Pakistan J. Zool., vol. 51(5), pp 1783-1791, 2019. DOI: http://dx.doi.org/10.17582/journal.pjz/2019.51.5.1783.1791

Role of Salicylidene Acylhydrazide and Proteases in Biofilm Inhibition of *Desulfovibrio* spp.

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ABSTRACT

This study has highlighted that several compounds including salicylidene acylhydrazide, proteinase k, trypsin and chymotrypsin target bacterial virulence in *Desulfovibrio* spp. that causes bacteremia, periodontitis and abdominal infections. Ten soil samples were collected and screened by morphological and biochemical study. Only one strain DUV1 was confirmed by 16S rRNA gene sequencing as *D. vulgaris* (accession number: KY698020). It showed significantly reduced biofilm formation (52%; $p<0.05$) by test tube method compared to liquid interface coverslip assay (35%) and congo red assay. *D. vulgaris* was treated with various concentrations (5, 40, 80 and 100µM) of salicylidene acylhydrazide and 5-15 µM of proteases (proteinase K, trypsin, and chymotrypsin). Concentration dependent decrease was observed after 72 hours in biofilm formation of *D. vulgaris* on treatment with salicylidene acylhydrazide. Whereas 15 µM concentration of proteases produced significantly ($p<0.05$) reduced biofilm formation via test tube assay. Overall, while comparing all inhibitory compounds trypsin was most effective in decreasing biofilm formation followed by chymotrypsin > proteinase k > salicylidene acylhydrazides. Our study indicated that inhibitory compounds may disrupt the protein filament of flagellar apparatus producing significantly controlled biofilm formation which might also be helpful to control clinical infections associated with *Desulfovibrio* spp.

Article Information
Received 04 June 2018
Revised 30 July 2018
Accepted 06 August 2018
Available online 24 June 2019

Authors' Contribution
IL conceived, designed the study and experimental protocols. IL, NMA and MZ executed the experiments and analyzed the data. IL and NA interpreted the data.

Key words
***Desulfovibrio* spp., Salicylidene acylhydrazide, Proteinase K, Trypsin, Chymotrypsin, Biofilm formation.**

INTRODUCTION

Desulfovibrio spp. are Gram-negative, obligate anaerobe, non-spore forming vibrio-shaped bacteria, present in soil, animal intestine, and feces, fresh and even in aerobic marine water biofilms. Due to their slow growth, conventional approach without using any molecular technique makes it difficult to isolate from clinical samples. It is commonly present in anoxic sub surface biofilms for instance, region fashioned by microbial disintegration (Zhou *et al*., 2011). In anaerobic environment, *Desulfovibrio* spp. use sulfate as terminal electron acceptor for respiration that effects the production of sulfide, a greatly toxic and reactive compound (Ramel *et al*., 2013). *Desulfovibrio* spp. presents health implications by acting as opportunistic pathogen causing primary bacteremia and abdominal infections including abscesses and cholecystitis. Furthermore, some *Desulfovibrio* spp. may be associated with periodontitis, rapidly progressive periodontitis, adult periodontitis, and refractory periodontitis (Berry and Reinisch, 2013). Little is known about the inhibitory effect of synthetic compounds on *Desulfovibrio* spp. biofilms. Biofilms are aggregates of bacteria in aquatic ecosystem enclosed into a slimy glycocalyx polysaccharides matrix. Bacteria in biofilm mode form micro colonies and various assays including test tube assay, coverslip assay and congo red assay are used to assess biofilm forming ability. Test tube assay and air liquid interface assay are considered more reliable and precise as compared to congo red assay. Both methods produce biofilms but several past studies reveal that biofilm forming percentage of bacteria and accuracy is present greater in tube method (92%) than cover slip method (42%) and congo red method (11%) (Ruzicka *et al*., 2004; Mathur *et al*., 2006; Baqai *et al*., 2008).

Desulfovibrio spp. uses protein filament flagella not only for the purpose of locomotion but also for the formation of biofilm (Clark *et al*., 2007). The structure of protein filament helps to drive the type III secretion system. Type III secretion system is tool for spreading disease both by animal and plant pathogens. Type III secretion system works like flagellar apparatus which is associated with biofilm formation in *Desulfovibrio* spp. The use of drugs that specifically target virulence properties of bacteria

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0030-9923/2019/0005-1783 $ 9.00/0

without killing them is a way to combat the emerging bacterial resistance against these drugs. Previously, in many screening assay, small molecules such as caminosides, unique glycolipids, guadinomines, 2-imino-5-arylidene thiazolidinone that inhibit virulence properties of T3SS harboring strains without affecting the viability were identified (Kimura *et al*., 2011). In subsequent years, these and other substances such as salicylidene acylhydrazides, protease K, trypsin and chymotrypsin were found to inhibit secretion of T3SS-related proteins and to affect host cell infection by *Yersinia*, *Chlamydia* and *Salmonella* (Tsou *et al*., 2013).

