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**Original Article**

# Twitching, swimming, swarming in biofilm forming strains in response to chemical and physical factors

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**STA:** perform the experiments and draft the manuscript, **ANS:** Supervise the research and improved the manuscript

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Temperature
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Proteinase K
Tobramycin

**Abstract**

Motility is a dynamic and remarkable element of bacterial physiology and determines the ability of bacteria to form biofilms. There is a need to control bacterial motility. Present study determined swimming, swarming, and twitching motilities in response to different physiochemical factors. Twenty-two morphologically different bacterial strains were collected from a drain of sewage water located in the vicinity of Jinnah hospital Lahore, Pakistan. Six biofilm forming bacterial strains were selected on the basis of their motility and biofilm forming ability firstly by growing them on congo red agar medium and then by performing ring test. By 16S rRNA sequencing, bacterial strains were identified as *Exiguobacterium aurantiacum, Bacillus subtilis, Bacillus megaterium*, *Bacillus endophyticus*, *Pseudomonas fragi* and *Bacillus subtilis*. The optimum temperature and pH for swimming, swarming and twitching motilities was 37°C and pH 7. All the bacterial strains showed reduction in diameter of zones of all motilities when the concentration of NaCl increased in medium. Among chemical factors, tobramycin was proven to have excellent efficiency to reduce bacterial motilites whereas proteinase K enzyme showed weak antimotility effect against all tested bacterial strains. From this study we proposed that instead of biofilm detachment by expensive commercial detergents, we could change the physical environment of sewage systems as well as flooding some specific chemicals, which disrupt bacterial motilities and biofilm formation.

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## INTRODUCTION

The ability of microorganism to promptly reach and colonize to biotic and abiotic surface to form new microniches is called their motility. Motility is an essential and remarkable element of bacterial physiology and determines the competitive success of bacteria for host invasion, biofilm formation (Harshey, 2003) and different mechanisms for the movement of bacteria in aqueous medium across surfaces. The swimming motility occurs when bacteria with disorganized morphological pattern swim along the surface of an adequately thick aqueous layer. When aqueous layer on a surface is thin in natural habitat or when bacterial cells are inoculated on agar surface in laboratory, the unorganized cells begin to differentiate into elongated, multinucleated, and hyper flagellated cells. These cells are called as swarm cells and their movement on surfaces called swarming motility (Harshey, 2003). In response to environmental factors, bacteria adopt an organized and reversible mode of living called swarming behavior (Senesi *et al.,* 2010). This behavior allows bacteria to travel to nutritionally rich environment and retreat from source of possible damage. It protects bacteria from harmful effects of antibiotics and other antimicrobial agents. It allows bacteria to circumvent competition and involves them to form biofilms (Harshey, 2003).Type IV pili on bacterial cell surfaces are responsible for twitching and adhesion and hence biofilm formation (Salzer*et al.,* 2014). Flagellum independent motility in *N. gonorrhoeae* and *P. aeruginosa* are the best illustrations of the twitching motility (Shi and Sun, 2002).

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Attachment of bacterial cells to the available matrix/surfaces is determined by the micro and macro environmental factors. Among them surface hydrophobicity, cations/ anion concentrations present, pH, temperature and redox conditions are of due importance. It also depends on flagellation and motility of microorganisms. (Bonaventura *et al.,* 2008; Fakhar *et al.,* 2017). Curatolo *et al.* (2017) studied the mechanism that how two species or different types of bacteria have mutual control of their motilities. Depending upon the type of species, type of motility, one species can hinder, lower or enhance the motility of other species. Initially in colonization mixed colonies grow in variety of pattern but further maturation depends upon the competitive motilities of the species in controlled fashion. Different techniques, motion video analysis and softwares are currently available to monitor the bacterial motilities in response to repellants, chemotaxis induced chemicals or pseudo taxis responses especially the swimming behavior matrices can be analyzed (Pottash *et al.,* 2017). Taking in view of importance motilities in biofilm formation and dispersion in the presence of different temperature, pH and antibiotics/antimicrobials present study was conducted. Presently responses of different types of motilities in biofilm forming bacteria are reported in association with different chemical and physical factors.

## MATERIAL AND METHODS

### *Sample collection*

Sampling of waste water was done from a sewerage drain (also contaminated with hospital waste) in sterilized bottles located in the vicinity of Jinnah hospital Lahore, Pakistan. Physicochemical parameters like temperature and pH of sewage water were measured at the site of sample collection. These samples were then taken to laboratory of MMG department PU, Lahore under sterilized conditions and processed at the same day.

### *Isolation and characterization of purified strains*

Bacterial strains after isolation and purification were maintained on L-agar plates. Purified bacterial strains were characterized morphologically, biochemically, physiologically (Cappuccino and Sherman, 2007). Gram staining and motility assays were performed.

### *Selection of biofilm formers*

Among the purified bacterial strains, biofilm formers were selected by using congo red method (Mathur *et al.,* 2006), and biofilm formation by test tube method (Liaqat *et al.,* 2009).

### *Identification of biofilm forming bacterial strains*

In order to find their taxonomic position, biofilm forming bacterial strains were sent to Macrogen Inc. Seoul South Korea for 16S rRNA gene sequences. Obtained data of sequencing was then analysed by ChromasLite software and compared with the known sequences in GenBank database.

### *Response of motility to physical and chemical factors*

Response of swimming, swarming, and twitching motilities were monitored under different physical and chemical conditions. Among physical factors, effect of varying temperature (25, 37 and 45°C), varying pH (5, 7, 9), and different salt concentrations (1.5, 5.5, and 10.5 %) was checked. Among chemical factors, effect of SDS (0.1, 0.5, and 1%), proteinase K (25, 50, and 100 µg/ml), and tobramycin (0.001, 0.0015, and 0.002 %) was checked. Analysis of variance was performed on data of zone of bacterial growth in milimeter.

