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EFFECT OF ENVIRONMENTAL FACTORS ON BIOFILM FORMATION

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ABSTRACT

Biofilms formed in food-processing environments are more hazardous with their potential to act as a persistent source of microbial contamination leading to food spoilage or transmission of diseases. Bacteria in biofilms are resistant to environmental stress, disinfectants, antibiotics and so extremely difficult to eradicate Several environmental factors, such as glucose, nutrients, osmolarity, ethanol, temperature, anaerobiosis, etc have been reported to affect biofilm formation. This study focuses on the effect of biofilm formation by 4 food-borne pathogens *Staphylococcus aureus*, *Salmonella typhi*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*. Under four environmental conditions of salt(2%, 4%, 6%, 8%, 10%), glucose (2%,4%,6%,8%,10%), pH range of 5-9, and different temperatures (Fridge (4°C), Room temperature and Incubator 37°C). As per the study none of the factors inhibited biofilm formation by *P. aeruginosa* followed by *S. aureus*. High salt concentration of 8% and above, low temperature and alkaline pH can prevent biofilm formation by *Klebsiella* and *S. typhi*. Biofilm formation is complex; hence prevention is the best measure. Thus in-depth knowledge on the formation, physiology and molecular signalling in biofilms can contribute to prevent and control food-related spoilage by pathogenic bacteria.

KEYWORDS : Biofilm, Antibiotics, *S. aureus*

Foodborne diseases are an important cause of morbidity, mortality, and significant impediment to socio-economic development worldwide. For the global estimates, among the thirty-one foodborne disease causing agents 11 are diarrhoeal disease agents (1 virus, 7 bacteria, 3 protozoa), 7 invasive infectious disease agents (1 virus, 5 bacteria, 1 protozoan), 10 helminths and 3 chemicals. (Website 1)

The World Health Organization (WHO) cites food safety as one of the top 11 priorities and challenges of this century .Currently, foodborne diseases are a primary public health concern in both developing and developed countries. In the USA every year, it has been estimated that 48 million people suffer from food-borne diseases, with 2,612 related deaths. Biofilms are involved in over 65% of all microbial diseases according to the US National Health Institute (NIH) and the Centers for Disease Control and Prevention (CDC).It is now well-documented that food-borne pathogens could form biofilms on raw and ready-toeat vegetables, fruits, or goods made from these, food processing environment which are a major cause of outbreaks.

Different sources suggest that the most prevalent pathogens on produce are *Norovirus, pathogenic Escherichia coli, Salmonella spp., Listeria monocytogenes, Shigella spp.*, *Yersinia enterocolitica*, and *Campylobacter spp*. (Jahid et al., 2012).

Pseudomonas and related genera are aerobic, gram-negative soil bacteria, some of which can degrade a wide variety of unusual compounds. They generally require a high water activity for growth (0.95 or higher) and are inhibited by pH values less than 5.4. Some species grow at refrigeration temperatures (psychrophilic) while other are adapted forgrowth at warmer, ambient temperatures. contact with equipment and storage environment. The most common pathogens causing rots in vegetables and fruitsare fungi such as *Alternaria, Botrytis, Diplodia, Monilinia, Phomopsis, Rhizopus, Pencillium, Fusarium, etc. Amongbacteria Ervinia, Pseudomona*s, etc. cause extensive damage (Rawat, 2015).

Kim et al., 2008 isolated *Bacillus cereus, Staphylococcus aureus, Vibrio parahaemolyticus, Clostridium perfringens, Yersinia enterocolitica*, and from *L. monocytogenes* from various food samples. (Kim et al, 2008). Another study reports, *Klebsiella pnuemoniae* being isolated from retail meat products. *Klebsiella*, an biofilm forming organism can cause pneumonia when inhaled and can cause urinary tract infections and infections in the lower biliary trace and in wounds . Whole genome sequencing found that the *Klebsiella* isolated from meat products and from patients were nearly identical. In other words, people can be exposed to the pathogen from contaminated meat as well. (Manges, 2015).

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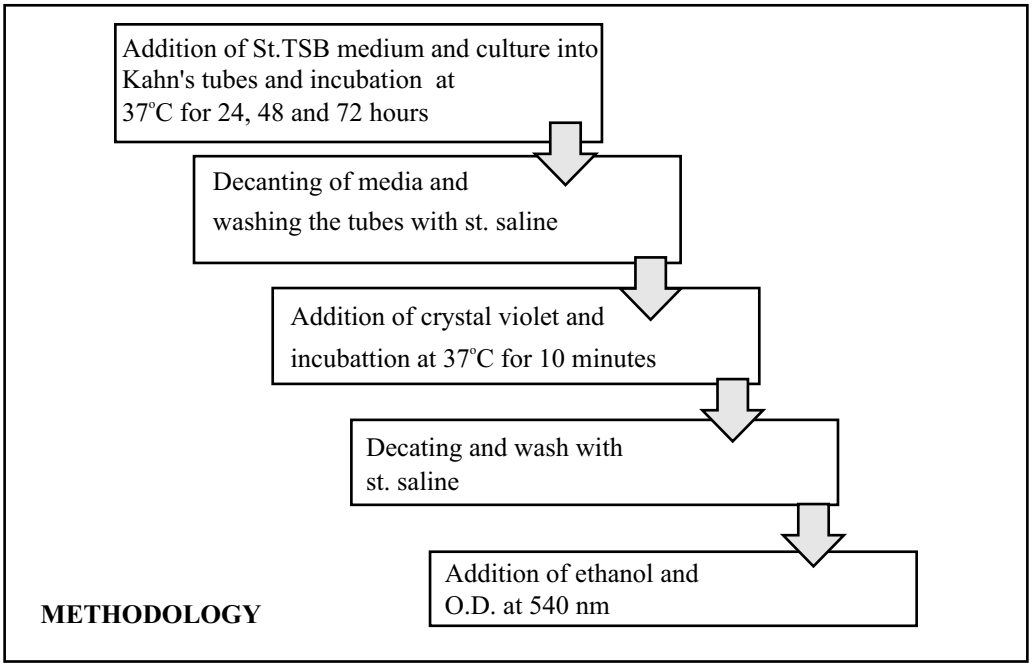
A biofilm is a complex aggregation of micro-organisms growing on a solid substrate. Mechanisms of biofilms formation is a stepwise, dynamic process and different physical, chemical, genetic, and biological processes are involved in the final maturation of biofilms. Though it is not clearly defined, more or less 5 steps are involved in biofilms formation. The steps are: (i) reversible attachment to a produce surface, (ii) irreversible attachment through producing quorum sensing and EPS, (iii) microcolonyformation, (iv) colonization or maturation steps, and (v) dispersal. (Jahid et al., 2012).