The first account on salicylidene acylhydrazide and its derivatives came in 2003 by the work of chemists from Umea University, Sweden (Duncan *et al*., 2012). Salicylidene acylhydrazide becomes a strong suppressor of biofilm formation by pathogenic bacteria when it is mixed with gallium ion and affects both T3SS secretion and motility without generally affecting transcription. Salicylidene acylhydrazide complex with gallium is effective in targeting Fe metabolism in tumors and can be used as anticancer agent in future (Rzhepishevska *et al*., 2014). Many Bioisosters (2-(2-Aminopyrimidine)-2,2-difluoroethanols are synthesized and used as inhibitors of type III secretion system in several Gram negative pathogens (Dahlgren *et al*., 2010). The inhibitory compounds are better choice as compared to antibiotics as some bacteria gained resistance against antibiotic not against inhibitory compounds (Duncan *et al*., 2012).

Proteinase K is a broad-spectrum serine protease and isolated from the fungus *Engyodontium album* (formerly *Tritirachium album*). Proteinase K is able to digest hair protein (keratin) hence, the named "Proteinase K". It is commonly used to check extracellular proteolytic activity *e.g*., swarming motility exhibited by protease deficient strains. Activated by calcium, the enzyme digests proteins preferentially after hydrophobic amino acids (aliphatic, aromatic and other hydrophobic amino acids) (Hungtington *et al*., 2000).

Trypsin and chymotrypsin, like most proteolytic enzymes, are synthesized as inactive zymogen precursors (trypsinogen and chymotrypsinogen) to prevent unwanted destruction of cellular proteins, and to regulate when and where enzyme activity occurs. The inactive zymogens are secreted into the duodenum, where they travel the small and large intestines prior to excretion. Zymogens are converted to mature, active enzyme by proteolysis to split off a pro-peptide, either in a subcellular compartment or in an extracellular space where they are required for digestion. Trypsin and chymotrypsin are structurally very similar, although they recognize different substrates. Trypsin acts on lysine and arginine residues, while chymotrypsin acts on large hydrophobic residues such as tryptophan, tyrosine and phenylalanine, both with extraordinary catalytic efficiency. Both enzymes have a catalytic triad of serine, histidine and aspartate within the S1 binding pocket, although the hydrophobic nature of this pocket varies between the two (Ye *et al*., 2001).

While it has been proposed that flagella might act in biofilm formation both as surface adhesions and as providers of force-generating motility, in at least *Escherichia coli* and *Vibrio cholerae* it is motility itself that is critical (Watnick *et al*., 2001). To date, the published data on the role of *D. vulgaris* biofilm formation and maturation suggests the involvement of proteinaceous material considering it as flagella (Clark *et al*., 2007, 2012). Hence , in this study, comparing wild-type bacteria to flagellum-minus and paralyzed-flagellum mutants created using salicylidene acylhydrazides, proteinase K, trypsin, and chymotrypsin, we established that flagellum-mediated motility is critical for biofilm formation on abiotic surfaces and flagella role as surface adhesions is dependent upon motility.

MATERIALS AND METHODS

Sample collection and purification of isolated strain

Ten samples of both soil and water were collected from different metal contaminated or sewage sites (River Ravi, Shalimar gardens, Canal bank, Jinnah garden and Sewage water) of Lahore. Samples were homogenized and subjected to different dilutions (10^{-1} …. 10^{-10}) using PBS. API sulfate agar medium was used to isolate and screen *Desulfovibrio* spp. strains (George *et al*., 2007). Morphologically different colonies were isolated and characterized biochemically. Briefly a small drop of a sample was spread on API agar plate placed in anaerobic chamber and colonies with black coloration were observed over two week period at 37°C. The general form of the colony, its color and shape, margins and texture was observed. Gram staining technique was used to differentiate the gram negative bacteria from Gram positive bacteria. Acid fast technique, endospore staining and motility test were performed (Jousimies-Somer *et al*., 2002). Pure cultures were maintained as stock cultures in 120-ml screwcap bottles containing liquid medium supplemented with 1 ml of a growth factor solution per liter, pelleted (to reduce carryover of glycerol) and stored at −80°C in cryovials.

