Journal of Population Therapeutics & Clinical Pharmacology

RESEARCH ARTICLE
DOI: 10.53555/jptcp.v30i18.3469

ENZYMATIC DEGRADATION OF E. COLI BIOFILMS BY S. AUREUS EXTRACTED ENZYMES: A PROMISING APPROACH FOR BIOFILM DISRUPTION

Shehzad Zafar[1], Shahzaib Khan[2], Fariha Ahsan[3], Iqbal Nisa[4], Muhammad Nazir Uddin[5], Fawad Khan[5], Shahida Sadiqqi[6], Sumia Khan[6], Didar Ali Shah[6], Maha Jawariya[7], Sidra Farooq[1*], Muhammad Ishfaq Khan[1#]

[1]Department of Health and Biological sciences, Abasyn university Peshawar, Pakistan
[2]Department of Microbiology Abbotabad University of Science and Technology, Pakistan
[3]Institute of Microbiology, University of Sindh , Jamshoro 76080, Pakistan
[4]Department of Microbiology, Women University Swabi, Pakistan
[5]Center for Biotechnology and Microbiology, University of Swat, Pakistan
[6]Department of Microbiology, Hazara University Mansehra, Pakistan
[7]Department of Biotechnology University name: SBK Women's University Quetta

[*]**Corresponding Author:** Sidra Farooq
sidra.farooq@abasyn.edu.pk

Co-Corresponding Author: Muhammad Ishfaq Khan
Ishfaqafridi300@gmail.com

ABSTRACT
Biofilms are complex communities of microorganisms encased within a matrix of extracellular polymeric substance (EPS) that they produce. These biofilms consist of microbial cells adhering to each other and to surfaces, whether living or non-living. Bacterial biofilms, in particular, are often associated with pathogenicity and can lead to nosocomial infections. In this study, we collected twenty samples from the industrial state of Hayatabad and employed pure culturing techniques along with biochemical tests to isolate and identify various bacterial species. The identified species included *E. coli, S. aureus, Salmonella, Shigella, P. aeruginosa, Yersinia enterocolitis, Providencia, P. mirabilis,* and *B. subtilis*. For our investigation into biofilm formation and degradation, we chose *E. coli* as the target bacterium and extracted enzymes from *S. aureus*. To assess biofilm formation, we utilized the microtiter plate assay. The optical density (OD) measurements for the control group were 1.749, and for replicate 1, they were 1.698 when treated with 20 μL of protease enzymes. For replicate 2, treated with 30 μL of enzymes, the OD was 1.582, and for replicate 3, treated with 50 μL of enzymes, the OD was 0.89. Our findings suggest that enzymatic treatment is a promising method for degrading biofilms, as evidenced by the substantial reduction in OD measurements. This research paves the way for further studies on a larger scale, focusing on the isolation and degradation of biofilms using enzymes. Understanding the potential of enzymatic degradation could lead to innovative strategies for combating biofilm-related infections.