The ability to stick to surfaces and to engage in a multistep process leading to the formation of a biofilm is almost ubiquitous among bacteria. Therefore, biofilm formation has substantial implications in fields ranging from industrial processes like oil drilling, paper production and food processing, to health-related fields like medicine and dentistry. The cellular mechanisms underlying microbial biofilm formation and behaviour are beginning to be understood .Hence novel targets for specific intervention strategies to control problems caused by biofilm formation in different fields and in particular for the food-processing environments is the need of the hour.Bacterial biofilms are ubiquitous in nature, and the food industry does not escape from the problems they can cause. Food spoilage and deterioration not only results in huge economic losses, food safety is a major priority in today's globalizing market with worldwide transportation and consumption of raw, fresh and minimally processed foods. In particular, biofilms formed on food-processing equipment and other food contact surfaces act as a persistent source of contamination threatening the microbiological quality and safety of food products, and resulting in food-borne disease and economic losses. Biofilm prevention and control is therefore a priority in the food industry (Van Houdt and Michiels, 2010).

Environmental factors, including temperature, sugar, salt, pH, and nutrients that are common in foods and food-processing environments, have been demonstrated to play significant role in adhesion and biofilm formation.

MATERIALS AND METHODS

The objectives of this study were to investigate and compare biofilm formation between different foodborne pathogens undera variety of environmental conditions, including different temperatures, pH and varying concentrations of salt and glucose.



Materials

Cultures and Medium

The foodborne pathogens chosen were *Staphylococcus aureus, Salmonella typhi, Pseudomonasaeruginosa, and Klebsiellapneumoniae*. They were grown on nutrient agar slants for 24 hours and then inoculated into the growth media. The media selected for biofilm construction was *Typtic Soya* Broth (TSB).

Construction of Biofilms

The construction of biofilms was carried out using glass as the adhering surface. The growth medium used was st TSB and 0.2 ml of the culture (24 hours old, 0.1 OD) was inoculated into each of the tubes.

The media composition varied in different tubes. For study of effect of NaCl and glucose, different concentrations of NaCl (2%, 4%, 6%, 8% and 10%) and Glucose (2%, 4%, 6%, 8% and 10%) were added in TSB. For pH, the media was adjusted to different pH (5, 6, 7, 8, and 9) and for temperature study; TSB (normal composition) was stored at different temperatures (Fridge, 4°C, Room temperature approx. 27°C and Incubator 37°C). The readings were recorded at intervals of 24 hours, 48 hours and 72 hours.

Measurement of Biofilm formation

The biofilm formation was studied using Crystal Violet Assay. The tubes were first decanted and washed with saline (which removed the planktonic cells). 2 ml of 0.1% crystal violet was added to each tube and were incubated at 37°C for 10 minutes which allowed the crystal violet to attach to the biofilms. The tubes were then decanted and 2 ml of Ethanol (95%) was added to each tube.Ethanol absorbed all the crystal violet that was attached to the cells of biofilms. The optical density was then measured at 540 nm (Pillai S. K. et al., 2004).

RESULTS AND DISCUSSION

Effect of NaCl

There was decrease in biofilm formation for *S. aureus* beyond 8% salt concentration. However since Saureus can tolerate high NaCl levels initial growth was seen upto 24 hrs upto 10% NaCl as well (figure 1).

Among the Gram negatives Pseudomonas aeruginosa was least affected by high salt concentration growing upto 10% NaCl. As the salt concentration increased, the culture growth and biofilm formation of Salmonella and Klebsiella was inhibited at 8% and 6%

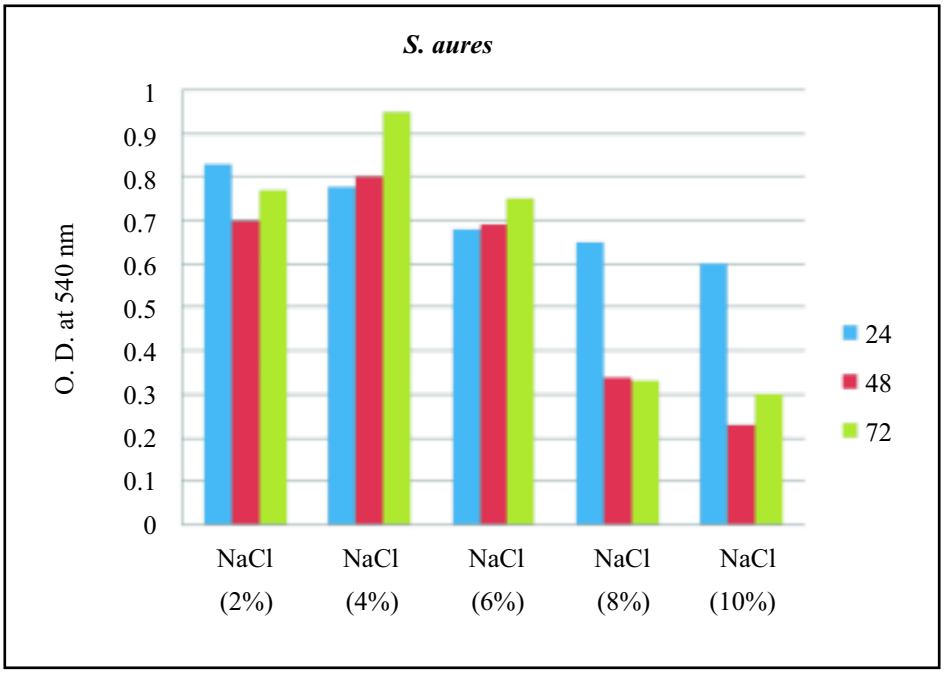


Figure 1 : Effect of NaCl on the Biofilm Formation of *S. aureus* Measured Using Crystal Violet Assay