Biochemical characterization and 16S rRNA gene sequencing

Biochemical tests were performed to identify bacterial strains till genus level as described in Berge's Manual of Systematic Bacteriology in an anaerobic

chamber (COMSAT, off Defence Raiwind Road, Lahore). These tests include catalase test, citrate utilization test, methyl red test, carbohydrate fermentation test, hydrogen sulfide production test, indole test, urease test (Liaqat *et al*., 2015), nitrate growth test and tryptophan deaminase (TDA) test (Jousimies-Somer *et al*., 2002).

Genomic DNA was extracted using phenol chloroform method (Wright *et al*., 2017) and PCR was performed under standard conditions using forward primer (5' GTTTGATCCTGGCTCAG 3') and reverse primer (5'TACCTTGTTACGACTT'3) to amplify 16S rRNA gene (Liaqat *et al*., 2015). Partial sequence (650 bp) was deposited in GenBank databases (KY698020) and phylogenetic tree was constructed using Clustal Omega.

Physiological characterization

Precultures were prepared as mentioned above. Physiological characterization was done on the basis of growth curve, temperature and pH. Briefly, for each individual batch experiment, cryovials stored at −80°C were streaked onto API agar plates. After incubation at 37°C and pH 7, one colony was picked and loop inoculated into a 500-ml Erlenmeyer flask containing 200 ml of API broth in anaerobic chamber. Bacterial growth curve was measured by withdrawing samples (1 ml) periodically and measuring an optical density at 590 nm (OD_{590}) spectrophotometrically. Linear regression of natural log cell number versus time data and slope of this line to measure specific growth rate was used to calculate the specific growth rate.

Optimum temperature (25 -45°C) and pH (4-9) was determined by incubating flasks in anaerobic chamber to late exponential or stationary phase and measuring OD_{590}. The experiments were run in triplicates. A graph between optical density and time (hrs) was plotted for each experiment.

Biofilm formation and quantification

Congo red assay, test tube assay (Liaqat *et al*., 2009) and air liquid interface cover slip assays were performed (Mathur *et al*., 2006) for biofilm formation and quantification. For test tube assay and liquid interface coverslip assay, biofilm was grown for 3, 5 and 7 days. 5ml of glacial acetic acid was added to preformed and washed biofilm after 3 days and O.D was measured at 590 nm.

Salicylidene acylhydrazides and protease treatments

Effect of salicylidene acylhydrazides and protease was studied following Negrea *et al*. (2007). Salicylidene acylhydrazide was provided by Dr. Mikael Elofsson, Department of Chemistry, Umea University, Sweden. The compound was solubilized in dimethyl sulfoxide (DMSO; Sigma) to a concentration of 20mM and added to 3 days old biofilm to a concentration of 5µM, 40µM, 80µM and 100 µM applying test tube assay. 10.6 mM Ga $(NO_3)_3$ solution was prepared (Rzhepishevska *et al*., 2014) and mixed with above mentioned concentrations of inhibitory compound. The DMSO was used as negative control in all experiments. The solvent concentration never exceeded 0.2% (vol/vol).

To study the effect of protease, biofilm was grown 3 days post inoculation in test tubes (Liaqat *et al*., 2017). Samples were rinsed with 10ml of 60mM PIPES and placed it in enough 50mM PBS to cover the formed biofilm in test tube. The PBS contained 5, 10 and 15µgml^{-1} of trypsin, proteinase K and chymotrypsin was added to biofilms followed by incubation at 30°C for 15 min. After incubation, 5ml of 0.03 % crystal violet was added to tubes and measured spectrophotometrically at 590 nm.

Motility assay of wild type and treated strains

Wild type and treated strains were compared and analyzed for motility on API sulphate motility agar plates. These plates contained either 1% agar or 0.7% (w/v) agar. Plates were inoculated by broth culture at 37°C in an anaerobic glove chamber. Plates were visually inspected after 48h of inoculation for differences in motility halos between the wild type and mutant strains (Clark, 2007).