### *Swimming, swarming and twitching motility assay*

Following Deziel *et al.* (2008), swimming and swarming motilities were checked on swimming and swarming agar base. By using sterile needle, single colony was transferred to the swimming and swarmining agar surface. Plates were then incubated for 48 hours at respective temperatures and pH and separate swimming and swarming agar plates were prepared for each concentration of NaCl, SDS, proteinase K and tobramycin. After incubation, results were stated as diameter of zone of growth (mm) made around the inoculation point. Twitching motility was checked on twitching agar. Single colony was inoculated to the bottom of twitching agar plate. Incubation was done as in swimming and swarming motilities. After incubation, plates with removed agar were flooded with crystal violet. CV-stained zone of migration and growth was measured (mm).

## RESULTS AND DISCUSSION

***Physicochemical properties of sewage water***

The temperature and pH of different sewage water were recorded (Table II)

***Isolation and characterization of purified strains***

Twenty-two morphologically different bacterial strains were isolated from two sewage water samples. After purification, Gram staining and motility tests were performed.

**Table I: Physicochemical properties of sewage water samples**

| Sample No. | Sample type | Locality | pH of sample | Temperature of sample | No of strains isolated |
|---|---|---|---|---|---|
| 1 | Sewage water | Jinnah hospital drain opening, Lahore, Pakistan | 7.3 | 41°C | 13 |
| 2 | Sewage water | Almost 1km ahead from Jinnah hospital drain, Lahore, Pakistan | 7.6 | 39°C | 9 |

**Table II: Gram staining and screening for biofilm formers**

| Bacterial isolates | Gram staining | Slime production | | Biofilm ring on test tube |
|---|---|---|---|---|
| | | BHI+ Glucose | BHI+ sucrose | |
| S1 | +ve, bacilli | +ve | +ve | +ve |
| S2 | +ve, cocci | -ve | -ve | -ve |
| S3 | +ve, cocci | +ve | -ve | -ve |
| S4 | +ve, bacilli | -ve | -ve | -ve |
| S5 | +ve, coccobacilli | -ve | +ve | +ve |
| S6 | -ve, bacilli | +ve | +ve | -ve |
| S7 | +ve, bacilli | -ve | +ve | -ve |
| S8 | +ve, bacilli | +ve | +ve | +ve |
| S9 | +ve, bacilli | +ve | -ve | -ve |
| S10 | +ve, cocci | -ve | +ve | -ve |
| S11 | +ve, bacilli | -ve | +ve | -ve |
| S12 | +ve, bacilli | -ve | +ve | -ve |
| S13 | +ve, coccobacilli | +ve | +ve | +ve |
| S14 | +ve, cocci | -ve | -ve | -ve |
| S15 | +ve, cocci | -ve | +ve | -ve |
| S16 | -ve bacilli | -ve | -ve | +ve |
| S17 | +ve, bacilli | -ve | -ve | -ve |
| S18 | +ve, cocci | -ve | -ve | -ve |
| S19 | +ve, bacilli | -ve | +ve | -ve |
| S20 | +ve, bacilli | -ve | +ve | +ve |
| S21 | +ve, cocci | -ve | -ve | -ve |
| S22 | +ve, cocci | -ve | +ve | -ve |

***Selection of biofilm formers***

Biofilm formation was qualitatively determined by checking slime production by congo red method. Slime production was done in the presence of glucose and sucrose. Majority of strains showed black colonies in the presence of sucrose indicating positive results for biofilm formation. Quantitative analysis of biofilm formation was done by ring test for all purified strains. All the results were shown in Table II.On the basis of these results, six biofilm forming bacterial strains were selected.

***Identification of biofilm forming bacterial strains***

Six biofilm forming bacterial strains were thensent to Macrogen Inc. Seoul South Korea for 16S rRNA gene sequences. Bacterial strains were identified as *Exiguobacterium aurantiacum* (AC: KU054327), *Bacillus subtilis* (AC:KU054331), *Bacillus megaterium* (AC:KU054332), *Bacillus endophyticus*

(AC:KU054328), *Pseudomonas fragi* (AC:KU054330). (AC:KU054329) and *Bacillus subtilis*