Keyword: biofilm, enzyme, bacteria, EPS , nosocomial infections

INTRODUCTION

Biofilms can be defined as communities of microorganisms attached to a surface. It is clear that microorganisms undergo profound changes during their transition from planktonic (free-swimming) organisms to cells that are part of a complex, surface-attached community. These changes are reflected in the new phenotypic characteristics developed by biofilm bacteria and occur in response to a variety of environmental signals. Recent genetic and molecular approaches used to study bacterial and fungal biofilms have identified genes and regulatory circuits important for initial cell-surface interactions, biofilm maturation, and the return of biofilm microorganisms to a planktonic mode of growth. Studies to date suggest that the planktonic-biofilm transition is a complex and highly regulated process. The results reviewed in this article indicate that the formation of biofilms serves as a new model system for the study of microbial development (Stoodley *et al*., 2001). Biofilms are comprised of microbes on a matrix of extracellular polymeric substances. Biofilm development in flow cells is monitored by Confocal Scanning Laser Microscope (CSLM), which aids in examining the biofilm without disrupting the microbial community. The composition of biofilm has been determined successfully using CSLM. 15% of the biofilm is composed of microbial communities and the rest 85% is extracellular polymers. The extracellular polysaccharides (EPS) are mainly comprised of polysaccharides, which are neutral or anionic. The anionic properties of EPS are confirmed by the presence of uronic acids (mannuronic; D-galacturonic; D-galacuronic) or ketal linked pyruvate. These anionic linkages in combination with divalent cations like calcium or magnesium help in crosslinking and confer greater binding force in a developed biofilm. These extracellular polymeric substances (EPS) The bacteria which inhabit the biofilm are sheltered by the EPS. The EPS matrix acts as an anion exchanger hence preventing the contact of antimicrobial substances. It also limits the diffusion of harmful substances from the environment into the biofilm. Furthermore, PS sequesters toxins, metal ions, and cations. It also protects the microbes from different environmental stresses like UV radiation, pH shift and osmotic shock (Beloin *et al.,* 2017). The water channels are an effective means for nutrient exchange and the transfer of metabolic intermediates with the main part of the aqueous phase, which enhances the nutrient accessibility and elimination of potential toxins. The small colonies in biofilm usually comprise a wide variety of microbial populations, which can be metabolically cooperative, therefore, their closeness enables interspecies substrate transport, exchange, and removal of metabolic intermediates (Miron *et al.,* 2001) Biofilm offers an ideal situation for the formation of syntrophic relations. Horizontal gene transfer is a vital feature in a biofilm where the transmission of DNA between different genomes occurs in a manner other than traditional reproduction. Furthermore, it has been suggested that biofilms play a significant role in the transmission and persistence of human disease. For human pathogenic bacteria, biofilms offer protection to the bacteria from the host immune system and allow those bacteria to withstand killing doses of antibiotics (Huq *et al*., 2008). Cellular proliferation and exopolysaccharide production increase the biomass of biofilm, whereas cell destruction, dispersion of biofilm, and “grazing” by benthic microorganisms decreases the biomass, which gives rise to cyclic life of biofilms. Removal of biofilms is difficult. In industrial settings, both the inactivation and removal of biofilms are of huge concern. If only disinfection without the removal of attached biofilms occurs, the inactivated biofilm cells may provide an ideal environment for further adhesion and growth, resulting in a complex matrix. Microbial resistance to biocides and their negative environmental impact are the main reasons for finding alternative biofilm control strategies. Enzymes may offer such an alternative (Molobela *et al.,* 2010).

METHODOLOGY

Sample collection

The twenty water and effluent sample were collected from industrial state Hayatabad Peshawar, the sample was collected from different industries including textile, glass, pharmaceutical, Pepsi, juices, milk pack and dairy product industries in sterile bottles. The source of sample collection was pipe line

of tape water and effluents of industries.The collected sample was stored at 4 ºC till further use (Ragin *et al*., 1998).

Sample Processing

All samples were cultured on nutrient agar plate by pour plate method. Pour plate method is usually the method of choice for counting the number of colony-forming bacteria present in a liquid specimen. In this method, fixed amount of inoculum (generally 1 ml) from a broth/sample is placed in the center of sterile Petri dish using a sterile pipette and circulate plate such that sample is completely spread over media. After the solidification of the agar, the plate is inverted and incubated at 37°C for 24-48 hours and furthered process for gram staining and biochemical test (Kumar *et al*., 2019).

Preparation of Biofilm

Commonly used media for biofilm assays is nutrient agar. Inoculate bacteria into 3–5 mL appropriate growth medium in sterile culture tubes and grow to stationary phase. Typically, bacteria are grown overnight at 37 °C with shaking. Aliquot 100µL of diluted culture into 96-well microtiter plate. Be sure to include a blank well containing uninoculated medium as a control. Cover plate and incubate at appropriate temperature for desired length of time. An incubation of 8–10 h is suggested for most purposes (Makari *et al*., 2004). Allow stained biofilm plate to air-dry for several hours or overnight. Pipette 150µL 30 % acetic acid into each well. This will solubilize the biofilm. Allow to sit for 10 min. Pipette up and down to assure that the biofilm is well solubilized, and then transfer 125µL of each sample to a 96-well optically clear, flat-bottom plate. Measure optical density of all samples in plate at 600 nm.

Extraction of protease enzymes from *S. aureus*

The activity of proteolytic bacteria was tested qualitatively on skim milk media, indications that microbes are able to integrate protein (casein) which are shown through clear zones around the colonies. Pure colonies that gave a clear zone are calculated for their proteolytic index, bacterial as the highest proteolytic index (IP) is a bacteria that is chosen for protein isolation step. Colonies that produces clear zones on MSA media were collected in Eppendorf tube and lysozymes enzymes has been added to it. These lysozyme enzymes help in lysis of cell, such that total enzymes come out of S. *aureus*, further it is centrifuge at 10,000 rpm for 10 minutes at 37 ºC. After centrifugation supernatant is transfer to precipitation column where it is treated with ethanol for washing. Again, it is centrifuge at same rpm and time. In precipitation column enzymes were precipitated and are collected in pure form. These enzymes were diluted with normal saline and were stored at 4 ºC for further use.

