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RESEARCH ARTICLE

EFFECT OF ENVIRONMENTAL FACTORS ON BIOFILM FORMATION BY *SERRATIA MARCESCENS* ISOLATES

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ABSTRACT

Majority of microbial infections occurring in the human body are attributed to be biofilm-mediated. Biofilms formations in industrial settings are associated with many problems. Formation of biofilm is controlled by environmental factors. This study is carried out to determine the biofilm formation in *Serratia marcescens* under different environmental condition. Biofilm assay in nutrient medium by microtitre plate methods under different test conditions were performed. Using a microplate assay with crystal violet staining, we examined biofilm formation by 30 strains in nutrient broth with different temperatures (fridge temperature, Room temperature and 37 C), at different pH (4.5, 7.0 and 8.5) and with varying concentrations of sodium chloride (0.5%, 1% and 2%). The synergistic effect on biofilm formation was observed for temperature, pH and salt concentration. The strains produced more biofilm at fridge temperature than at RT and 37 C. Biofilm production at pH 4.5, 7 and 8.5 was comparable but significantly higher at pH 4.5. This study also demonstrated the influence of NaCl on biofilm formation.

INTRODUCTION

Biofilms are a community of microorganisms attached on biotic or abiotic surfaces. In food industry, biofilms are a potential source of product contamination and may lead to food spoilage and serious fouling problems in equipment. The ability of bacteria to attach to surfaces and develop in to a biofilm depends on many factors, which may include cellular recognition of specific or non-specific attachment sites on surface, nutritional cues, CO2, pH, osmolarity and temperature (Marinho *et al.,* 2013; Fisher and Phillips, 2009). *Serratia marcescens* is a gram-negative, enteric bacterium that is able to inhabit a wide variety of ecological niches and cause disease in plant, vertebrate and invertebrate hosts (Grimont *et al.,* 1977). It is an opportunistic human pathogen and is responsible for an increasing number of serious nosocomial infections, a problem exacerbated by the resistance of many strains to multiple antibiotics (Hejazi and Falkine, 1997; Auken and Pitt, 1998). *S. marcescens* is a well-known cause of hospital-acquired infections, including nosocomial pneumonia, wound infections, urinary tract infections and septicemia (Liu *et al*., 1995). Treatments of these infections are often very difficult, which to a big extent is due to the widespread natural and acquired resistance of the organism to antimicrobials (Acar, 1986). The abilities of *S. marcescens* to cause nosocomial infections and survive in the environmental are attributed to its ability to form biofilms, its broad metabolic capacity, and its high natural resistance to antimicrobials and cleaning agents (Kalvioda *et al.,* 2010). One common survival strategy employed by bacteria pathogens is to form a biofilm, an amorphous and dynamic structure that is not only resistant to antibiotics, but also resistant to host immune clearance (Chen and Wen, 2011). Bacteria regulate gene expression in response to different environmental signals, such as temperature, oxygen and carbon dioxide concentrations, pH, and nutrient availability (Guiney 1997; Dancer 1999; Harjai *et al*., 2005). While information on the bacterial and fungal biofilm formation had been published (Stepanovic *et al*., 2001, 2003 a, b; Cernohorska and Votava 2004; Ruzicka *et al.,* 2004; Biswas and Chaffin 2005) no data are available in this regard for *S.marcescens.* The aim of the present study was to describe the effect of environmental factors (temperature, pH and salt concentration) that regulate biofilm formation in *S. marcescens.*

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MATERIALS AND METHODS

Collection and isolation of *Serratia* sp.

Serratia sp. was isolated from water as well as sewage, food, soil and clinical samples. In the present study, *Serratia marcescens* was targeted by enriching on nutrient broth as well as selectively grown on nutrient agar medium (HiMedia, Mumbai). Phenotypic and genotypic identification methods were employed to characterize the isolated strains. The Biochemical profiles of the isolated strains were studied by the standard methods with reference to the Bergey's manual of

bacterial classification. A total of 30 strains of *Serratia marcescens* were selected to study the influence of various factors on biofilm formation. Biofilm formation was analyzed individually for each strain by using microplate assay.

Microtitre plate methods of biofilm assay

S. marcescens were grown overnight in nutrient broth at 37 C. Transferred 200µl of the culture to the wells of sterile microtitre plate. One well with sterile nutrient broth served as the control. After incubating for 24 hours at 37 C, the wells were gently washed 3 times with 200µl phosphate buffered saline (PBS), dried in an inverted position and stained with crystal violet was 15 minutes. Then wells were rinsed again with distilled water and crystal violet was resolubilised in 200µl of 33% glacial acetic acid. The OD at a wavelength of 620nm was determined using a ELISA auto reader (Cyberlab). These OD values were considered as an index of bacteria adhering to surface and forming biofilms. Isolates were categorized based on the approach of Mohamed *et al*., (2004) as strong OD620 >2; medium OD620 1 to 2; or week OD620 greater than 0.5 but less than 1.