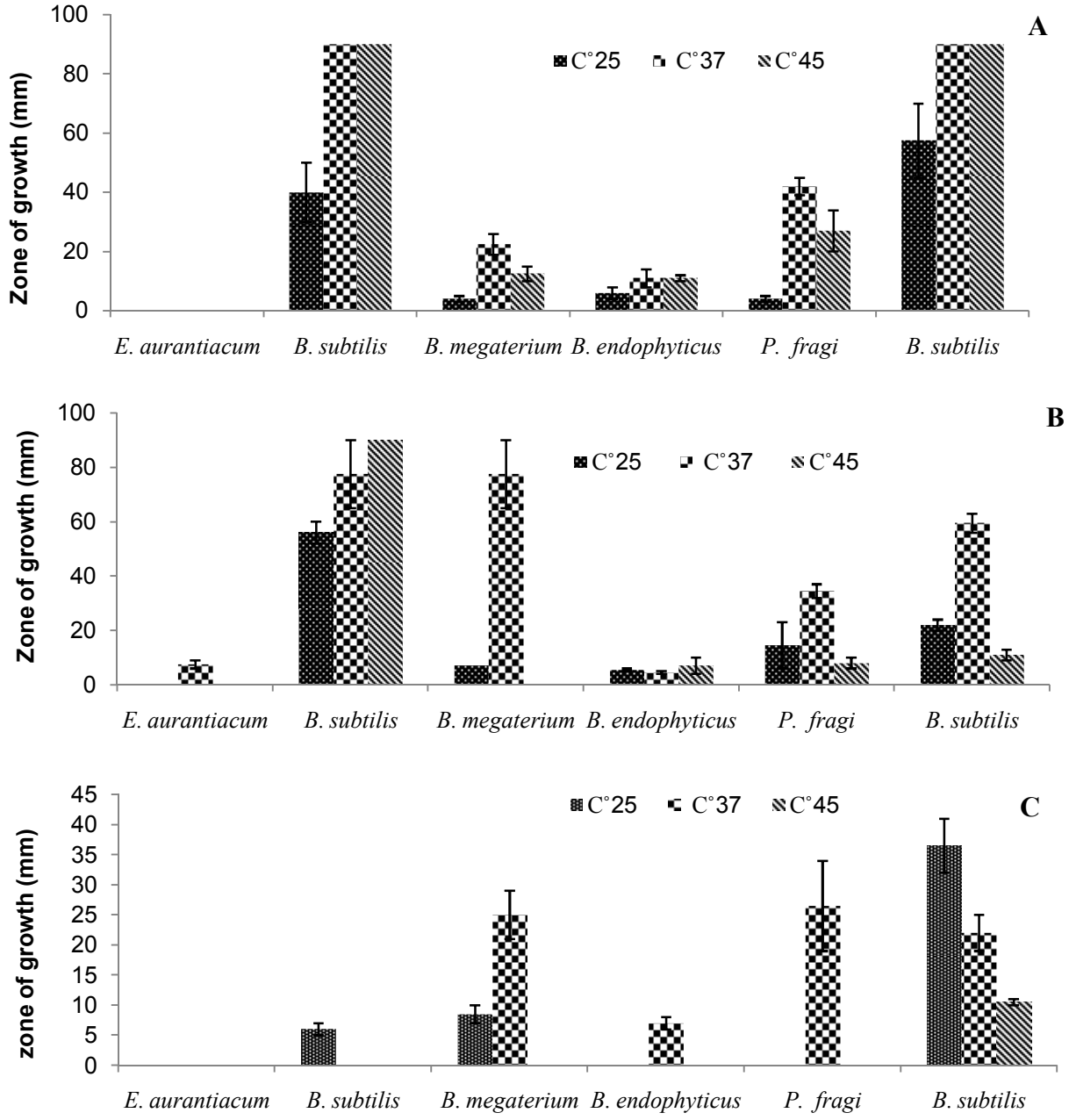


**Figure 1. Effect of temperature on motility assays. (A) swimming motility (B) swarming motility (C) twitching motility.Values are stated as mean+SE. All experiments were carried out in triplicate and repeated in two independent sets of experiments**.

***Effect of physical and chemical factors on motility assays***

Swimming, swarming, and twitching motility assays were done under different conditions. To apply low and high states of moisture, pH, temperature, chemicals and salt are among the strategies to target and control biofilms (Sandasi*et al.,* 2010; Kostakioti*et al.,* 2013; Nguyen and Burrows, 2014).Presently here the effects of a different physical factors *i.e.,* temperatures, pH and salinity and chemical factors such as SDS (an ionic detergent),

Proteinase K (a serine protease enzyme) and tobramycin (the aminoglycoside antibiotics), were investigated on swimming, swarming, and twitching motilities of bacteria.