RESULTS

A total of 20 distinct samples were meticulously collected from various industrial sources, which encompassed effluent and wastewater samples. These samples were subjected to a comprehensive culturing process, beginning with their placement on Nutrient agar medium. Following this initial culturing step, further procedures were implemented on selective media to validate and confirm the presence of specific bacterial species within the samples. To gain a deeper understanding of the microbial composition of these samples, Gram staining was employed as a fundamental technique. This staining procedure involved the application of crystal violet and iodine, followed by ethanol washes and safranin counterstaining. It allowed for the categorization of bacterial cells into Gram-positive or Gram-negative based on differences in cell wall structure. In addition to Gram staining, a series of biochemical tests were meticulously conducted. These tests were designed to explore various metabolic and physiological characteristics of the isolated microorganisms. By analyzing their reactions to specific biochemical assays, we were able to gather valuable information about the identity and functional traits of the bacterial species present in the industrial samples.

Table 1. show bacterial species, gram staining results and their biochemical tests.

S.No	Species	**G. stain**	**URE**	**OXI**	**CIT**	**IND**	**TSI**	**CAT**	**COG**	**GAS**	**H2s**
1	*E. coli*	Rod, -	-	-	-	+	A/A	+	-	+	-
2	*P. aeruginosa*	Rod, -	-	+	+	-	K/K	+	-	-	-
3	*Yersinia enterocolitis*	Rod, -	-	-	-	+	A/A	+	-	+	-
4	*B. subtills*	Rod, +	-	V	+	-	Nc/K	+	-	-	-
5	*Salmonella*	Rod, -	-	-	-	-	K/A	+	-	-	+
6	*S. aureus*	Cocci, +	+	-	+	-	K/K	+	+	-	+
7	*B. megaterium*	Rod, +	+	+	+	-	A/Nc	+	-	-	-
8	*P. mirabilis*	Rod	+	-	+	-	K/A	+	-	+	+
9	*Providencia*	Cocci	-	-	+	+	K/A	+	-	-	-
10	*Shigella*	Rod	-	-	-	V	K/A	+	-	-	-

KEY: += Gram positive, - = Gram negative, V = Variable, A = Acidic, K = alkaline, Nc = No change

Percentage of identified species

The samples obtained from industrial sources exhibited a diverse composition of identified bacterial species, each contributing to varying percentages of the total microbial population. The predominant species in the samples was *E. coli*, constituting the largest proportion at 60%. Following closely behind was *S. aureus*, comprising 30% of the identified species. Additionally, the samples contained several other bacterial species, each contributing to the overall microbial diversity. These included *Salmonella* (2%), *Shigella* (2%), *P. aeruginosa* (1%), *Yersinia enterocolitis* (1%), *Providencia* (1%), *P. mirabilus* (1%), and *B. subtilis* (2%). This distribution of bacterial species highlights the heterogeneous nature of the industrial samples, with E. coli and *S. aureus* being the most prevalent, while other species make up smaller but still noteworthy portions of the microbial community. Understanding this composition is essential for further research and applications in industrial settings, as different species may have distinct roles and impacts on various processes.

Table.2 Shows percentages of identified species.

S.NO	**Species**	**%**
01	*E. coli*	60
02	*S. aureus*	30
03	*Salmonella*	2
04	*Shigella*	2
05	*P. aeruginosa*	1
06	*Yersinia enterocolitis*	1
07	*Provindcia*	1
08	*P. mirabilus*	1
09	*Subtills*	2
10	*B. megaterium*	1

Treatment of Biofilm with Proteases Enzymes of *S. aureus*

The results indicate that enzymatic treatment with protease enzymes extracted from *S. aureus* has the potential to effectively disrupt *E.coli* biofilms. As the quantity of enzymes increased, there was a corresponding decrease in biofilm formation, with the highest reduction observed in replicate 3 (50 µL of enzymes). These findings highlight the promise of enzymatic treatment as a method for biofilm degradation and suggest that further research on a larger scale is warranted to explore its practical applications in combating biofilm-related infections.