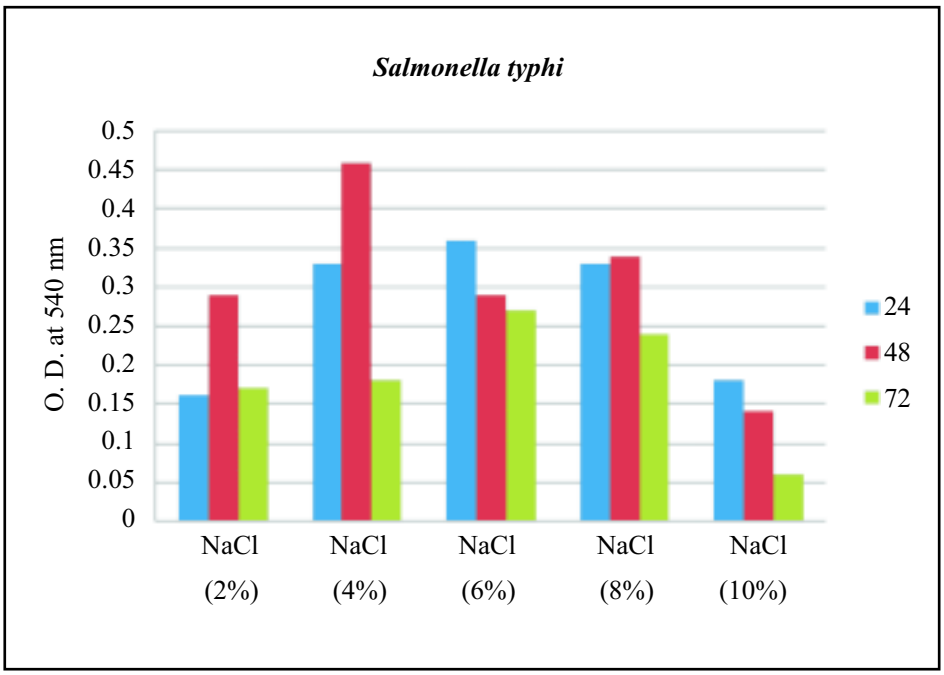


Figure 2 : Effect of NaCl on the Biofilm Formation of *P. aeruginosa* Measured Using Crystal Violet Assay

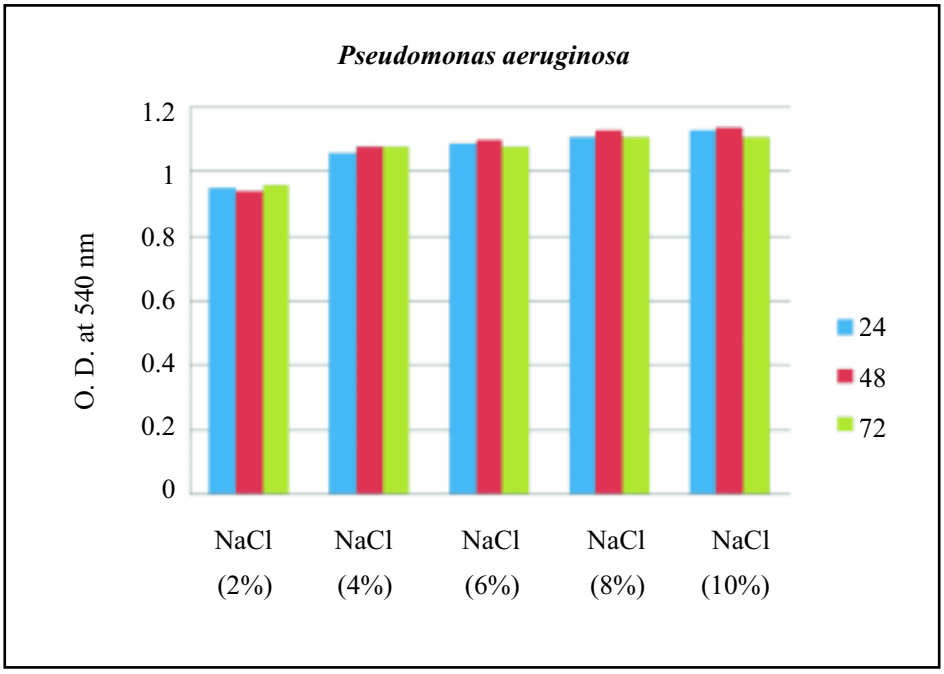


Figure 3: Effect of NaCl on the Biofilm Formation of *K. pneumoniae* Measured Using Crystal Violet Assay

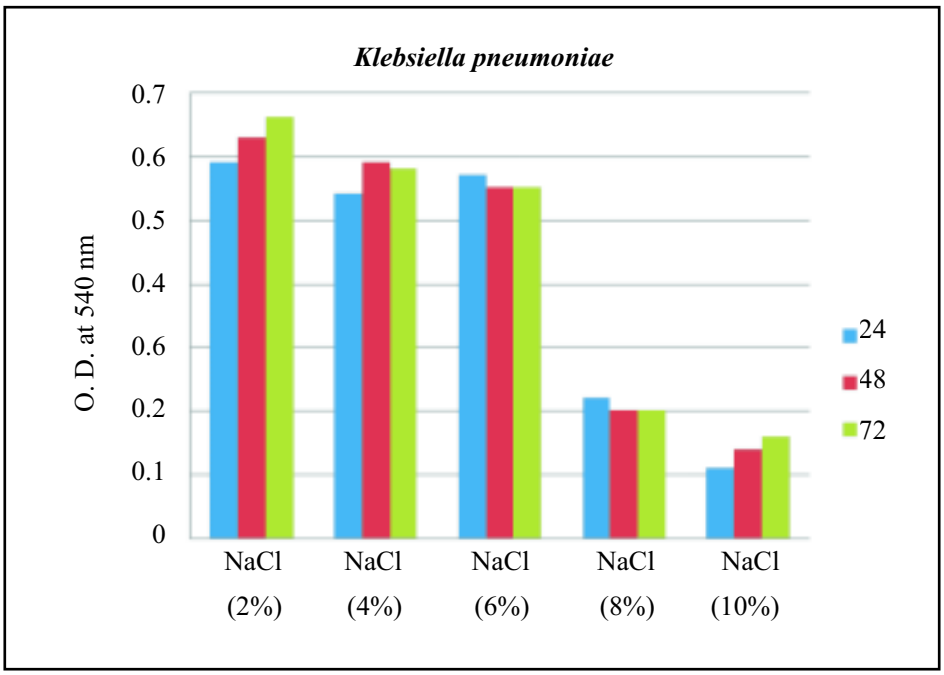


Figure 4 : Effect of NaCl on the Biofilm Formation of *S. typhi* Measured Using Crystal Violet Assay