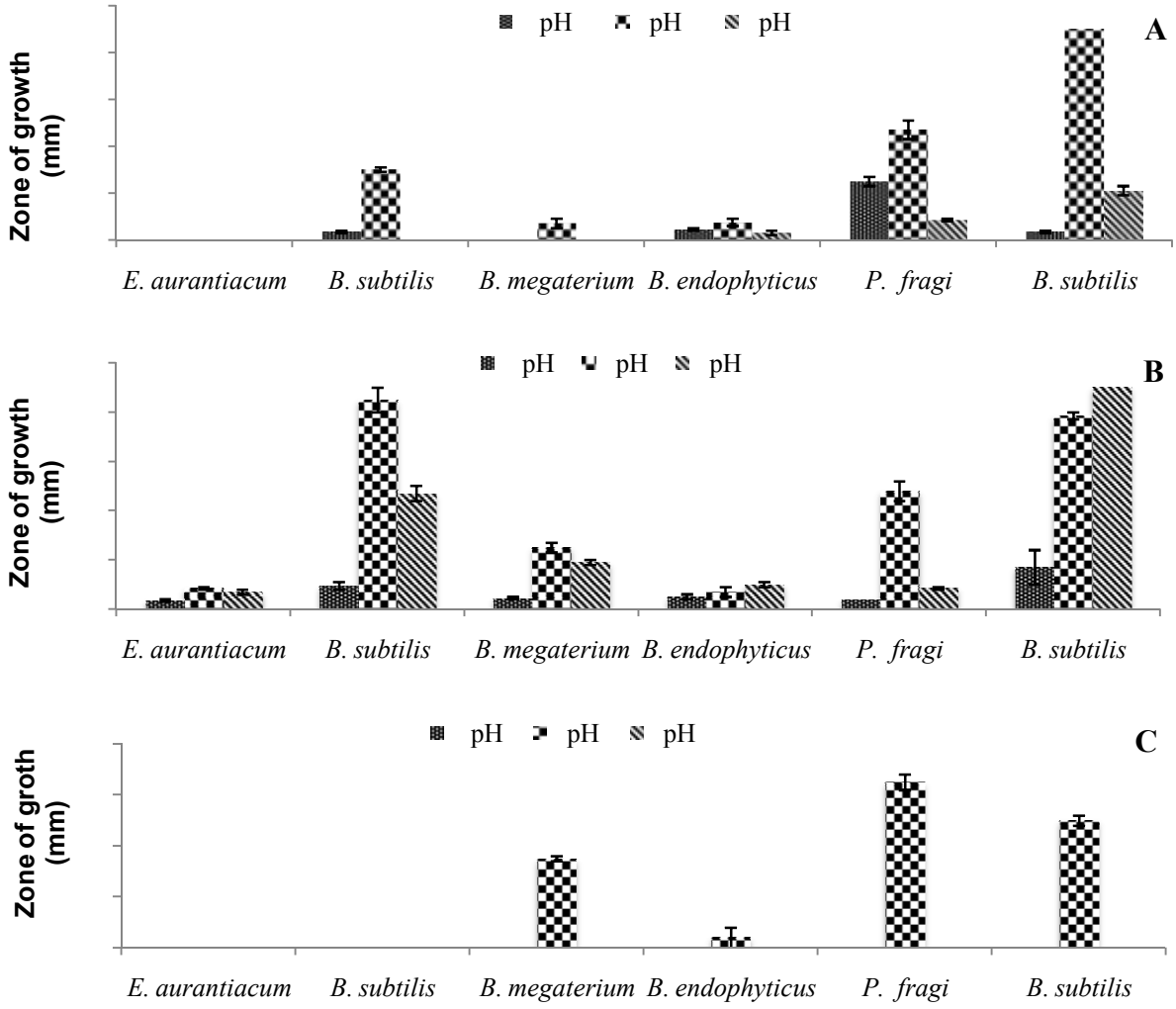


**Figure 2. Effect of pH on motility assays. (A) swimming motility (B) swarming motility (C) twitching motility. Values are stated as mean+SE. All experiments were carried out in triplicate and repeated in two independent sets of experiments.**

***Effect of temperature on motility assays***

The optimum temperature for swimming, swarming and twitching motilities was 37°C which was also the preferred optimal growth temperatures for these bacterial strains with few exceptions. The overall, swimming motility was reduced at 25°C but at 45°C the swimming motility was increased as compared to 25°C. These results showed that despite of few exceptions, the general ability of bacteria to swim with flagella in liquid media improves with rise in temperature (Fig. 1A).Increase in about 10°C temperature results in a two to three fold increase in motility of *Escherichia coli* (Larsen *et al.,* 2004). Swimming speed of *V. anguillarum* and *Salmonella enteric* ovar *Typhimurium* are temperature dependent (Maeda *et al.,* 1976; Larsen *et al.,* 2004). Swimming behavior of the bacteria depends on the intensity of stimulus changes with time. The swimming behavior of bacteria was significantly disturbed by temperature. At constant temperatures the swimming speed increased with temperature (Maeda *et al.,* 1976). The overall trend of best temperature for swarming motility was recorded as 37°C with an exception. At 45°C, most of the strains reduced their ability to swam and even few strains lost their ability to swarm through media at that temperature (Fig. 1B).Swarming behavior in microorganisms is a coordinated and

a reversible changes in response to the environmental factors. This response helps bacteria to travel towards nutrition source, to retreat from source of possible damage, to defend microorganism from the harmful action of antibiotics. (vicario *et al.,* 2015) Many bacterial genera such as *Aeromonas, Bacillus, Proteus, Azospirillum, Escherichia* and *Pseudomonas* are documented to have swarming behavior in response to different environmental factors (Daniels *et al.,* 2004; Vicario *et al.,* 2015). The optimum recorded temperature for twitching motility for all strains was 37°C with few exceptions. The extreme difference in twitching ability of bacteria was documented below and above optimum temperature (Fig. 1C).In previous studies, it has been documented that T4P of *L. pneumophila,* and bundle-forming pili of *Escherichia coli,* have temperature dependent regulations. This type of regulations was found to be essential for pili production and adhesion to the surfaces of the host cells (Salzer *et al.,* 2014).

**Effect of pHon motility assays**

The overall optimum range of pH for swimming motility for all bacteria was documented as neutral with an exception (Fig. 2A).