Statistical analysis

One way/2-way analysis of variance (ANOVA) and Student "t" test were performed using SPSS 13.0 to determine the statistical significance of all experimental data at $p<0.05$. Biofilm formation was measured after treatment with various concentrations of salicylidene acylhydrazide and controls. Similarly the comparison of variance by proteinase K, trypsin, and chymotrypsin and salicylidene acylhydrazide was checked. Asterisk (*) indicate values that are significantly different from control group by ANOVA/t test at $p<0.05$.

RESULTS

Morphological characteristics

Ten strains were studied morphologically, biochemically and physiologically. Three strains were vibrio and black in color showing convex elevation and smooth surfaces. Five strains were cocci and white/yellow/black in color showing raised margins and convex elevation with smooth surfaces. Rest of the two strains was curved rod in shape and black in color showing entire margins with raised elevation and smooth surface. All strains were Gram negative and motile showing negative result for acid fast and endospore staining. The size of the colonies measured 3-5 µm in diameter (Table I).

Table I.- Morphological characteristics of isolated bacterial strains.

Sr. No.	Strains	Form	Color	Texture	Margin	Elevation	Surface	Size (µm)
1	DUV1	Rod	Black	Moist	Entire	Raised	Smooth	5
2	DUV2	Rod	Black	Moist	Entire	Raised	Smooth	5
3	DUV3	Vibrio	Black	Moist	Lobate	Convex	Smooth	5
4	DUV4	Cocci	Black	Moist	Entire	Convex	Smooth	5
5	DUV5	Vibrio	Black	Moist	Entire	Convex	Smooth	3
6	DUV6	Vibrio	Black	Moist	Lobate	Convex	Smooth	5
7	DUV7	Cocci	Yellow	Dry	Lobate	Raised	Rough	5
8	DUV8	Cocci	Black	Moist	Entire	Raised	Rough	3
9	DUV9	Cocci	Yellow	Moist	Entire	Convex	Smooth	4
10	DUV10	Cocci	White	Moist	Entire	Convex	Shiny	4

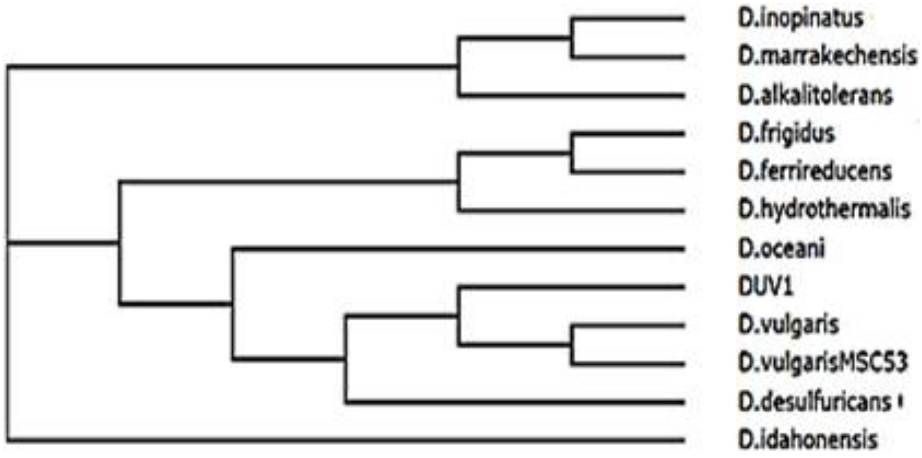

Fig. 1. Dendrogram of *Desulfovibrio* spp. constructed using Clustal Omega showing homology with *Desulfovibrio* spp. MSC53.

Biochemical characteristics and identification

All strains were catalase positive, indole positive, motile and produced H_2S gas. Six out of 10 strains metabolize citrate for their energy needs. All bacteria showed negative results for sucrose and lactose metabolism. Only three strains showed positive results for urease test. Six strains indicated methyl red negative results.

Genomic DNA was obtained successfully from two strains only and only one was successfully PCR amplified for 16S rRNA gene. Sequencing result of the 16S rRNA gene of DUV1 deposited in the GenBank databases had accession number, KY 698020. Phylogenetic tree showed maximum homology with *Desulfovibrio* spp. (Fig. 1).

Physiological characteristics

Growth curve of *Desulfovibrio* spp. indicated that it has 2 days long lag phase, 3 days long log phase (specific growth rate, $\mu = 0.103\ h^{-1}$ and doubling time, T_d of 6.7 h followed by 3 days long stationary phase (Fig. 2A) optimum temperature was found to be 37°C (Fig. 2B) and optimum growth was observed at pH 7 (Fig. 2C).

