microbiological free products.

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