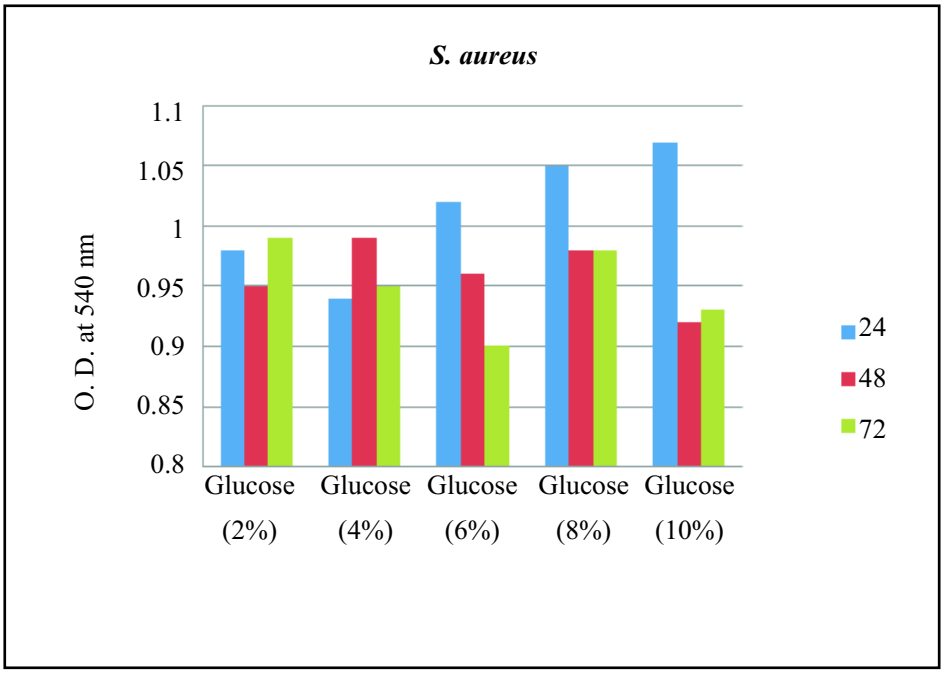


Figure 5 : Effect of Glucose on the Biofilm Formation of *S. aureus* Measured Using Crystal Violet Assay

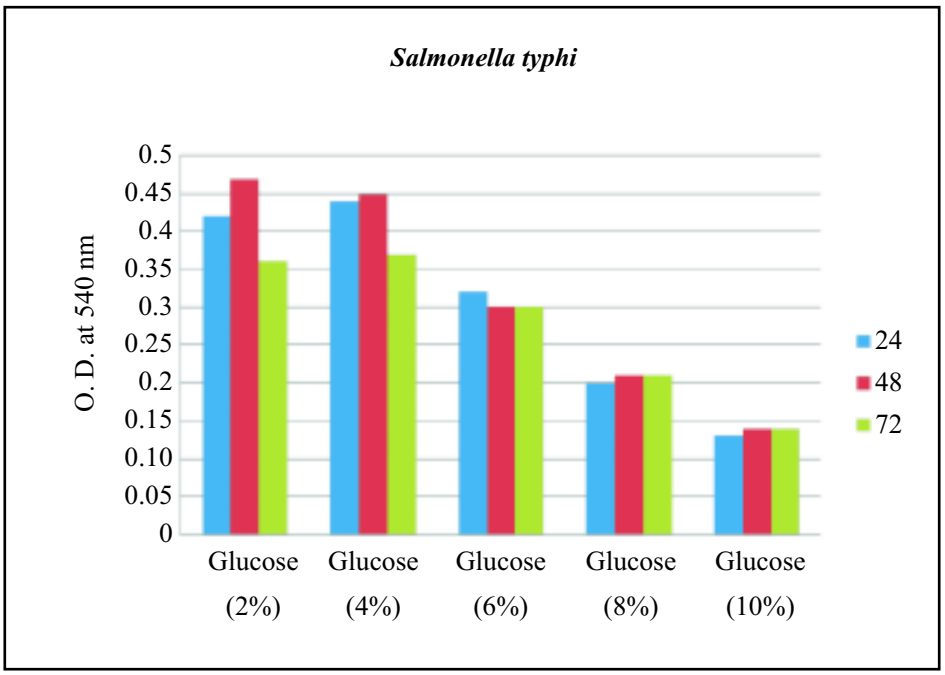


Figure 6 : Effect of Glucose on the Biofilm Formation of *S. typhi* Measured Using Crystal Violet Assay