Biofilm formation curve and effect of inhibitory compounds

Congo red assay showed variation in biofilm forming capacity of strains. Colonies color varying from light black to light brown indicating weak/ light biofilm formation was observed, while control (wild type) and mutant strains showed black and red colonies, respectively (Table I).

Test tube assay showed strong and significant biofilm formation (52%; $p<0.05$) for all strains compared to liquid interface coverslip assay (35%) (Fig. 3). Hence further experiments to monitor biofilm inhibition with salicylidene acylhydrazide, trypsin, proteinase K and chymotrypsin were carried out using test tube assay only. Treatment of *D. vulgaris* with salicylidene acylhydrazide resulted in concentration dependent significantly reduced biofilm formation after 72 h (Fig. 4). Likewise, trypsin, proteinase-K, and chymotrypsin showed no significant effect on biofilm formation upto $10\mu gml^{-1}$ concentration (Fig. 5A, B). However, significantly ($p<0.05$) reduced biofilm formation was observed at $15\mu gml^{-1}$concentration of trypsin, proteinase K and chymotrypsin compared to wild type (Fig. 5C).

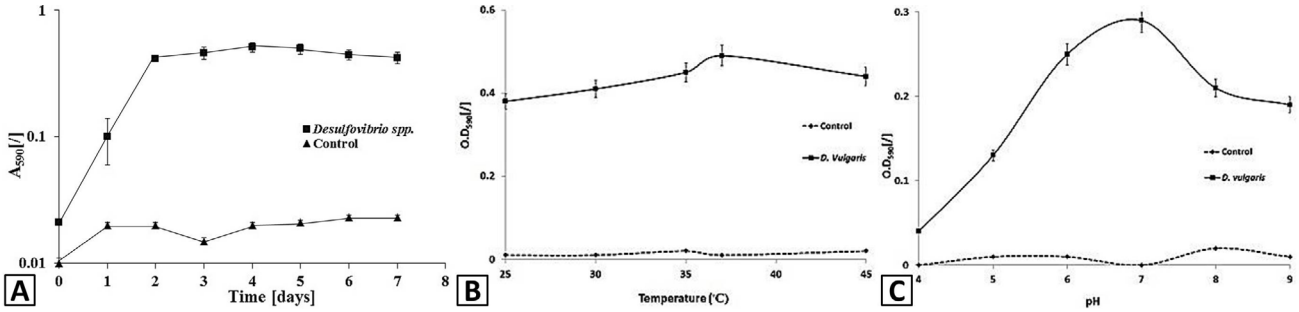

Fig. 2. Growth curves of *Desulfovibrio* spp. A, *Desulfovibrio* spp. was grown in lactate sulfate broth for 7 days at pH 7, incubated at 37°C. O.D was determined at 590 nm. Maximum growth was observed after 3 days. B, Effect of temperature: *Desulfovibrio* spp. was grown in lactate sulfate broth for 72 h at different temperatures (25-45°C). O.D was determined at 590 nm. Highest growth was observed at 37°C. C, Effect of pH: *Desulfovibrio* spp. was grown in lactate sulfate broth for 72 hours at different pH, incubated at 37°C. O.D was determined at 590 nm. Maximum growth was observed at pH 7. Error bars in figures represent standard deviation (n=3).

Table II.- Congo red assay of treated *Desulfovibrio* spp.

Sr. No.	Strains	Conc. used	Colony color	Inference
1.	DUV1+ve C*	Untreated/wild	Dark black	Thick biofilm formation
2.	DUV1 –ve C*	Mutated *Desulfovibrio* spp.	Red	Absence of biofilm formation
3.	DUV1 Try*	10 µM	Light Brown	Very reduce biofilm formation
4.	DUV1 Chymo*	10 µM	Moderate Brown	Moderately reduce biofilm formation
5.	DUV1 Pro. K*	10 µM	Brown	Reduced biofilm formation
6	DUV1 S. acyl*	80 µM	Light Black	Weak biofilm formation

*, +ve C, positive control; -ive C, negative control, Pro. K, Proteinase K; Tryp, Trypsin; Chym, Chymotrypsin; S. acyl, Salicylidene acylhydrazide.