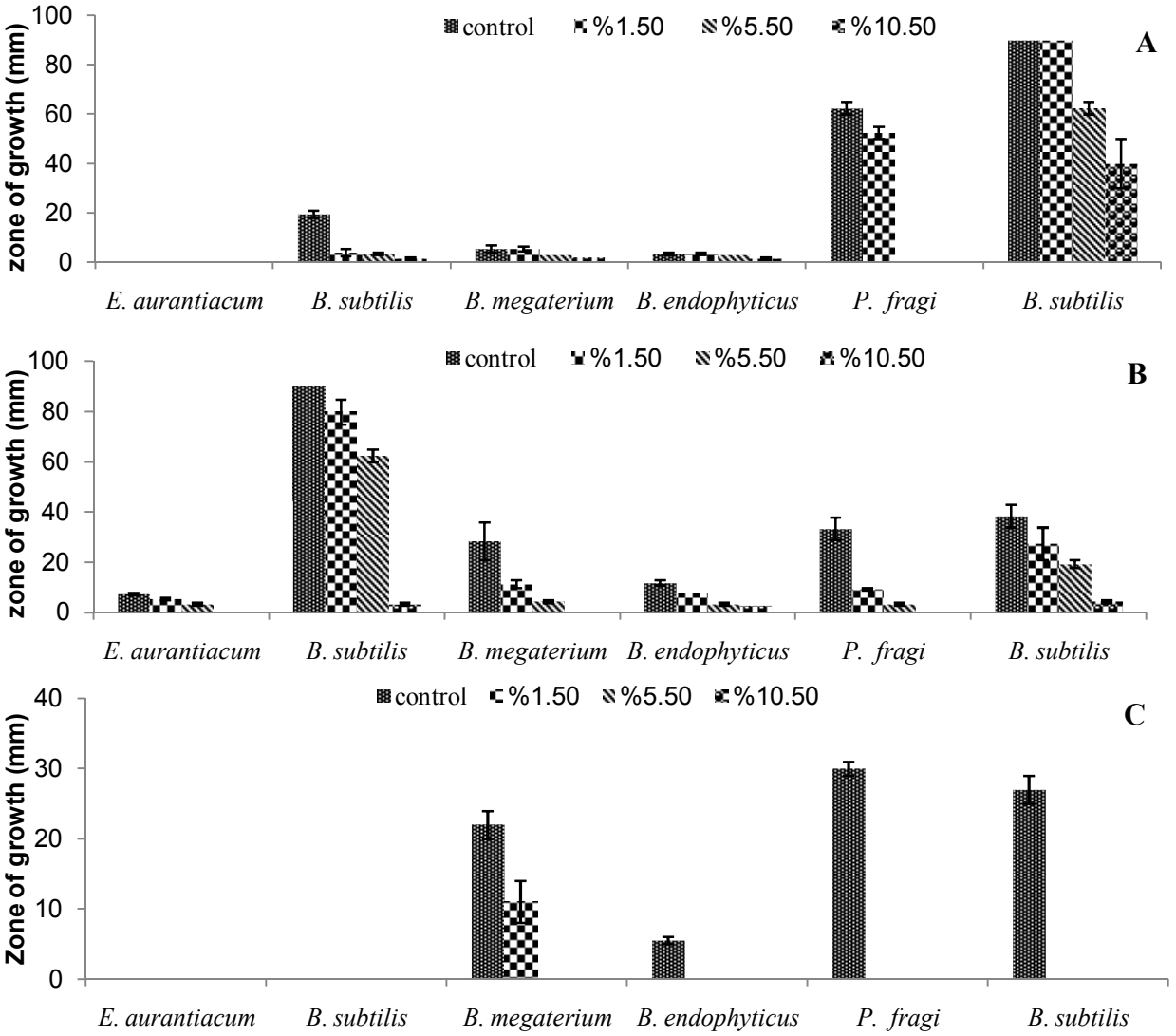


**Figure 3. Effect of NaCl on motility assays. (A) swimming motility (B) swarming motility (C) twitching motility.Values are stated as mean+SE. All experiments were carried out in triplicate and repeated in two independent sets of experiments.**

The acidic and alkaline pH disturbed the capability of bacterial flagella to swim in aqueous media. Acidic environment suppresses both the gene responsible for flagellar movement and ultimately reduces motility (Stancik*et al.,* 2002). In another study, Polen*et al*. (2003) reported an evidence for reduction in chemotaxis and motility at high pH of aqueous medium. The overall optimal range of pH for swarming motility for all bacteria was recorded as neutral with few exceptions. Second preferable pH rang for swarming motility for all above strains was pH 9 (Fig.2B). Change in pH had drastic effect on twitching motility of all bacterial strains. All bacterial strains showed no zone of twitching motility at acidic and alkaline pH (Fig. 2C). These types of motility responses were also found *Salmonella* and *E. coli* (Maurer *et al.,* 2005).

***Effect on salinityon motility assays***

The optimum swimming motility was observed in controls (0.5 % NaCl) for all bacterial stains. There was reduction in swimming motility of bacterial strains as the concentration of salt increased (Fig. 3A).

