# COMPARATIVE STUDY OF BIOFILM INHIBITION OF *AEROMONAS HYDROPHILA* BY MUTAGENESIS OF PILMNOPQ GENES AND ESSENTIAL OIL COMPONENT ISOEUGENOL

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**Abstract**

Eugenol exhibits antibacterial activity against several bacteria, such as *Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli,* etc. Also eugenols and its derivatives are known to reduce the biofilm at sub MIC levels. In the current study we evaluated the effect of sub MIC levels of isoeugenol on biofilm formation against *Aeromonas hydrophila* strain. Biofilm forming capability was reduced in a concentration dependent manner at all tested subMIC values of isoeugenol. Additionally, β-galactosidase assay suggests isoeugenol down regulates the expression of putative type IV pili operon *pilMNOPQ* genes. Similarly reduced biofilm formation phenotype was also observed in PilMNOPQ operon mutant strain. Thus the study suggests that the phenotype of PilMNOPQ mutant strain of reduced biofilm forming ability appears comparable to that of use of isoeugenol to inhibit biofilm formation of *Aeromonas hydrophila*.

**Keywords:** *Aeromonas hydrophila*, Biofilm, Pili, Isoeugenol.

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## INTRODUCTION

Eugenol is a phenolic aromatic compound. It is the primary ingredient in clove oil and is often derived from the natural essential oils of plants from the Lamiaceae, Lauraceae, Myrtaceae, and Myristicaceae families *(Syzygium aromaticum)*. It occurs in various concentrations in different plant species (Table-1). The richest source is *S. aromaticum*, which contains between 9381.7 mg and 14,650 mg per 100 g of fresh plant material and is chiefly accountable for its distinctive aroma. Many antioxidant, analgesic, antimutagenic, anti-platelet, anti-allergic, anti-swelling, and anti-inflammatory properties of eugenol have been proven. Moreover, it has shown antibacterial properties against a variety of Gram-positive and Gram-negative bacteria, fungi, and parasites like *Giardia lamblia, Fasciola gigantica,* and *Haemonchus contortus* [1-3].

TABLE-1
OCCURRENCE OF EUGENOL AND ITS CONCENTRATION
IN PARTICULAR PARTS OF PLANTS

| **Plant** | **Part** | **Concentration (mg/g)** |
|---|---|---|
| Clove, clovetree | Flower Leaf, stem | 1809 |
| Clovepepper | Fruit | 36 |
| Beetel pepper | Leaf | 17.85 |
| Tulsi | Leaf | 4.3-4.7 |
| Carrot | Seed | 7 |
| Ceylon cinnamon, cinnamon | Bark | 3.52 |
| Turmeric | Leaf, essential oil | 2.1 |
| Bay, Bay laurel | Leaf | 1.34 |
| Chinese ginger | Rhizome | 0.4 |
| Nutmeg | Seed | 0.32 |
| Small flowered oregeno | Shoot | 0.055-0.125 |

*Aeromonas hydrophila* is a Gram negative opportunistic pathogen widely existing in variety of aquatic environments. It not only infects fishes and other aquatic animals causing huge loss to aquaculture industry but also infects humans leading to skin infections, necrotizing fasciitis and gastroenteritis. The pathogenicity of the bacterium is a result of assortment of virulence factors like enterotoxins, hemolysins, cytotoxins, aerolysin and production of type IV pilus (T4P) [4,5]. Numerous studies on *A. hydrophila* have focused on pathogenesis process aiming to identify more serviceable virulence factors and potential vaccine candidates [6,7]. Bacterial biofilms are characteristic of various pathogenic bacteria like *Haemophilus influenzae* known to cause chronic otitis and *Escherichia coli* responsible for urinary tract infections. Mechanisms that bacteria employ to form biofilms vary widely among different species. Biofilms are major causes of persistent infection and drug resistance due to its ability to attach to visceral organs and other material surfaces. Different genes play important roles in each phase of biofilm formation. For *e.g.* pili and flagella are involved in surface attachment [8], surface proteins like biofilm associated protein, extracellular matrix binding protein and fibronectin binding proteins affect matrix formation [9-11]. In *Pseudomonas aeruginosa* T4P and rhamnolipid are involved in dispersal of biofilms [12]. The search for biofilm inhibitors is essential to control infections associated with biofilms. Eugenol is known to possess these properties.