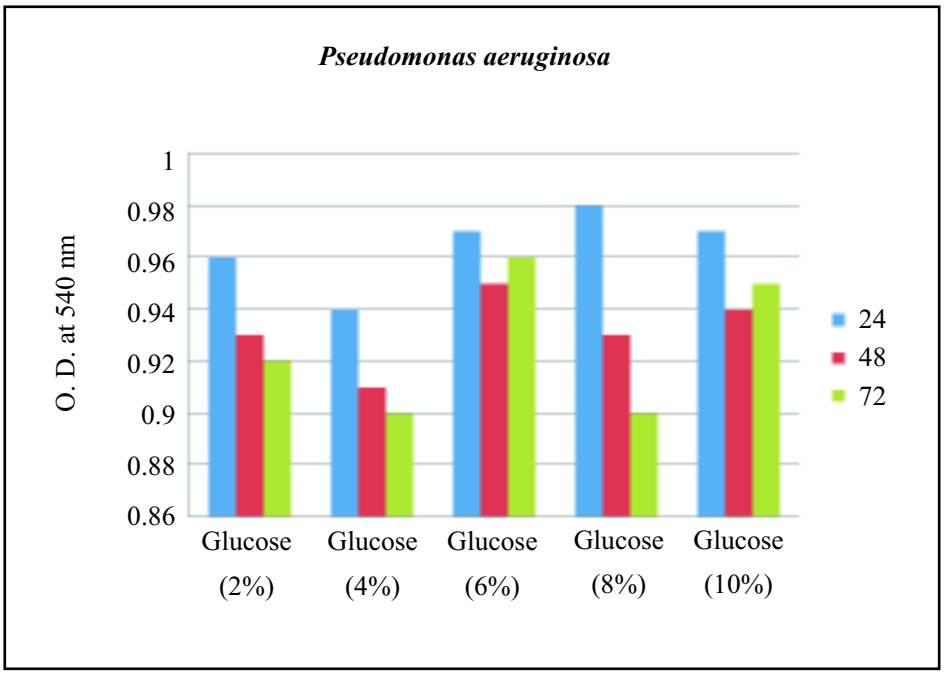


Figure 7 : Effect of Glucose on the Biofilm Formation of *Ps. aeruginosa* Measured Using Crystal Violet Assay

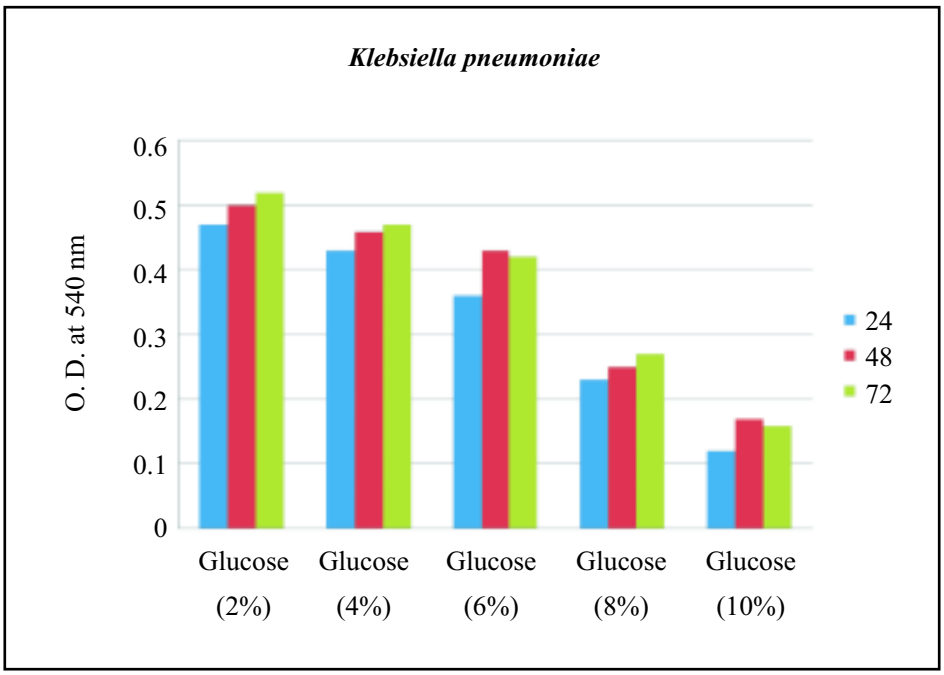


Figure 8 : Effect of Glucose on the Biofilm Formation of *K. pneuomiae* Measured Using Crystal Violet Assay

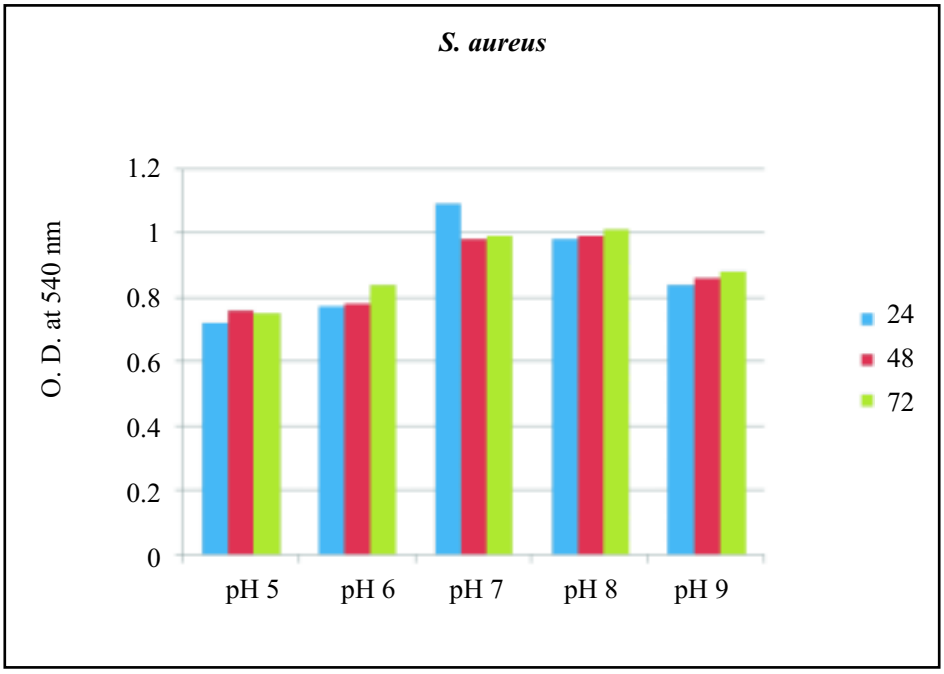


Figure 9 : Effect of pH on the Biofilm Formation of *S. aureus* Measured Using Crystal Violet Assay