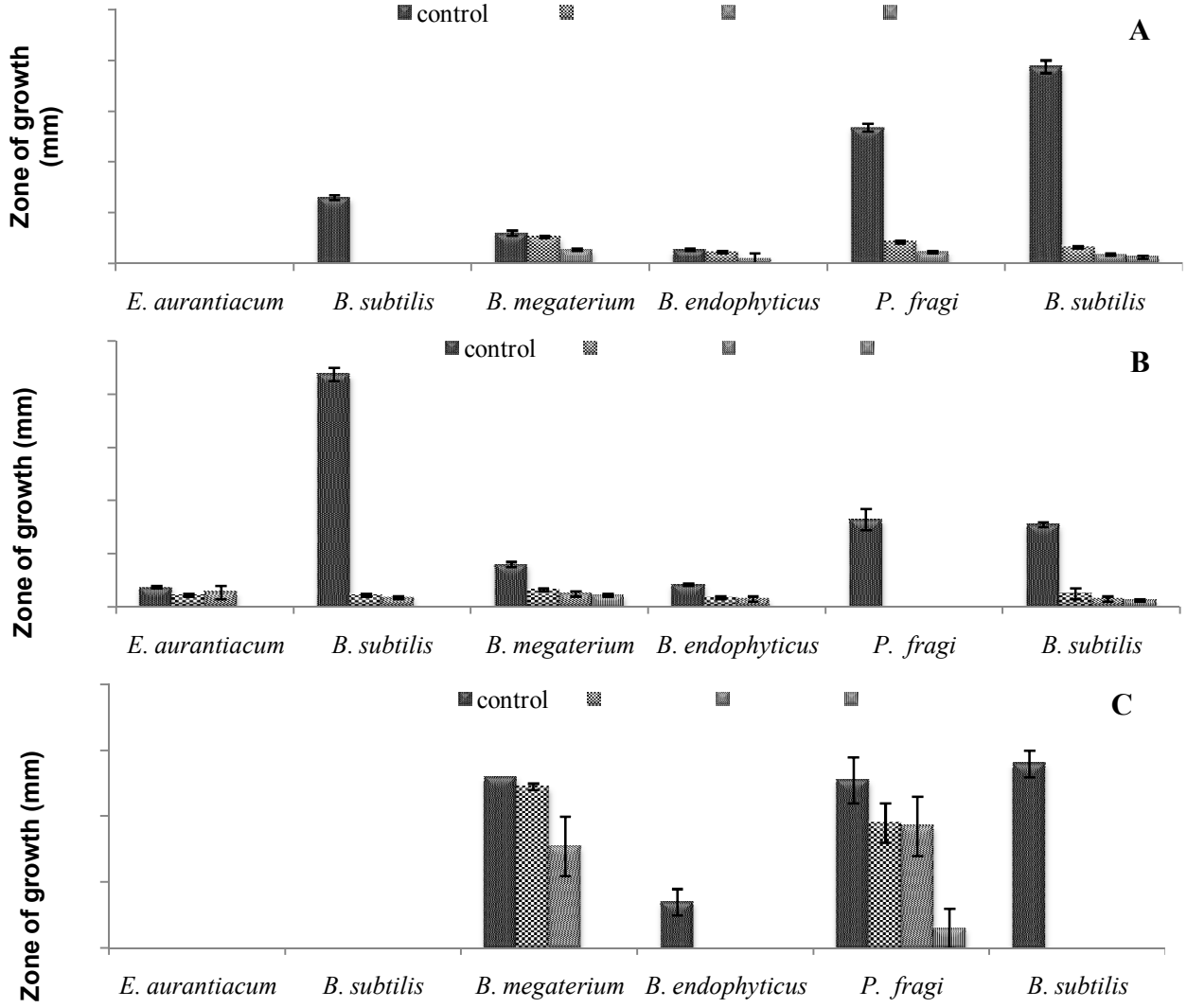


**Figure 4. Effect of SDS on Motility Assays. (A) Swimming motility (B) Swarming motility (C) Twitching motility. Values are stated as mean+SE. All experiments were carried out in triplicate and repeated in two independent sets of experiments.**

High concentration of salt had severe impact on swarming motility of bacterial strains. All the bacterial strains showed reduction in diameter of zones when the concentration of NaCl increased in medium (Fig. 3B). The twitching motility was only observed in controls (0.5% NaCl) and

completely prohibited in all twitch positive strains at all concentrations of NaCl except *Bacillus endophyticus* that had zone of twitching motility only at 1.5% NaCl (Fig. 3C).In a study it has been documented that in *V. alginolyticus*, swimining movement is not affected by the NaCl concentration between 0.8 and 3.6% in an aqueous medium. The swimming motility of bacteria was disturbed when the concentration of NaCl increased to the recommended level (Larsen *et al.,* 2004). High salt concentration resulted in impairment in the motility of *P. aeruginosa* and disrupted the structure and function of flagella (Havasi *et al.,* 2008).