The genome sequence of *A. hydrophila* also shows presence of PilMNOPQ system (AHA_3190-AHA_3194) [13]. The *pilMNOPQ* gene cluster is a crucial group of genes needed for the surface expression of Tap and related motility [14,15]. Previous studies in *Aeromonas schubertii* have reported that *tapM/N/O/P/Q* mutants were deficient in cell and tissue adhesion [16]. Also in *Pseudomonas aeruginosa* both T4P assembly and twitching motility, a type of bacterial movement necessary for pathogenicity, are dependent on genes in the *pilMNOPQ* operon [17]. T4p mutants leads to impairment in colonization process suggesting they contribute toward the infection process. This study aims to compare the biofilm inhibition of *A. hydrophila* by mutagenesis of *pilMNOPQ* gene cluster and essential oil component isoeugenol.

## MATERIALS AND METHODS

**Bacterial strains, plasmids and growth conditions:** Plasmids and bacterial strains used are listed in Table-2. *Aeromonas hydrophila* strain was cultured overnight (16 to 18 h) at 28 °C whereas all the *E. coli* strains were grown aerobically overnight by shaking at 37 °C. Cultures were grown in Lysogeny broth (LB) and LB Agar supplemented with appropriate antibiotics. Cultures were maintained on LB agar plates at 4 °C for a maximum of 1 weeks and glycerol stocks were stored at -80 °C. Gentamycin and chloramphenicol were used at a final concentration of 70 µg/mL and 25 µg/mL respectively.

TABLE-2

LIST OF STRAINS AND PLASMID USED IN THE STUDY

| **Strain** | **Feature** | **Source** |
|---|---|---|
| ***A. hydrophila*** | | |
| *A. hydrophila* ATCC 7966 | Wild type, isolated from tin of milk with a fishy odour | ATCC |
| 3194i | AHA_3194 insertional inactivated mutant derived from ATCC 7966 | This study |
| 3194C | 3194i with full length 3190-3194 operon from *A. hydrophila*) in pJB3Cm6 $Cm^r$ | This study |
| ***Escherichia coli*** | | |
| EC100 | *E. coli* EC100 derivative, pir+ | Epicenter technologies |
| β2155 | pir::RP4, $Km^r$ | [19] |
| **Plasmids** | | |
| pER21 | Suicide vector containing *sacB* gene of *Bacillus subtilis*; $Gen^r$ | [18] |
| pJB3CM6 | Broad host range Vector $Cm^r$ | [20] |
| pMC10 | Promoter less *lacZ* gene; $Cm^r$ | [22] |
| pER3194i | pER21 containing 278 bp XmaI-XmaI fragment for insertional inactivation of AHA_3194 | This study |
| pJB3194C | pJB3Cm6 containing full length gene for complementation of AHA_3194 | This study |
| pPilM | pMC10 containing the promoter region of AHA_3194 | This study |

**Generation of insertional inactivation mutants:** Chromosomal insertional mutant of *pilM* (AHA_3194) was generated using the suicide vector PER21. Briefly, approximately 490 bps of the internal sequence of the *pilM* gene (AHA_3194) was amplified using primers with Xma1 restriction sites (Table-3). This amplified fragment was cloned in pER21 [18] which has R6k origin of replication and a gentamycin resistance cassette. The resulting plasmid was transferred from *E. coli* β2155 [19] to *A. hydrophila* strain *via* conjugation. The mutant strain was identified by gain of gentamycin resistance. Mutant is designated as 3190i.

TABLE-3

LIST OF PRIMERS USED IN THE STUDY

| Purpose | Primer name | Sequence 5'-3' |
|---|---|---|
| Amplification of internal fragment of pilM gene from *A. hydrophila* ATCC 7966 | 3194i1 Xma1 site included | gatccccgggGCCCTCAAGCGCCTCATCTCGG |
| | 3194i2 Xma1 site included | ccgacccgggGCGCCTGACTATATTGATCGC |
| Amplification of full length pilMNOPQ from *A. hydrophila* ATCC7966 | 3194c1 Xba1 site included | gatctctagaCTGCATCGATCATGGACTG |
| | 3194c2 Xba1 site included | catgtctagaTCAGAAGGCGTCGGTGACTATC |
| Construction of full-length pilM (AHA_3194) promoter fragment for β-galactosidase assay | pA1 Hind III site included | gatcaagcttGGAAGAACTACCAATC |
| | pA2 BamH1 site included | catgggatccCATCCATGCGTTATTG |

**Complementation of mutant:** A DNA fragment carrying the complete sequence of the operon along with upstream 500bp region was amplified by PCR using *A. hydrophila* ATCC7966 total genomic DNA, Phusion polymerase (New England BioLabs) and the primers listed in Table-3. The fragments were cloned into the Xbal-digested broad host-range vector pJB3Cm6 [20] to generate the plasmids pJB3194C. The ligation mixture was used to transform *E. coli* EC100 cells by electroporation followed by blue-white selection and PCR to confirm the presence of the desired insert. The pJB3194C plasmid was used to transform *E. coli* β2155 and then transferred to *A. hydrophila via* conjugation.

