SCREENING OF MARINE BACTERIUM *VIBRIO ALGINOLYTICUS* STRAIN AS05 FOR THE PRODUCTION OF *N*-ACYL HOMOSERINE LACTONE-BASED QUORUM SENSING SIGNALING MOLECULES

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ABSTRACT: Marine environments, including aquatic and coastal environments, are highly prevalent of marine bacteria with the highest levels of *Vibrio* species. Vibrios play a vital role in marine ecology associated with carbon and energy acquisition. The density-dependent quorum sensing (QS) system may regulate certain biological activities in marine bacteria. QS is a conversation system utilized by many bacterial communities to communicate and coordinate through different signalling molecules. *N*-acyl homoserine lactones (AHLs) are the most important QS signalling molecules widely produced by Gram-negative bacteria. This study aimed to investigate the detection and Identification of AHL-based QS signalling molecules produced by marine bacterium *V. alginolyticus* strain AS05 isolated from marine water of the Arabian Sea, Karachi, Pakistan. Marine medium Zobell-2216 was used to isolate bacterial strains. Moreover, 16S rRNA analysis was applied to identify AS05 strain. Agar plate bioassay was used to detect the production of AHL signalling molecules using *Chromobacterium violaceum* CV026 as a biosensor. The Identification of AHLs was made by reversed-phase thin-layer chromatography (RP-TLC) analysis. The NCBI-blast results revealed the identification of the isolated bacterial strain as *Vibrio alginolyticus* strain AS05 (OQ130030) member of the family of *Vibrionaceae* under the class of Gammaproteobacteria. The results of agar plate bioassay using CV026 as a biosensor strain revealed highly positive reactions for producing AHL signalling molecules. Moreover, two AHL molecules produced by AS05 bacterial strain were identified as C6-HSL and C-8HSL based on TLC analysis. This study reveals the detection and Identification of two different AHL signalling molecules produced by *V. alginolyticus* AS05 isolated from marine water of the Arabian Sea, Karachi, Pakistan. This study provides insight into investigating quorum-sensing signalling molecules in the Arabian Sea, Karachi, Pakistan, and marine bacterial species.

Key words: Quorum-sensing, AHLs, TLC, and CV026.

INTRODUCTION

The Ocean is deemed the vigorous reservoir for biogeochemical cycling, predominantly the carbon cycle, which is involved in extenuating the impacts of global warming (Jatt *et al*., 2015). Among marine heterotrophic bacterial groups, members of the genus *Vibrio* are known as the best models and play a crucial role in biogeochemical cycling through the remineralization of carbon, nitrogen, and phosphorus compounds (Zhang *et al*., 2018). This genus has been classified into more than 120 species (Wang *et al*., 2019). Some species of the genus *Vibrio* may cause severe infections in marine organisms and humans (Jesser & Nobel, 2018). However, many of the marine Vibrio species are non-virulent (Takemura *et al*., 2014). The main features of the *Vibrio* species are fast generation time, motile with polar flagella, halophilic in nature, and production of a wide range of extracellular enzymes (Wang *et al*., 2019). They can produce high biomass contents, which may impact biogeochemical cycles (Rizzo *et al*., 2016).

Vibrio alginolyticus is a Gram-negative, motile, non-sporing, curved rod-shaped bacterium associated with the family of *Vibrionaceae* of Gamma-proteobacteria. Previously, based on similar genotypic characteristics, *V. alginolyticus* was considered a biotype of *V. parahemolyticus* (Siddiqui *et al*., 2012). However, these two species can be differentiated based on the fermentation of sucrose carried out by *V. alginolyticus*. It is highly halophilic and grows best at higher concentrations of salts (10% NaCl) (Bunpa *et al*., 2016). *V. alginolyticus* is found in various environments, including marine water, diseased fish, sponges and human patients and is reported as an opportunistic pathogen (Bunpa *et al*., 2016). *V. alginolyticus* and some other *Vibrio* species such as *V. harveyi* and *V. anguillarum* produce several extracellular enzymes such as protease, chitinase, gelatinase and lipase. The virulent strains of *Vibrio* species may use these extracellular

enzymes as virulence factors and cause infections in marine organisms, including fish and sponges. *V. alginolyticus* has been reported as a highly dominant bacterial species in the marine environment (Liu *et al*., 2020). It produces higher concentrations of extracellular enzymes and other exopolysaccharides, and it is speculated that a cell density-dependent mechanism might regulate the production of these polysaccharides, including other virulence factors. The cell density-dependent mechanism, also called quorum sensing (QS), was first reported in *Vibrio* species of *Vibrio fischeri* a marine bacterium associated with the light organ of Sepiolid squid, in which light bioluminescence was regulated by QS system (Gram *et al*., 2002; Eberhard *et al*., 1981). However, identifying QS signalling molecules of different types in *V. alginolyticus* is unclear.

QS is a communication mechanism in which bacterial species may communicate with each other by producing extracellular signalling molecules called autoinducers. The bacterial species have distinct chemical signalling molecules and may produce more than a single QS molecule. Gram-negative bacteria are typically characterized by two different types of signalling molecules, also called autoinducers, such as *N*-acyl homoserine lactones (AHLs) (autoinducer-1) and furanose borate diester (AI-2). In contrast, modified peptide signalling molecules are produced by Gram-positive bacteria (Jatt, 2021). Moreover, AI-2 signalling molecules can be produced and utilized by Gram-negative and Gram-negative bacteria. Several types of marine microorganisms utilize QS system to regulate important biological functions such as bioluminescence, biofilm formation, and algicidal functions. Antibiotic production, control of secondary metabolism, and enhancement of extracellular enzyme production (Chen *et al*., 2020). Thus, the QS system plays a vital role in symbiotic and pathogenic bacterial communities. These marine bacteria may utilize QS to regulate extracellular enzymatic activities and virulence. The enzymatic activity in marine bacteria consists of various environmental factors, micro-environment, and ecosystems, predominantly by QS systems.