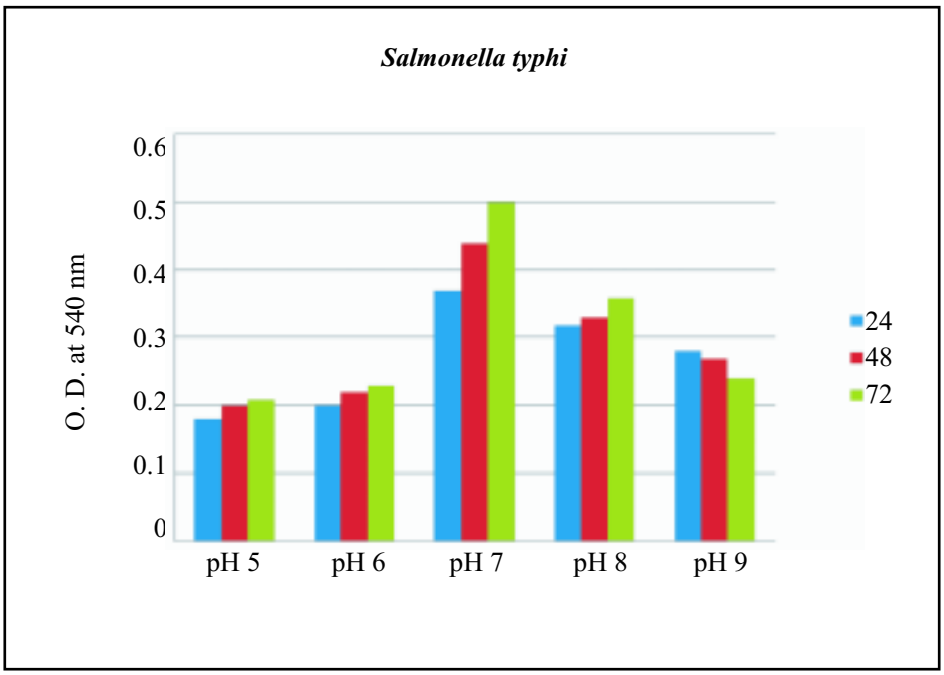


Figure 10 : Effect of pH on the Biofilm Formation of *S. typhi* Measured Using Crystal Violet Assay

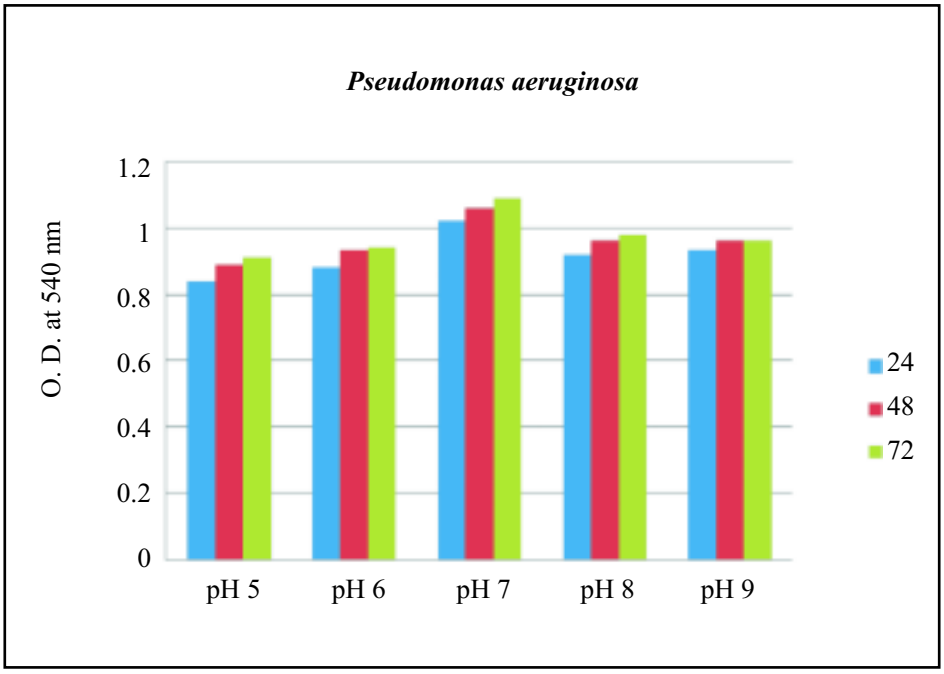


Figure 11 : Effect of pH on the Biofilm Formation of *P. aeruginosa* Measured Using Crystal Violet Assay

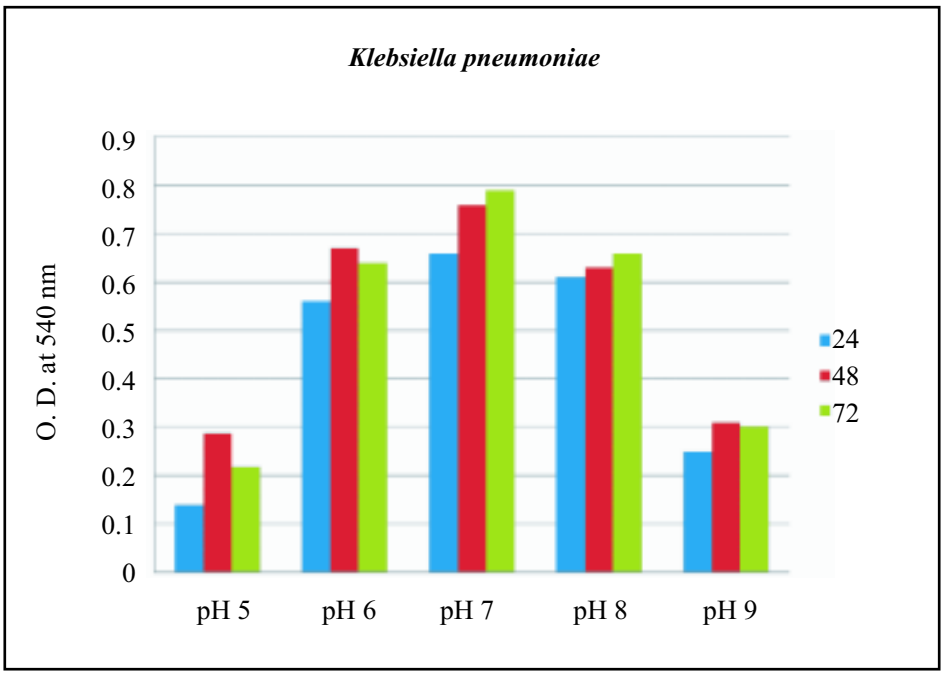


Figure 12 : Effect of pH on the Biofilm Formation of *K. pneumoniae* Measured Using Crystal Violet Assay