**Growth curve analysis:** The growth curve analysis was performed to test the antibacterial activity of sub-MICs of isoeugenol against WT, *3194i*, and 3194C strains. Briefly, cells were inoculated into 50 mL LB broth and cultivated in the presence or absence of test concentrations of isoeugenol. The culture setup was incubated at 37 °C and the $OD_{600}$ was monitored at 2 h intervals for up to 24 h.

**Antibacterial assay:** The minimum inhibitory concentration (MIC) of isoeugenol for cells were determined using the microdilution method in accordance with the guidelines of the Clinical and Laboratory Standards Institute, document M31-A3 (CLSI, 2008), following the methodology described by Dal Pozzo *et al.* (2011)[21].

**Biofilm formation assay:** Biofilm assay was performed in a 96 well plate in medium AB prepared by mixing solution A and solution B. Solution A comprises 2.0 g of $(NH_4)_2SO_4$, 6.0

g of $Na_2HPO_4$,183.0 g of $KH_2PO_4$, 3.0 g of NaCl, and 0.011 g of $Na_2SO_4$, dissolved in 200 mL of distilled water. Solution B contains 0.2 g of $MgCl_2$, 0.010 g of $CaCl_2$, and 0.5 mg of $FeCl_3 \cdot 7H_2O$ dissolved in 800 mL of distilled water. Finally the media was supplemented with 0.5% (w/v) glucose solution and sterilized by autoclaving. Bacterial strains were cultured aerobically overnight in Luria bertani broth at 28 °C. These overnight cultures were then diluted in AB's medium to $O.D_{600}$ of 0.6. 100 µL of each diluted culture was then transferred to 96 well plated and incubated at 28 °C overnight without shaking. Non adherent cells were removed and remaining adherent bacteria were stained with 0.1% crystal violet. After 30 min wells were washed with distilled water and dried in 37 °C incubator. Retained crystal violet was dissolved in 200 µL of ethanol-acetone (80:20 v/v), and the absorbance was measured at 590 nm. Replicates of six wells per culture were examined in each experiment and a broth control containing only media.

**β-Galactosidase assays:** *pilM* gene putative promoter was amplified and cloned into pMC10 [22] to generate the plasmid pPilM. Plasmid was introduced into *A. hydrophila* wild type (WT) after transformation of *E. coli* β2155 cells. The transformed strains were grown at different subMiC concentration of isoeugenol. The transformed strain was then tested by β galactosidase assay. 1 mL of culture was diluted with Z buffer to a total volume of 5 mL for the assay. The absorbance at 600 nm was used to determine cell density. The $OD_{600}$ of this cultures was normalized to 0.6. The cells were lysed by mixing them with 50 µL of 0.1 percent SDS and 100 µL of chloroform. 250 µL of ONPG (9 mg ONPG in 10 mL of 1 mM $MgCl_2$ and 0.1 mM β-mercaptoethanol) was added, then the mixture was briefly mixed. The samples were incubated for 1 h at 30 °C. The amount of o-nitrophenol (yellow colour) produced was proportionate to the amount of β-galactosidase enzyme present, indicating the level of promoter expression. The reaction was halted by adding 0.5 mL of 1 M $Na_2CO_3$ to the mixture. The absorbance was measured at 420 nm and 550 nm. The units of β-galactosidase activity were calculated using the formula mentioned below: The assay was performed thrice and in triplicates.

$$\text{Units} = 1000 \times \text{OD}_{420} - \frac{1.75 \times \text{OD}_{550}}{\text{t} \times \text{v} \times \text{OD}_{600}}$$

where, t = time of the reaction (min); V = volume of the culture used in the assay (mL).