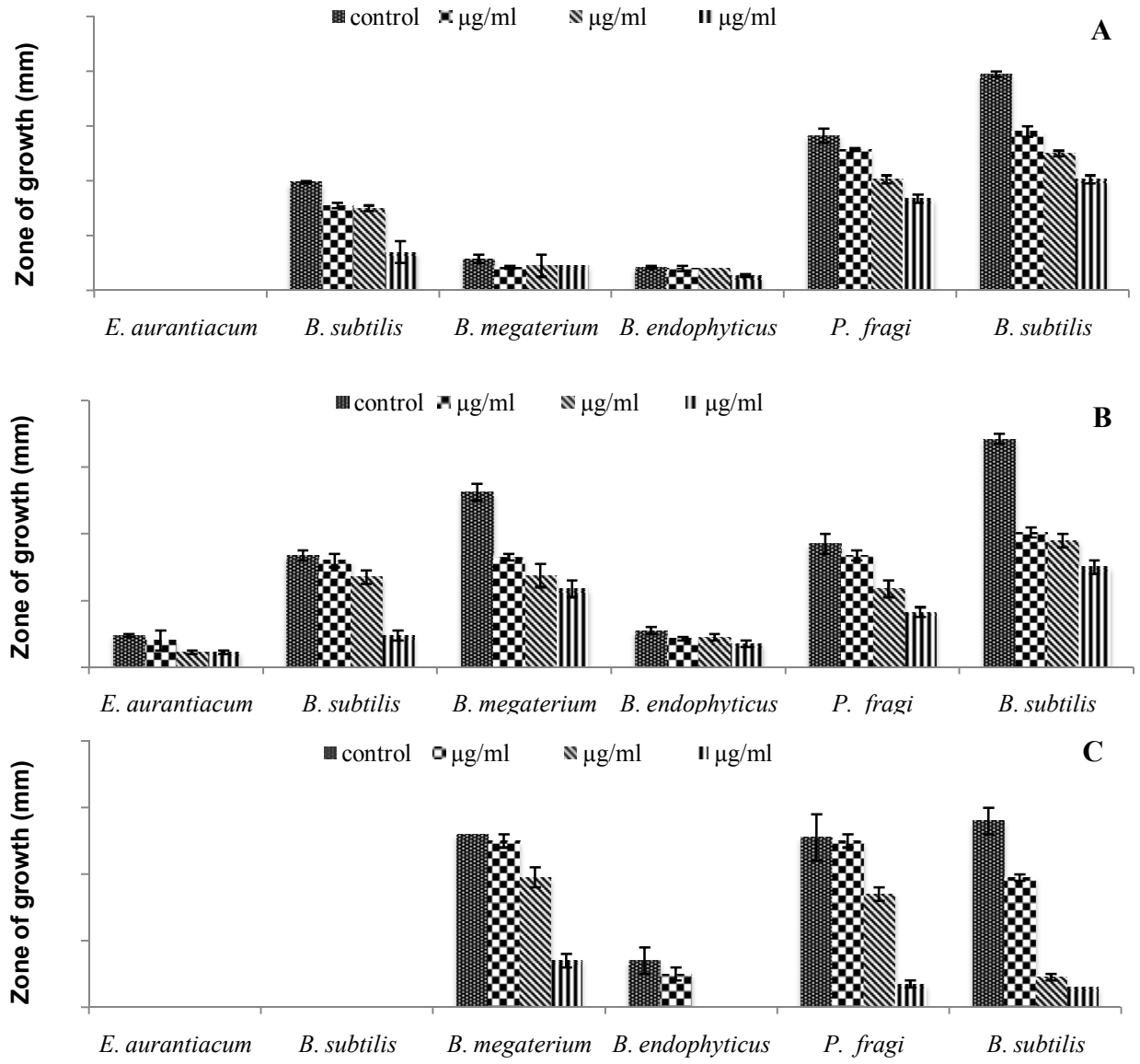


**Figure 5. Effect of Proteinase K on motility assays. (A) swimming motility (B) swarming motility (C) twitching motility.Values are stated as mean+SE. All experiments were carried out in triplicate and repeated in two independent sets of experiments.**

**Effect of SDSon motility assays**

In the absence of SDS, all the strains showed motility zones with an exception. Overall, there was reduction in swimming motility of each bacterial strain when the concentration of detergent increased as compared to the controls. Swimming motility of *Bacillus subtilis* (AC:KU054331)was completely inhibited by SDS because it did not show zones at all concentrations of SDS when compared to

control(Fig. 4A).Sodium dodecyl sulfate can be responsible for the denaturing of membrane proteins(Seddon *et al.,* 2004). All bacterial strains showed swarming motility zones in the absence of SDS. In general, the diameter of zones of swarming motility reduced to great extent in the presence of SDS when compared to controls (Fig. 4B). SDS significantly disrupted the twitching motility of bacterial strains. Among twitch positive strains, *Bacillus megaterium* and *Bacillus endophyticus* showed zones at 0.1 and 0.5% SDS but did not show at 1% of SDS. The twitching motility of *Pseudomonas fragi* was completely prohibited at all concentration of SDS (Fig. 4C).