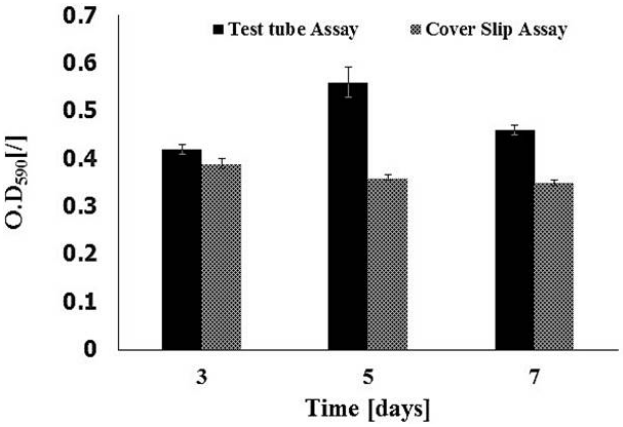

Fig. 3. Comparison of biofilm formation by *Desulfovibrio* spp. using test tube and cover slip assay using 2-way ANOVA analysis. *Desulfovibrio* spp. was grown in lactate sulfate media for 72 h at 37°C and pH 7. OD was measured at 590 nm. Test tube assay showed significantly strong biofilm formation after 5 days compared to coverslip assay ($p<0.001$). Error bars represent standard deviation (n=3) and * is significantly different.

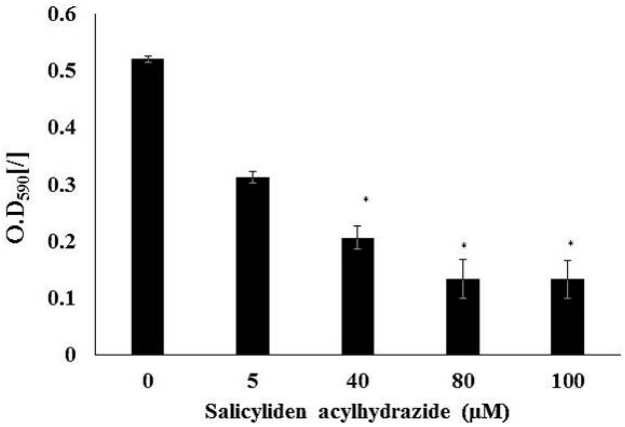

Fig. 4. Effect of salicyliden acylhydrazide on *Desulfovibrio* spp. biofilm formation. *Desulfovibrio* spp. was grown in lactate sulfate medium for 72 h at 37°C and pH 7. Various concentrations (5, 40, 80 and 100 µM) of salicylidene acylhydrazide were added. OD was measured at 590nm. Significant reduction in biofilm formation was observed at 40, 80 and 100 µM ($p<0.001$). Error bars represent standard deviation (n=3) and * = significantly different.

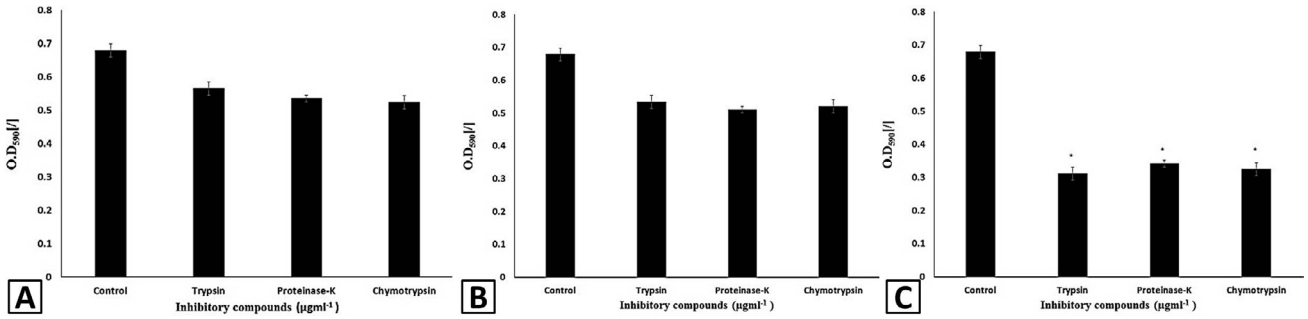

Fig. 5. Effect of 5µM concentration (A), 10µM concentration (B) and 15µM concentration (C) of trypsin, proteinase K and chymotrypsin on *Desulfovibrio* spp. biofilm formation. *Desulfovibrio* spp. was grown in lactate sulfate medium at 37°C and pH 7 for 72 h. OD was measured at 590 nm in A and B. Significant reduction in biofilm formation was observed in C using all compounds ($p<0.01$). Error bars represent standard deviation (n=3) and * is significantly different.