## RESULTS AND DISCUSSION

Eugenol a component of essential oils, has been tested for its effectiveness in eliminating bacterial biofilm. Bacterial biofilm is a three-dimensional macrocolony of bacteria isolated by an extracellular matrix, produced by these microorganisms. Bacterial biofilms are a significant problem. For example, in the food industry, they form on the surface of food products, contaminating the food and can cause disease development. Therefore, effective measures are being sought to solve this issue. Eugenol affects bacterial biofilms generally through two mechanisms: it inhibits biofilm development and reduces the vitality of cells that produce biofilms. Additional outcomes included cell dispersion within the biofilm matrix, bacterial cell inactivation and gene expression suppression linked to biofilm**s.** Previously it has been reported that eugenol inhibits and eradicate the biofilms formed by *Staphylococcus aureus* [23], *Candida albicans* [24], *C. dubliniensis* [25], *Porphyromonas gingivalis* [26], *S. aureus* ATCC25923 [27], *Escherichia coli* [28], and *Streptococci* [29].

*A. hydrophila* infection causes a motile *Aeromonas septicemia*, red fin disease in fishes and severe gastroenteritis in humans. This bacterium has become a major threat to aquaculture industries as leads to huge economic loss each year [30]. To date, many studies have reported the interactions between *A. hydrophila* and aquatic animals so that the pathogenic mechanisms of this bacterium can be clearly understood. T4P pose to be critical virulence factors for many pathogenic bacteria. In the current study we have investigated the effect of isoeugenol on pilin operon AHA_3190-AHA_03194 that predicts to encode type IV *pilMNOPQ* gene cluster. Since pili are often involved in bacterial adherence, we hypothesized that the Tap pili of *A. hydrophila* were associated its pathogenicity.

According to NCBI conserved domain search AHA_3190 is predicted to be pilQ which is

known to be involved in type IV pilus formation, competence for transformation and is a member of secretin family. It forms a channel to export T4p out of the cell. AHA_3191 is annotated as pilP which is a periplasmic lipoprotein involved in biogenesis of type IV pilus. Signal peptide of pilP possess a highly conserved lipobox. AHA_3192 encodes T4a biogenesis protein PilO, it contains unstructured regions which are essential for protein-protein interaction with PilM and PilN. AHA_3193 is a type IV biogenesis pilN. PilN and PilO are integral membrane proteins with a periplasmic domain which forms a stable heterodimer. AHA_3194 is annotated to be a cytoplasmic pilus assembly protein. PilM which is required for pilus biogenesis and competency has conserved actin like ATP binding domain. Additionally, the development and stability of the outer membrane PilQ secretin channel is impacted by inner membrane complex formed by the PilMNOP proteins.

To study the comparative effect of isoeugenol on biofilm formation in *A. hydrophila* and role of *pilMNOPQ* operon in *A. hydrophila* we generated insertionally inactivated *pilM* gene mutant strain of *A. hydrophila*. This being the first gene of the operon may be a polar mutation thus inactivating the other genes of the *pilMNOPQ* operon as well. Characterization of mutant (3194i), wildtype and complemented strain (3194C) showed no difference in their growth rate (Fig. 1).

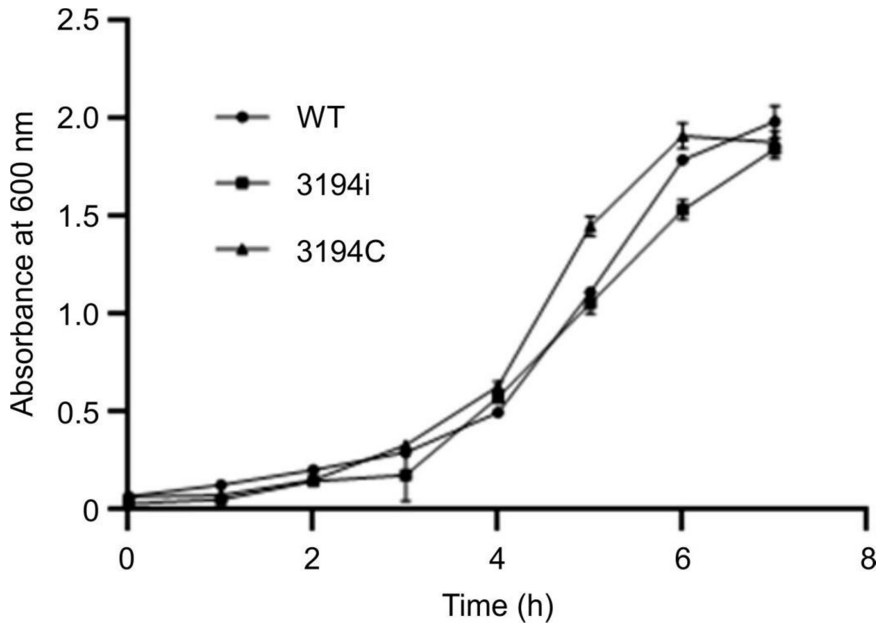


Fig. 1. Growth assay of wild type *A. hydrophila* (WT), pilin mutant with insertional inactivation of AHA_3194 (3194i), and *pilin* complemented strain (3194C). All the three strains showed similar growth pattern. Error bars depict the S.D. of three biological replicates