Table: 3 Show OD of treated biofilm

S.NO	REPLICATES	TREATMENT	OD/600nm
1	Control	----	1.749
2	01	20 *ul*	1.698
3	02	30 *ul*	1.582
4	03	50 *ul*	0.589

DISCUSSION

Twenty Samples were collected from industries and investigated under standard protocol to identify bacteria. Results showed that high percentage of *E. coli* (60%) followed by *S. aureus* (30%), *Salmonella* (2%), *Shigella*, (2%), *P. aeruginosa* (1%), *Yersinia enterocolitis* (1%), *Providencia* (1%) *P. mirabilis* (1%) and *B. subtills* (2 %) are present in samples. The bacteria used for biofilm formation were *Escherichia coli*, obtained from the industrial water. *E. coli* cells were grown in nutrient broth for overnight. Estimation of biofilm formation was performed according to microtiter plate method with slight modification. Overnight cultures were diluted 1:100 into fresh medium and grown in 96-well microtiter plates at 37 ºC for 24 hrs. Biofilm formation was estimated by measuring the crystal violet staining of the cells attached to the microtiter plate wells. After discarding the medium in the microtiter plate wells, wells were rinsed with normal saline (three times). Then, crystal violet solution 1% crystal violet solubilized with 33% acetic was added to the microtiter plate wells for staining of the attached cells. After discarding the crystal violet solution and rinsing the microtiter plate wells, crystal violet was extracted by 70% ethanol solution, and that solution was transferred to a new microtiter plate, and the absorbance at 600 nm was measured using a plate reader. Similarly, research conducted by Naokai *et al*., (2004). The bacteria used for biofilm formation were *E. coli* obtained from industrial water.

CONCLUSION

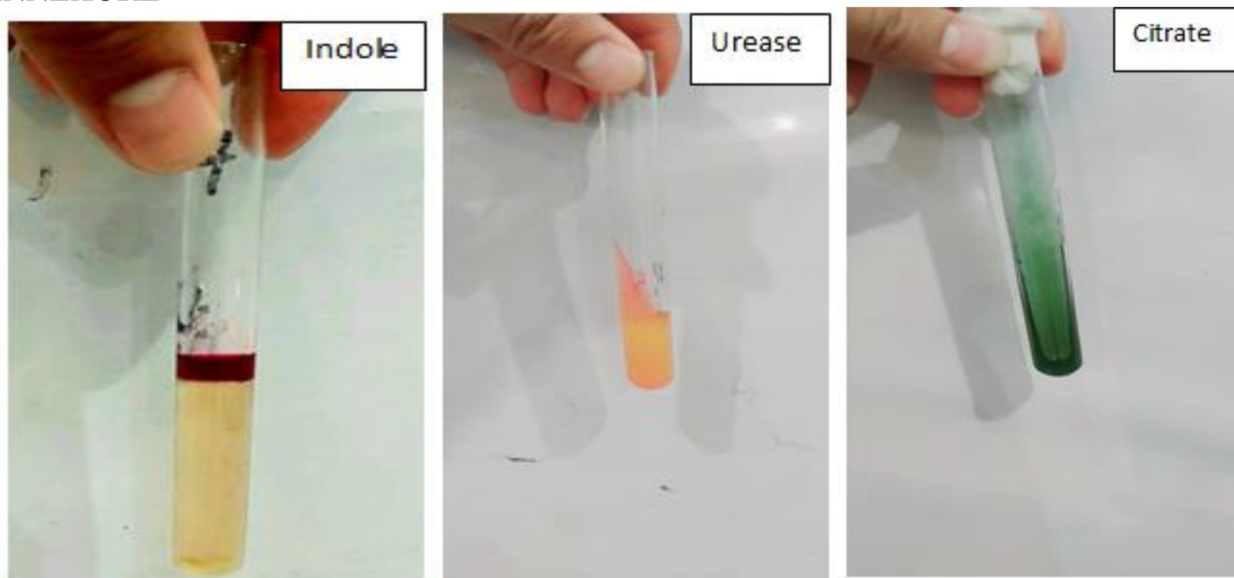
It is concluded from the current study that degradation of biofilm by enzymatic mean was suitable method. Enzymes extracted from *S. aureus* show best activity against biofilm formed by *E. coli.* As compared to other compounds enzymes are good to use for biofilm removal. In this study, we report purification and characterization of a biofilm degrading protease secreted by *S. aureus*. It showed optimum activity at 37 ºC and retained its activity in the presence of various salts and organic solvents. The enzyme was able to degrade biofilms efficiently at concentration lower than other known chemicals and detergents such as bleach, washing soda and acetic acid. Thus, this protease may serve as an effective tool for management of biofilms.