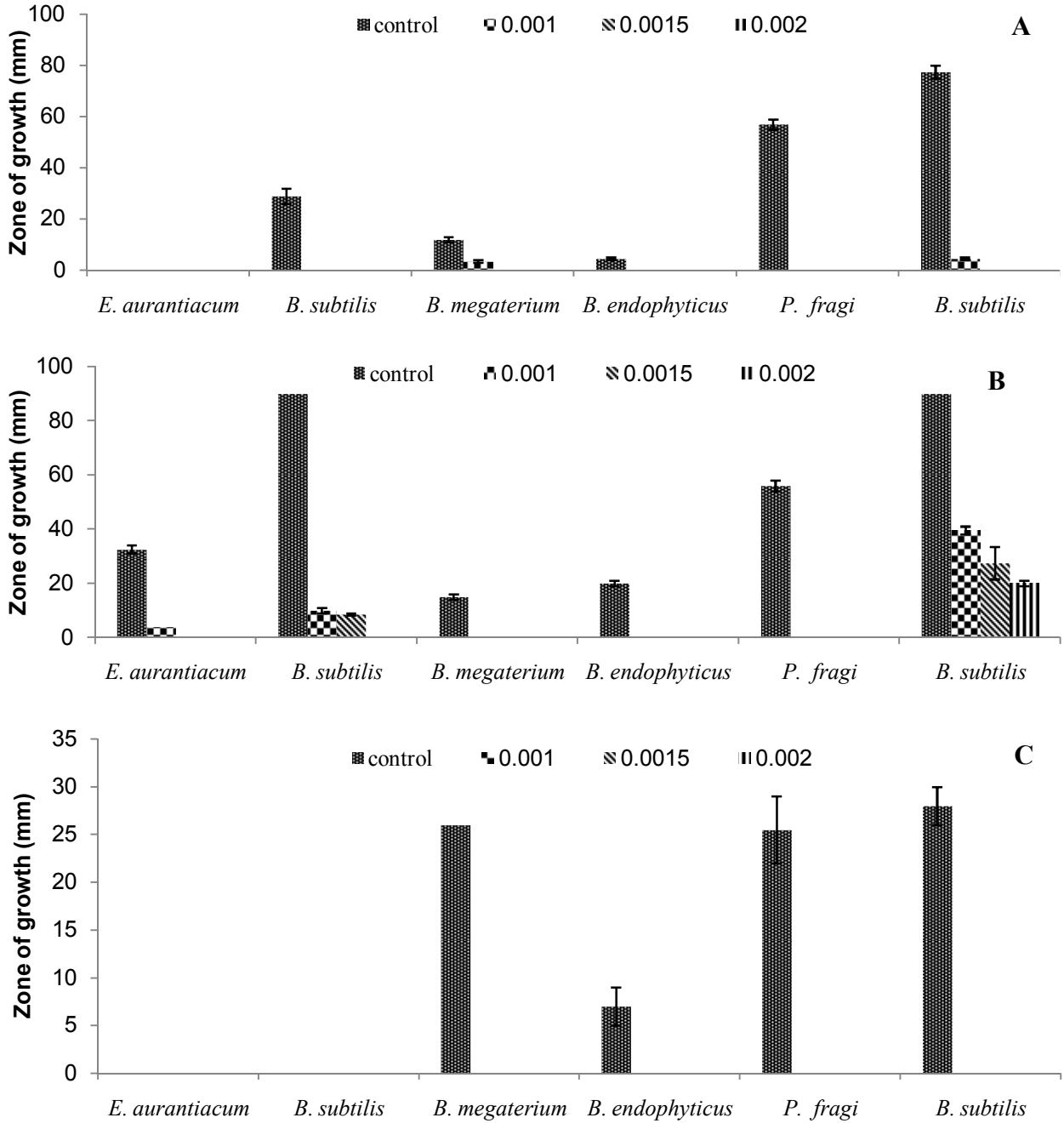


**Figure 6. Effect of tobramycin on motility assays. (A) swimming motility (B) swarming motility (C) twitching motility.Values are stated as mean+SE. All experiments were carried out in triplicate and repeated in two independent sets of experiments.**

***Effect of Proteinase K on motility assays***

All swim positive strains showed swimming motility zones at all concentrations of Proteinase K. By increasing concentration of enzyme, *Bacillus subtilis* (AC:KU054331), *Bacillus endophyticus* and *Pseudomonas fragi* showed gradual decrease in diameter of zones when compared to controls (Fig. 5A).In most of the strains, there was the gradual reduction in diameter of zones of swarming at 25, 50, and 100 µg/ml respectively when compared to controls (Fig. 5B). Proteinase K disturbed the twitching motility of bacterial strains to some extant. Most twitch positive strains showed a persistent reduction in twitching motility as the concentration of enzyme increased (Fig. 5C). Biofilm dispersal in *Bacillus, Pseudomonas* and *Staphyloccocus*is reported to be stimulated by addition of Proteinase K (Longhi *et al.,* 2008; Connelly *et al.,* 2004; Lequette *et al.,* 2010; Shukla and Rao, 2013). Cell surface proteins, which are involved adhesion and finally in biofilm development are disturbed in early phaseby proteinase K (Nguyen and Burrows, 2014). Our results were comparable with the previous researches, that the proteinase K disturbed the early adhesion of the bacterial cells the outcome of which was interruption in motility patterns of the bacterial cells (Shukla and Rao, 2013).

***Effect of tobramycinon motility assays***

*Bacillus subtilis* (KU054331), *Pseudomonas fragi,* and *Bacillus subtilis* (KU054330) did not show any zone at all three concentrations of tobramycin as compared to the controls. *Bacillus megaterium* showed swimming motility zones at all concentrations with a reduction in diameter of zones by increasing dosage of antibiotics. *Bacillus endophyticus* showed zones at 0.001% tobramycin and did not show any zones at 0.0015 and 0.002 % antibiotics (Fig. 6A).In general, the diameter of zones of swarming motility reduced to great extent in the presence of tobramycin when compared to controls (Fig. 6B).The twitching motility of bacterial strains was also completely prohibited by all concentrations of antibiotics except for *Bacillus megaterium,* which showed a small zone only at 0.001 % of tobramycin (Fig. 6C).Swimming and swarming are significant organizations of bacterial motility. In an investigation of antibiotics effect on these motilities assays, clear differences were experienced among different antibiotics. Subinhibitory concentrations of tobramycin enhanced both movements in *P. aeruginosa*.At that concentration, tobramycin did not cause a problem on bacterial physiology (Linares *et al.,* 2006). In present study, a decrease in swimming, swarming and twitching motilities were detected. Same was also reported by Luke *et al.* (2004). It might be possible that tobramycin induced different toxicities and responses in different bacterial strains when cultivated in different growth mediums. Medium composition affects the growth of cultivated bacteria (Babic *et al.,* 2010). Antibiotics differentially affect the motilities Harman *et al.* (2017) reported that addition of vancomycin for 24 hours at concentration of ≤2.0 *µ*g/mL resulted in change in cell shape, decreased speed of bacteria and affect cell wall. It reduces the stiffness of the cell wall and slow down the speed of bacteria. Bacteria due to motile nature have the ability to response nutrient by attracting towards them. Ultimately due to these bacteria are developed chemotactic strategies. Which brings reorientation in their movements *i.e.,* changes occur in flat straight run, tumble movement, Brownian movement or just have movement by natural flow dragging. It all is attributed to cell geometry (Guadayol *et al*., 2017). Guadayol *et al.* (2017) reported that when *Escherichia coli* is exposed to antibiotics, varying length motile cells with morphological variations were formed. According to them cell length controls the patterns, direction and speed of bacterial swimming.

***Conclusions***

In the developing countries, sewage water systems are main cause of water born diseases caused by biofilm forming bacteria. Excessive use of antibiotics in the hospitals develops high resistance and genetic adaptability in bacterial strains, which may lead to an alarming situation in health departments. It is difficult to treat existing biofilms by commercial antimicrobial products but it might be easier to manage biofilms by developing strategies to control the bacterial motilities. Motility is an important regulator during biofilm formation. The transition from motile bacterial state to biofilm state can be controlled by variety of ways. Our findings indicate that a change in the physical environment of sewage water systems may contribute to alter bacterial cell surface properties and attachment factors accessible to the bacteria. Overall low and high temperature, acidic pH and high salt

concentration of sewage water were effective measures to control biofilm formation. The present study clearly indicates that different detergents, antibiotics and protein digesting enzymes may disrupt bacterial motilities and eventually effect biofilm formation. Tobramycin response against motilities will become significant implication to manage sewage systems.

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