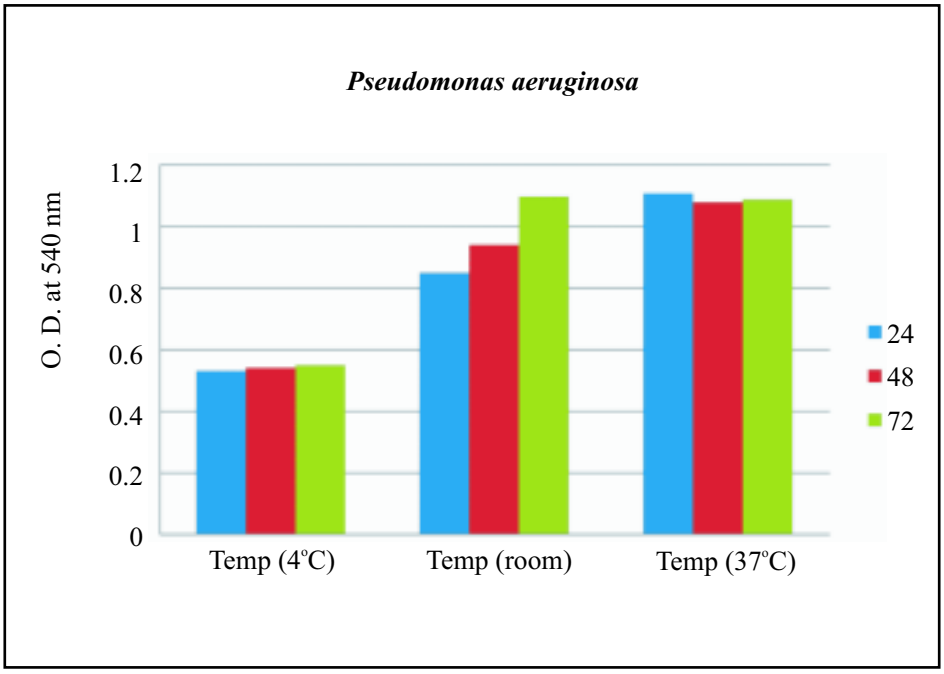


Figure 13 : Effect of Temp on the Biofilm Formation of *S. aureus* Measured Using Crystal Violet Assay

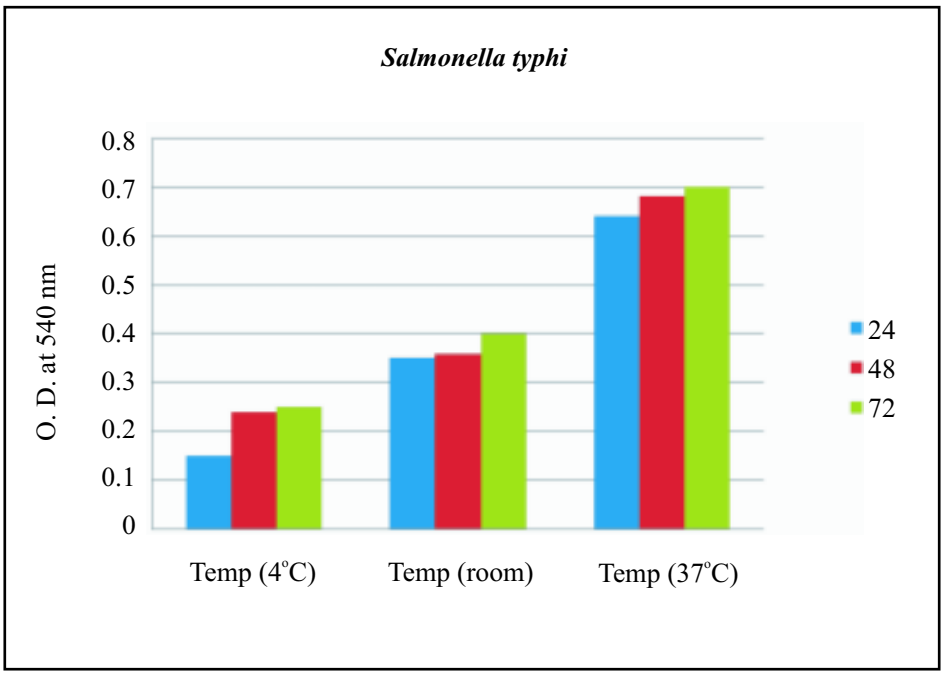


Figure 14 : Effect of Temp on the Biofilm Formation of *S. typhi* Measured Using Crystal Violet Assay

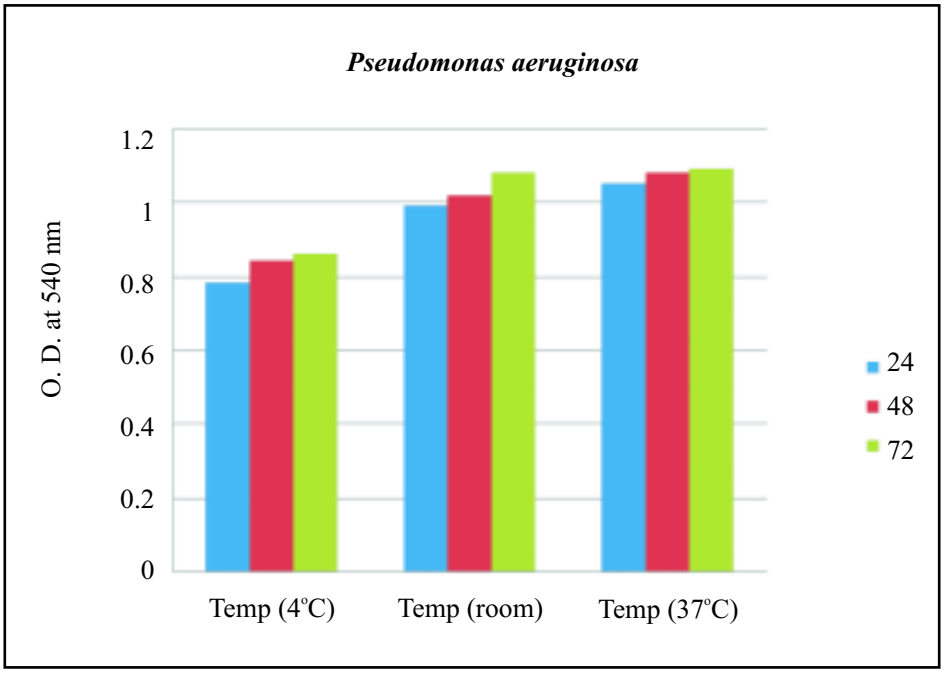


Figure 15 : Effect of Temp on the Biofilm Formation of *Ps. aeruginosa* Measured Using Crystal Violet Assay