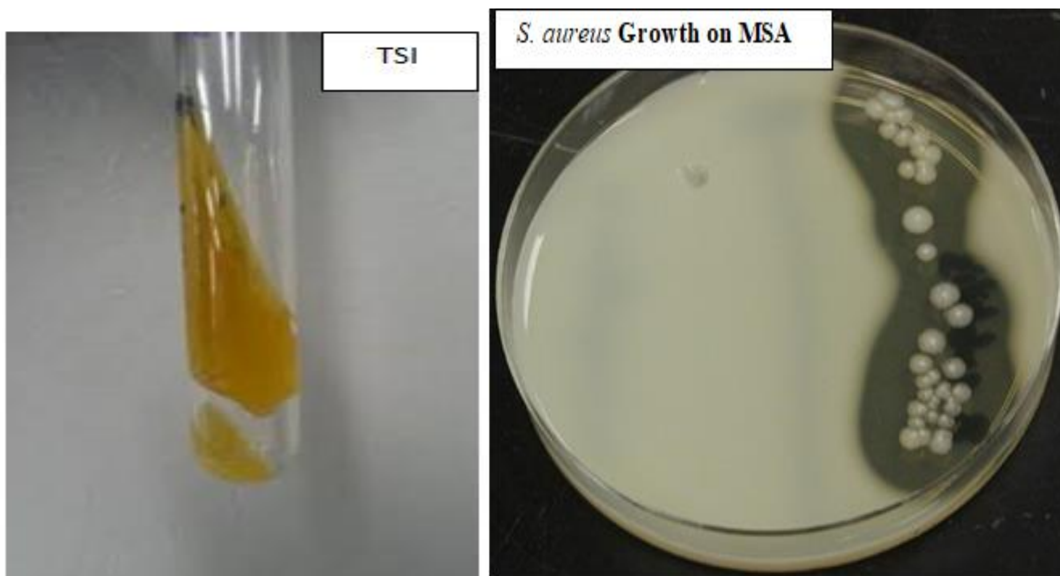
References

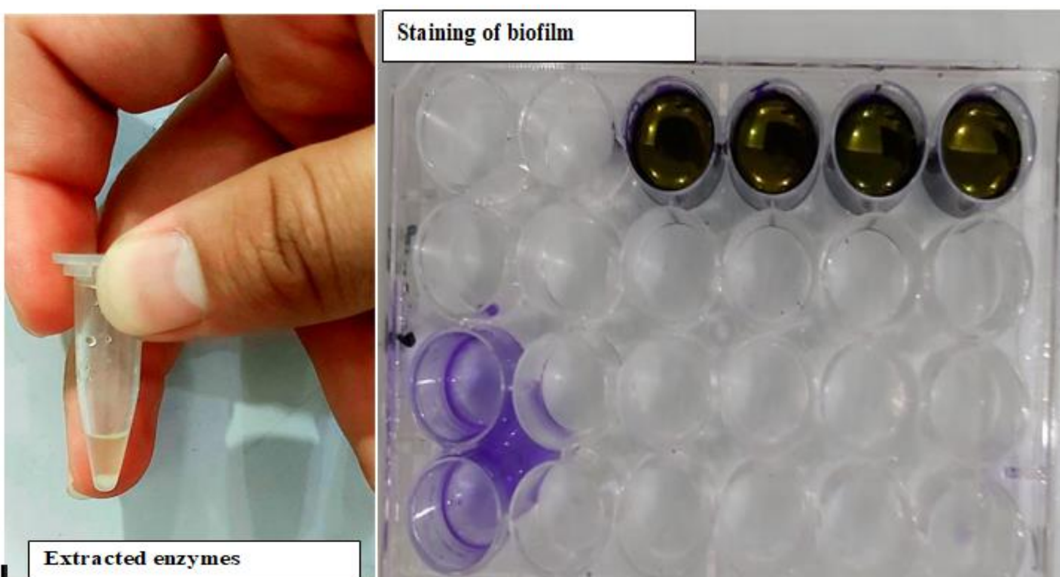
1. Giaouris, E., Heir, E., Desvaux, M., Hébraud, M., Møretrø, T., Langsrud, S., ... & Ölmez, H. (2015). Intra-and inter-species interactions within biofilms of important foodborne bacterial pathogens. Frontiers in microbiology, 6, 841.
2. Hancock, V., Dahl, M., & Klemm, P. (2010). Probiotic Escherichia coli strain Nissle 1917 outcompetes intestinalpathogens during biofilm formation. Journal of medical microbiology, 59(4), 392-399.
3. Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., ... & Calder, P. C. (2014). Expert consensus document: The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nature reviews Gastroenterology & hepatology, 11(8), 506.
4. Hong, S. H., Hegde, M., Kim, J., Wang, X., Jayaraman, A., & Wood, T. K. (2012). Synthetic quorum-sensing circuit to control consortial biofilm formation and dispersal in a microfluidic device. Nature communications, 3(1), 1-8.