Minimum inhibitory concentration of bioactive compound isoeugenol was calculated to be 1.25 mg/mL against *A. hydrophila*. Further, assays of *A. hydrophila* were conducted at calculated sub-MIC value of isoeugenol. The growth curve study showed no significant change in cell densities between treated and untreated *A. hydrophila* cultures, suggesting that isoeugenol at selected sub-MICs does not inhibit significant growth of the test strain.

Since biofilm formation is an important parameter contributing to pathogenicity of several opportunistic bacteria we studied effects of isoeugenol at different sub-MICs on the biofilm formation in WT, 3194i and 3194C strains. Our results suggest that *A. hydrophila* ATCC 7966 wildtype strain can attach to the microtiter plates to form biofilm. The 3194i mutant from *A. hydrophila* ATCC7966 was comparatively deficient in biofilm formation

and attachment to the microtiter plates while the complemented strain showed wildtype level of biofilm formation. Almost 18 folds difference was observed in biofilm forming capacity of 3194i and 3194C strains (Fig. 2). The investigation revealed a concentration-dependent decrease in biofilm formation in wildtype and 3194C bacterial strains when grown in the presence of isoeugenol. At 1.25 mg/mL concentration, isoeugenol showed a maximum reduction in biofilm forming capability of WT and 3194C strains. However no change was observed in biofilm formation capacity of the 3194i strain. These results suggest that isoeugenol at sub-MIC have a role in reduction of biofilm formation by down regulating the expression of *pilMNOPQ* operon genes. So to validate biofilm inhibition by isoeugenols we developed *LacZ* fusions to test the expression of *pilMNOPQ* operon promoter (*pilM*) in presence and absence of isoeugenol. The expression of β-galactosidase gene through pilM promoter in WT strain was 488.96 ± 12.72 Miller units. However, the expression through the promoter was downregulated in presence of isoeugenol. The maximum downregulation was observed at 1.25 mg/mL concentration and was 83.47 ± 7.777. This data indicates that isoeugenol has effect in biofilm inhibition by downregulating the expression of *pilMNOPQ* operon genes