Factors affecting biofilm formation

Effect of temperature

The effect of temperature was tested by incubating the microtitre plate cultures at three different temperatures Fridge, RT and 37 C for 72 hours and at the end of the incubation, plates were stained and analyzed as described above.

Effect of pH

The effect of pH on biofilm formation was done by microtitre plate method. The pH of the medium was adjusted to 4.5, 7 and 8.5 using NaOH or HCl before autoclaving. After autoclaving the pH was retested. Bacterial culture was inoculated, incubated and OD was measured as above.

Effect of NaCl

Microtitre plate assay were done with 0.5%, 1% and 2% salt incorporated medium and was inoculated with overnight broth culture of bacteria. It was incubated at 37ºC for 72 hours. After incubation the plates were stained and analyzed.

RESULT

In the present study, the various sources of *Serratia marcescens* like water, sewage, food, soil and clinical samples were collected from various locations of Coimbatore. The samples were processed immediately. The isolates were proved as *Serratia* based on morphological and biochemical identification tests. All the isolates showed convex, opaque centre effuse with almost transparent periphery and irregular with differencing pigmentation (colourless, orange and pink) was noticed. The cultures were retrieved and the single colony isolate was maintained on nutrient agar slants. The effect of temperature, pH and salt concentration on biofilm formation of the isolates were observed. Biofilm formation was observed at all temperatures studied and it was found to be elevated at fridge temperature in all species tested. Optical density of biofilm at fridge temperature was significantly more than that of high temperature. The effect of temperature on biofilm formation is shown in Fig. 1.

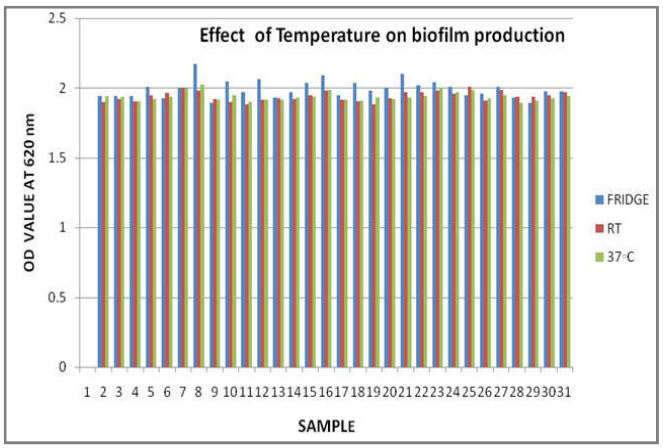

Fig. 1. Effect of temperature

An increase in the amount of biofilm production was observed with increase in pH. At pH 8.5 most of the strains displayed significantly more biofilm formation. The effect of pH on biofilm formation is shown in Fig. 2.

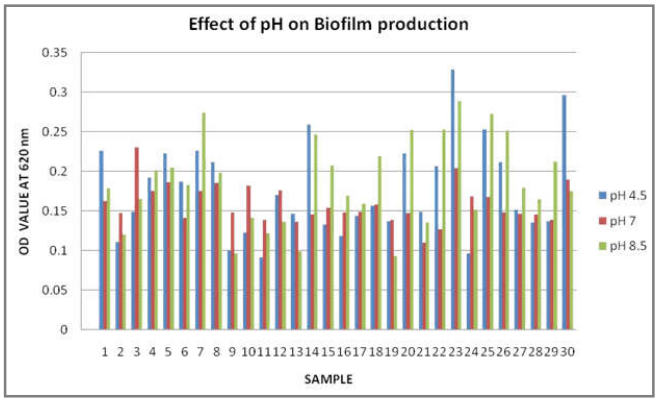

Fig. 2. Effect of pH

The biofilm formation was observed to be high at 1% NaCl concentration compared with that of 0.5% and 2% salt concentration. And the effect of pH on biofilm formation is shown in Fig. 3.