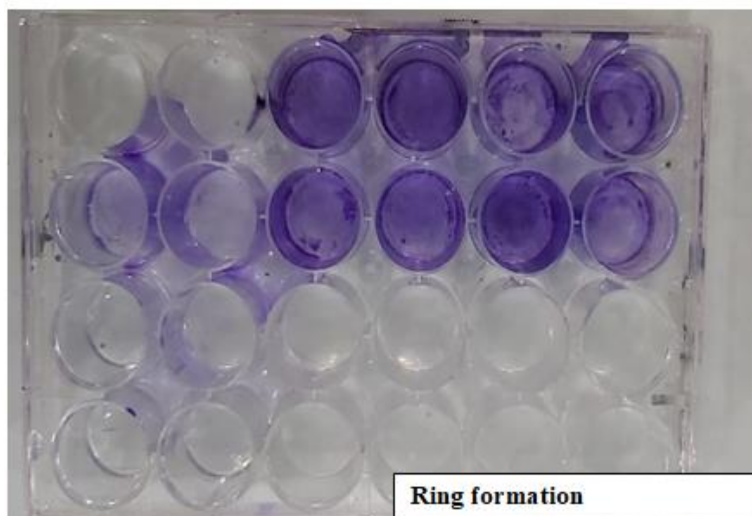
5. Hyland, N. P., Quigley, E. M., & Brint, E. (2014). Microbiota-host interactions in irritable bowel syndrome: epithelial barrier, immune regulation and brain-gut interactions. World journal of gastroenterology: WJG, 20(27), 8859.
6. Jamal, M., Ahmad, W., Andleeb, S., Jalil, F., Imran, M., Nawaz, M. A., ... & Kamil, M. A. (2018). Bacterial biofilm and associated infections. *Journal of the Chinese Medical Association*, *81*(1), 7-11.
7. Kay, M. K., Erwin, T. C., McLean, R. J., & Aron, G. M. (2011). Bacteriophage ecology in Escherichia coli and Pseudomonas aeruginosa mixed-biofilm communities. Applied and Environmental Microbiology, 77(3), 821-829.
8. Keogh, D., Tay, W., Ho, Y., Dale, J., Chen, S., Umashankar, S., ... & Kline, K. (2016). Enterococcal metabolite cues facilitate interspecies niche modulation and polymicrobial infection. Cell host & microbe, 20(4), 493-503.
9. Letoffe, S., Chalabaev, S., Dugay, J., Stressmann, F., Audrain, B., Portais, J. C., ... & Ghigo, J. M. (2017). Biofilm microenvironment induces a widespread adaptive amino-acid fermentation pathway conferring strong fitness advantage in Escherichia coli. PLoS genetics, 13(5), e1006800.
10. Lim, J. Y., Yoon, J. W., & Hovde, C. J. (2010). A brief overview of Escherichia coli O157: H7 and its plasmid O157. Journal of microbiology and biotechnology, 20(1), 5.
11. McBain, A. J. (2009). In vitro biofilm models: an overview. Advances in applied microbiology, 69, 99-132.
12. Oloketuyi, S. F., & Khan, F. (2017). Strategies for biofilm inhibition and virulence attenuation of foodborne pathogen-Escherichia coli O157: H7. Current microbiology, 74(12), 1477-1489
13. O'Toole, G. A. (2011). Microtiter dish biofilm formation assay. JoVE (Journal of Visualized Experiments), (47), e2437.
14. Rendueles, O., & Ghigo, J. M. (2012). Multi-species biofilms: how to avoid unfriendly neighbors. FEMS microbiology reviews, 36(5), 972-989.
15. Stressmann, F. A., Couve-Deacon, E., Chainier, D., Chauhan, A., Wessel, A., Durand-Fontanier, S., ... & Beloin, C. (2017). Comparative analysis of bacterial community composition and structure in clinically symptomatic and asymptomatic central venous catheters. Msphere, 2(5).
16. Vasudevan, R. (2014). Biofilms: microbial cities of scientific significance. J Microbiol Exp, 1(3), 00014.

ANNEXURE




TSI
S. aureus Growth on MSA


Extracted enzymes
Staining of biofilm


Ring formation