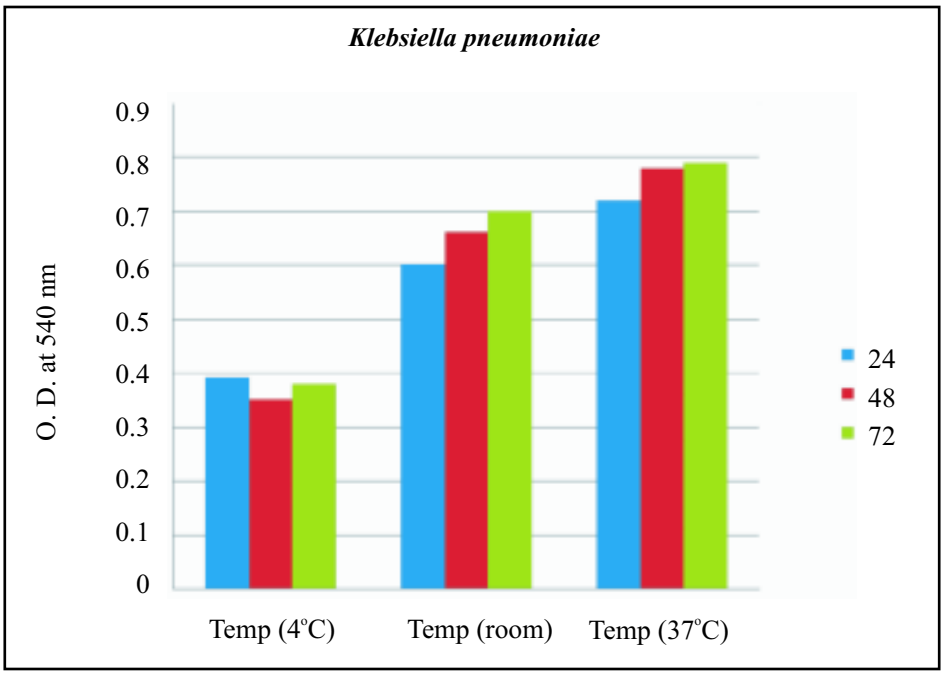


Figure16 : Effect of Temp on the Biofilm Formation of *K. pneumoniae* Measured Using Crystal Violet Assay

respectively. Also considering complexity of biofilm formation in certain cases growth was seen at higher concentration but no persistence was seen post 24-48hrs (figure1-4).

The increasing NaCl concentration was found to be most effective in controlling biofilms formed by *S. aureus*. *Salmonella typhi* and *Klebsiella pneumoniae*, while it had no inhibitory effect on *Pseudomonas aeruginosa* (figure 1-4).

Effect of Glucose

Increasing sugar concentration has been shown to stimulate biofilm formation in *S. aureus*. Though maxiumum amount of biofilm was produced at 8% glucose even upto72hrs and a slight decrease wasseen at 10% after 24hrs.

It was observed that, the increasing glucose concentration was most effective on the control of biofilms by *Salmonella typhi* and *Klebsiella pneumoniaboth* of which showed decline in biofilm formation from 8%. Once again among the Gram negatives *Pseudomonas aeruginosa* was least affected by high osmolarity,followed by S aureus (gram positive) with biofilm formation seen upto10% sugar concentration (figure 5-8).

Effect of pH

AtpH 5 decrease in biofilm formation was seen for both *Saureus*, *S. typhi* and *Klebsiella*. pH 6 had no significant impact on inhibition of biofilm formation by all organisms apart from *Salmonella typhi*. At pH 7 and 8 maxiumum biofilm formation was by all the organism. At pH9 too organisms reported no decrease in growth or biofilm formation apart for Klebsiella. Within the range of pH 5-9 *Psuedomonas* reported no decrease in biofilm formation (figure 9-10).

With the use of different temperatures, it was observed, that low temperature i.e. 4°C was most effective in the control of biofilm formation by all the test organisms. 37°C was observed to be the optimum temperature for biofilm formation. Overall, effect of low temperature in inhibiting biofilm formation was found to be least on *Pseudomonas aeruginosa* (figure 11-16).

CONCLUSION

From the above study one can conclude that hypertonic salt concentration of 8% and above can prevent biofilm formation by *Klebsiella* and *Salmonella*. This data is more significant as many times these two organisms enter human body via contaminated meat products, so the biofilm formation by these organisms could be controlled by preserving meat in high salt concentration. Alsolow temperature and high alkaline pHaffected biofilm production by *Salmonella typhi* and *Klebsiella pneumonia*. Among all the parameters used in study for *Saureus* only low temperature resulted in slight decrease in biofilm formation by *S. aureus*. The effect of pH in inhibiting biofilm formation was reported for *Klebsiella pneumoniae* and *Salmonella typhi* while there was not much effect observed on *S. aureu*s and *Pseudomonas aeruginosa*. None of the environmental factorsused in the study inhibited biofilm formation by *Pseudomonas aeruginosa*.

The study of biofilm formation by foodborne pathogens is of utmost importance, to the food industry. From the above study we can conclude that bacterial biofilms form as rapidly as within 24 hours under varied environmental conditions. Biofilm formed by *Pseudomonas* and *Saureus* are most difficult to manage. However due to the complexity, of biofilms, they are difficult to eradicate, and therefore, it is highly crucial to prevent biofilm formation.An in-depth study of the genetic basis of biofilm formation in these organisms needs to be understood to help devise novel control strategies.

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