Motility in treated and wild type strains

Since motility may contribute to biofilm formation, wild type and treated strain were grown on LS4D media agar plates. Wild type produced a diffused halo at 1% agar plates or confluent colony on 0.7% media plate. Treated strain was more sporadic and uneven compared to wild type (data not shown).

DISCUSSION

Biofilms are actually bacterial community enclosed in the protective but complex extracellular polymeric substances (EPS) (Beech, 2004). Biofilm formation is a tool for determining the bacterial pathogenicity. Extra polymeric substance in biofilm acts as protective shield for microorganisms against anti biotics (Chandki *et al*., 2011).

This study was planned to investigate the inhibitory effect of salicylidene acylhydrazide, proteinase k, trypsin and chymotrypsin on biofilm forming ability among the wild type and treated bacterial strains which was further confirmed by motility assays afterwards. Total ten bacterial strains were studied morphologically and biochemically following Bergey's Manual of Systematic Bacteriology, which depicted isolated strains similarity to *Desulfovibrio* spp. Biochemical tests demonstrated results like: catalase test (100%), citrate test (40%), H_2S test (100%), carbohydrate fermentation (40%) for glucose, (40%) for sucrose fermentation while (40%) positive results at lactose fermentation. TDA test (60%), indole test (100%), nitrate growth test (70%), urease test (70%), methyl red test (40%). Two of our strains DUV1 and DUV2 showed close resemblance to *Desulfovibrio* spp. as observed later by partial sequencing of DUV1. Previous studies also showed similar findings with only difference that *Desulfovibrio* spp. was catalase negative (Warren *et al*., 2005; Clark *et al*., 2007; George *et al*., 2007). Discrepancy in biochemical characterization might be due to differences in test methods. Warren *et al*. (2005) reported different biochemical reactions while characterizing four strains of *Desulfovibrio* spp. compared to what was observed by Loubinoux *et al*. (2000).

Traditional methods of identification and characterization of bacterial strains are difficult to operate and time-consuming. Moreover, variable phenotypic of strains belonging to the same specie result in character unique enough to be identified easily (Drancourt *et al*., 2000). Among modern techniques, 16S rRNA gene sequence data is the most reliable technique to determine bacterial phylogenetic relationship. In this study, only one strain DUV1 was successfully PCR amplified for 16S rRNA gene and submitted to M/S Bio science international for sequencing. The sequencing result was submitted to GenBank databases under accession number KY 698020. Clustal Omega was used to construct Dendrogram showing 96% homology with *D. vulgaris* which is below the average nucleotide identity of 98.65 % for relatedness between strains. Hence this strain was referred as *Desulfovibrio* spp. Previously, Warren *et al*. (2005) characterized four species of *Desulfovibrio* spp. using 16S rRNA gene sequences and biochemical tests including catalase, indole, nitrate, bile, urease, formate-fumarate stimulation, desulfoviridin, motility, and hydrogen sulfide production. All *Desulfovibrio* spp. produced H_2S and motile except for *D. piger*. The four *Desulfovibrio* spp. could be distinguished from each other using tests for catalase, indole, nitrate, urease and growth on bile with the following results (positive [+], negative [-], growth [G], and no growth [NG]): for *D. piger*, -, -, -, -, and G, respectively; for *D. fairfieldensis*, +, -, +, -, and G, respectively; for *D. desulfuricans*, -, -, +, +, and NG, respectively; and for *D. vulgaris*, -, +, -, -, and G, respectively.

Desulfovibrio spp. grows very slowly as observed by measuring growth curve, optimum temperature and pH. It took 3 days to reach log and 3 days to reach stationary phases respectively. Bacterium was mesophilic in nature, having optimum pH of 7. Our results are in accordance to with Ismail *et al*. (2014), who grew *Desulfovibrio* spp. at 37°C in modified Baar's medium which has almost same composition as of lactate sulfate media used in this research. The sulfate reducing bacteria showed maximum growth after 72 h. The growth started to decrease at 4, 5, 6 and 7 days.