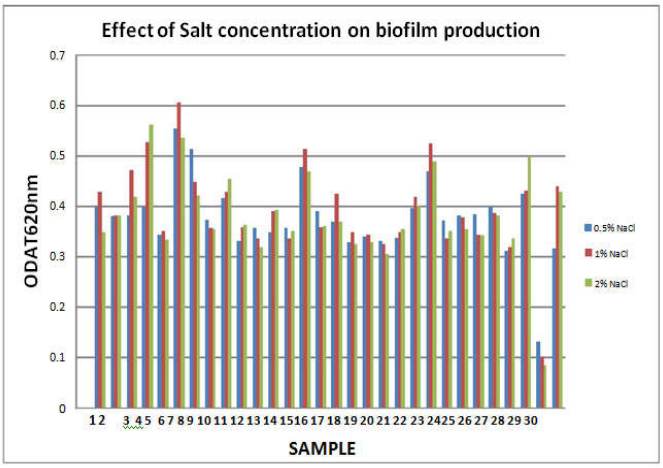

Fig. 3. Effect of NaCl

DISCUSSION

A number of environmental factors regulate, biofilm formation as bacterial attachment to surfaces is a prelude to biofilm formation which is influenced by physicochemical properties of environment and nutrient contents of the growth medium. Glucose, serum, CO2, osmolarity, pH and temperature influence biofilm production among different bacteria (Ramli *et al.,* 2012). In this study biofilm was produced in a wide range of temperatures. It was found that maximum biofilm production occurred at fridge temperature as compared to room temperature and 37ºC. There are data suggesting that a temperature of 37 ºC can induce more biofilm development since this temperature is optimum for the production of ESP molecules (Tendolkar *et al*., 2004). But contradictory report with high biofilm formation at low temperature was found (White-Ziegle *et al*., 2008). Anyhow biofilm formation occurred at low temperature in this study and this may be due to its psychrotolerant nature. Biofilm formation at low temperature may have a role in the contamination of refrigerated food and drugs. Biofilms if formed in food-processing environments and in water distribution systems acts as a persistent source of microbial contamination that may lead to food spoilage or transmission of diseases.

Since biofilm could be produced in all the tested temperatures conditions, it potentially provides a survival benefit in non-optimal growth condition to the organism. Increase in biofilm production at elevated temperatures can be due to increased hydrophobicity and thereby attachment ability and increased bacterial growth at this temperature (Tendolkar *et al*., 2004). Biofilm mediated chronic infections like ear, sinus infections and wounds in diabetic patients are challenges to physicians. Further research may be needed to understand how biofilm production is enhanced by environmental factors. In the present study biofilm production occurred at low pH and alkaline conditions yielded maximum biofilm production. Though there is a lack of literature on the effect of alkaline pH on biofilm procuction, there are studies revealing the tolerance of enterococcal biofilm to alkaline conditions (Yan *et al*., 2012). The electrostatic repulsion between cells to cell and substrate may weak at this pH and this favors aggregate formation. This indicates that biofilm formation in catheters and other prosthetic devices can predispose urine and blood infections (Guiton *et al.,* 2010).

The pH of the body, especially in fluids such as urine and blood, is influenced by diet, metabolic intermediates and the level of final catabolic products. As the biofilm production by *Serratia marcescens* at alkaline pH, we suppose that the colonization and biofilm formation on prosthesic devices (i.e. urinary catheters) is more probable and more rapid in situations, such as renal failure, urinary tract infections or sodium salt administration, causing metabolic alkalosis (Bonaventura *et al*., 2007). This study demonstrated the influence of NaCl on biofilm formation. Though at 1% NaCl, increased biofilm formation was observed, an increase in salt content up to 2% decreased biofilm density. A previous study revealed that an increase in salt concentration resulted in creased biofilm formation up to a level and a further increase in the NaCl concentration did not contribute to an increase in biofilm formation (Kristich *et al.,* 2004 and Peter *et al.,* 2013).

Conclusion

Biofilm production was present in environmental strains and hence it is becoming clear that biofilm formation seems to be a niche-fitness associated trait. It is assumed that these bacteria from the skin of patient, health care workers or even from the environment including tap water can colonize prosthetic devices and ultimately results in patient exposure. Nevertheless once biofilm is formed in the body, it may trigger drug resistance and inflammation, resulting in persistent infections. This is a matter of the utmost importance since *Serratia marcescens* has been recognized as a cause of hospital-acquired infection for the last two decades (Hejazi and Falkiner, 1997) and has been noted that bacterial biofilms may impair wound healing (Schierle *et al*., 2009). The main strategy to prevent biofilm formation is to clean and disinfect regularly before bacteria attach firmly to surfaces (Midelet & Carpentier, 2004). Biofilm detectors can also be used to control of biofilms in the early stages of development (Pereira *et al*., 2008; Philip-Chandy *et al*., 2000).

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