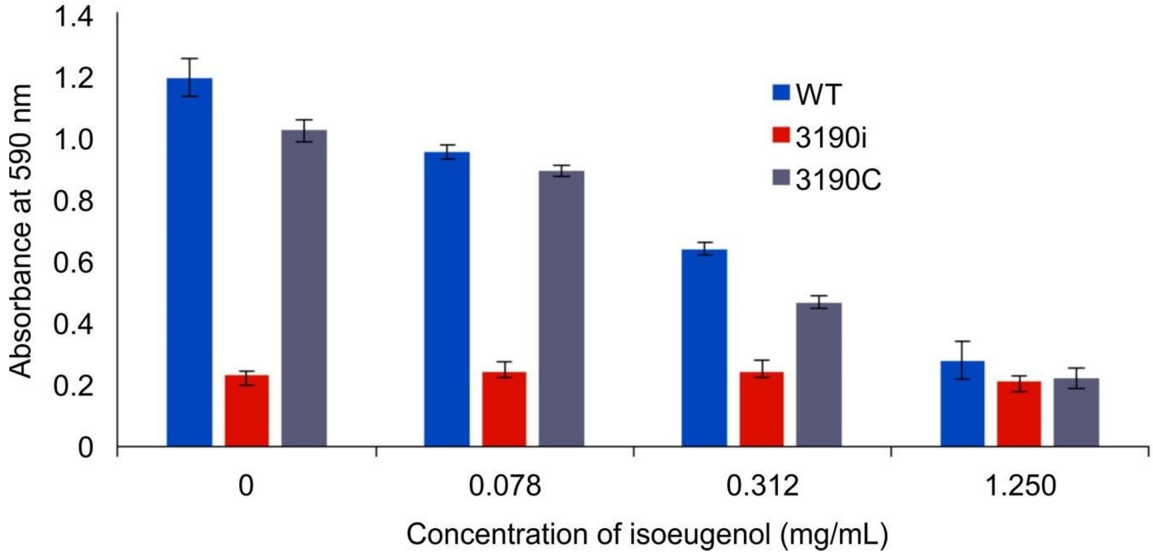


Fig. 2. Biofilm formation by wild type *A. hydrophila* (WT), pilin mutant with insertional inactivation of AHA_3194 (*3194i*), and pilin complemented strain (3194C) at different concentration of Isoeugenol. Inactivation of pili genes of *A. hydrophila* resulted in significantly less biofilm formation than the wildtype. Also with the increasing concentration of isoeugenol there is concentration dependent decrease in biofilm formation in WT and 3190C strains.

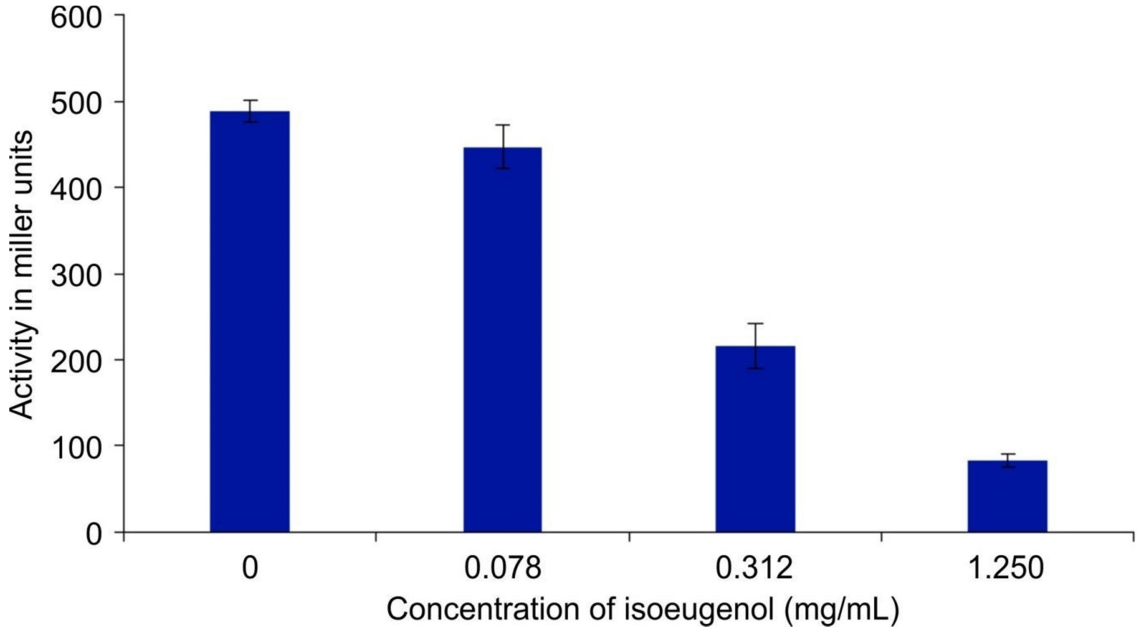


Fig. 3. Expression of *pilM* promoter in *A. hydrophila* (WT) at different concentration of isoeugenol. There was a concentration dependent decrease in expression of promoter with increasing concentration of isoeugenol

This study concludes that isoeugenol can be used at sub-MIC concentration for disposal of biofilms. Attenuation of biofilms can lead to decreased rate of infectivity and silencing of these pili genes can lead to attenuation of biofilms. Vaccines targeting these pilin proteins can be generated as a method to combat these infections. Alternatively addition of essential oils containing isoeugenols can also be supplemented in fish feed or aquaculture to control the infection process.

## Conclusion

Eugenol in subinhibitory concentrations promoted the inhibition of biofilm formation in *A. hydrophila* and downregulates the expression of *pilMNOPQ* operon genes. These results, along with biofilm inhibition by mutagenesis of *pilMNOPQ* gene cluster, validates the use of eugenol as biofilm inhibitor and antibacterial against *A. hydrophila* infections. Aquaculture industries may consider use of eugenol because of its wide spectrum of pharmacological and biological effects.

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## Declaration of Competing Interest.

The authors declare that they have no competing financial or personal interest related to this study.

## Ethical Approvals

This study does involve any human and animal studies.

## Data Availability Statement

The authors confirm that all the relevant data is included in the manuscript.

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