Our experimental data showed strong and significant biofilm formation (52%; $p<0.05$) by test tube method compared to liquid interface coverslip assay (35%) and congo red assay. Congo red method is however not very authentic method for biofilm formation (Taj *et al*., 2012). Similar observations were made by others (Mathur *et al*., 2006). Test tube assay and air liquid interface assay are considered more reliable and precise as compared to Congo red assay (Liaqat *et al*., 2009). Previously, Baqai *et al*., (2008), also reported significant biofilm production by strains using the above two methods. Their data revealed that biofilm forming percentage of bacteria and accuracy was high in test tube method (54%) than coverslip method (44%).

Treatment with inhibitory compounds revealed massive biofilm formation in control and significantly reduced biofilm formation ($p>0.001$) in *Desulfovibrio* spp. Salicylidene acylhydrazide chelated with gallium treatment upto 80µM showed significantly reduced biofilm formation after 72 h. Similar results were obtained by Rzhepishevska *et al*. (2014), who applied 3µM concentration of Ga (III) hydrazine complex on biofilm formation of *P. aeruginosa.* Likewise, Hudson *et al*. (2007) reported that salicylidene acylhydrazides were identified to affect flagellum mediated motility by inhibiting type III secretion system (T3SS) in *Yersinia, Chlamydia, Salmonella* and *Shigella* species. The protein nature of biofilm was confirmed by protease treatment using trypsin, proteinase-K and chymotrypsin. It was assumed that protease treatment could possibly control the formation of mature biofilm as observed by significant decrease ($p<0.05$) in biofilm formation at 15 μgml^{-1} concentration in this study. Our preliminary data indicated the role of extracellular protein in biofilm formation. This was further confirmed by motility assay. Decreased motility observed in treated strains compared to wild type might be due to defective and or absence of flagella, necessary for attachment and maintenance of biofilm. The present study indicated that *Desulfovibrio* spp. biofilm maturation may be under the control of specific proteins, however future work is required to identify those specific proteins and their role in biofilm maintenance of *Desulfovibrio* spp. Likewise, Clark *et al*. (2007) observed the similar inhibitory effect of proteases on the mixed biofilm and reported that *Desulfovibrio* spp. biofilm was dependent on extracellular proteins rather than carbohydrates.

Desulfovibrio spp. causes petroleum loss at industrial level by corrosion of petroleum oil and gas pipes, quality of petroleum products by degrading hydrocarbons increasing its viscosity which increase cost of petroleum extraction. *Desulfovibrio* spp. also causes uranium bioremediation by reducing U (VI) to U (IV). Furthermore, it reduces uranium in a fastest rate in sulfate reduction conditions in the absence of Fe (II) (Vannela *et al*., 2012). Salicylidene acyl hydrazide also shows *in vitro* anti-HIV-1 activity and thus these compounds can be fruitful in controlling many sexually transmitted diseases (Forthal *et al*., 2012). Likewise, proteinase K is used to purify the nucleic acid in the presence of EDTA. Chymotrypsin is found in pancreatic juice acting in duodenum where it is used for proteolysis (breakdown of proteins and polypeptides), act as digestive enzyme. Owing to increasing trends of bacteria gaining antibiotic resistance, inhibitory compounds usage is not only novel idea for research but also alternate to antibiotics for controlling diseases. Therefore, it is recommended that these compounds may have applied value for both pharmaceutical and petroleum industry (Duncan *et al*., 2012).

CONCLUSIONS

The result of this study suggested that salicylidene acylhydrazide, proteinase K, trypsin and chymotrypsin showed significant reduction (0.120*) in biofilm formation by *Desulfovibrio* spp. The usage of these compounds is better choice due to their interference with bacterial T3SS, hence without having any concern of increased resistance as observed in antibiotic treated strains. Petroleum industry may get rid of petroleum pest by reducing biologically induced corrosion due to disarming of bacteria from their flagellar apparatus, leading to boost up economy. The data in this study may be helpful to control the growth of *Desulfovibrio* spp. at ideal level both at *in vitro* and *in vivo* by the application of these inhibitory compounds. It would be effective and cost friendly as compare to drugs used in the pharmaceutical industry as well as controlling biocorrosion process, therefore mediating bioremediation.

ACKNOWLEDGEMENT

This study was supported by ORIC Grant (No. 90/ORIC/15,). The authors will like to express their gratitude to Department of Chemical Engineering Department,

COMSAT University, Lahore and Dr. Mikael Elofsson, Department of Chemistry, Umea University Umea, Sweden for facilitating the study.

Statement of conflict of interests

The authors have declared that there is no conflict of interests.

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