The present study was conducted to detect and identify chemical signalling molecules in marine bacterial isolate *V. alginolyticus* strain AS05 isolated from the Arabian Sea, Karachi, Pakistan. The present study represents the production of AHL-mediated QS signals in marine bacterial species.

MATERIALS AND METHODS

Isolation and identification of bacterial strain: Marine bacterium *V. alginolyticus* was isolated from marine water using marine medium Zobell agar 2216 as per the method described by Jatt *et al*. (2015). The bacterial strain was grown at 28^0C for 24-48hrs. Primarily, the bacterial strain was identified based on Gram's staining and biochemical tests, followed by molecular Identification using 16S rRNA analysis. DNA extraction, PCR, and sequencing for 16S rRNA were extracted at Macrogen, Geumcheon-gu, Seoul08511, Republic of Korea. The primers used to amplify the 16S rRNA gene from its bacterial DNA were 785F-5' (GGA TTA GAT ACC CTG GTA) 3' forward and 907R-5' (CCG TCA ATT CMT TTR AGT TT) 3'reverse primer. The obtained results of the gene sequencing were blasted against GenBank NCBI database (www.ncbi.nlm.nih.gov). MEGA, version 7.0 was used to construct a phylogenetic tree based on the neighbour-joining method (Fig. 1).

Screening of *V. alginolyticus* AS05 for producing AHL signalling molecules: V. alginolyticus strain AS05 was screened for producing AHL signalling molecules using crossing-feeding agar plate bioassay (Zhang *et al*., 2016). Chromobacterium violaceum CV026 was mainly applied as a biosensor bacterial strain to detect AHL molecules. A known AHL-negative bacterial strain was used as a negative control. The cross-feeding bioassay plate with test strain (AS05) and biosensor strain (CV026), along with the negative control plate, were incubated at 28^0C for 24-48hrs. After incubation, the bioassay plate was observed to produce violacein pigment with purple colour by CV026 biosensor strain of signalling molecules produced by AS05 test strain.

Extraction of AHL signalling molecules: AHL molecules were extracted by growing AS05 test bacterial strain in broth medium Zobell 2216 for 48-72hrs with shaking at 200 rpm. The cell suspension was centrifuged at 12,000 rpm for 10 min to obtain supernatant. AHLs were extracted by mixing the obtained supernatant with ethyl acetate equally and processed using a rotatory evaporator at 30^0C (Jatt *et al*., 2015) to obtain dried material. Subsequently, methanol was used to dissolve dried material and used for further process.

Thin layer chromatography (TLC): The ethyl extract of AS05 bacterial strain was processed to identify AHLs using reversed-phase thin layer chromatography (RP-TLC). Approximately 1µL of ethyl extract and specific AHL standards were loaded on TLC plate with the help of sterile capillary tubes. Methanol-Millipore water (60:40 v/v) was used to develop TLC plate (Zhang *et al.*, 2016; Jatt *et al*., 2015). TLC plate was air-dried and overlaid with soft LB medium (0.7% agar) containing

biosensor strain CV026. The plate was incubated at 28^0C for 24-48hrs and observed for change in color with purple spots

RESULTS AND DISCUSSION

Isolation and Identification of marine bacterial strain: Marine bacterial strain AS05 was isolated from marine water collected from the Arabian Sea, Karachi, Pakistan. The Identification through basic techniques such as Gram's staining and biochemical analysis showed the bacterial strain AS05 as Gram-negative, curved rod-shaped, motile and aerobic. Moreover, AS05 showed highly positive reactions for catalase and oxidase tests. Predominantly, the molecular Identification based on 16S rRNA gene sequence and NCBI-blast results revealed the bacterial strain as *Vibrio alginolyticus* strain AS05 with GenBank accession number OQ130030.

Phylogenetic tree: A phylogenetic tree for the strain AS05 was constructed using obtained 16S rRNA gene sequence through the neighbour joining method. The bacterial strain AS05 indicated the relatedness to the genus *Vibrio* and the species of *alginolyticus* under *Vibrionaceae* family, the class of Gammaproteobacteria (Fig. 1).

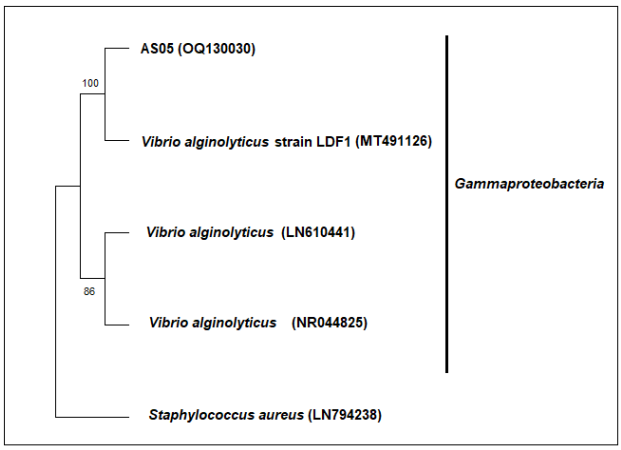

Figure 1. Phylogenetic tree constructed through MEGA version 7.0 by neighbor joining method of *V. alginolyticus* AS05(OQ430030) based on 16S rRNA gene sequence. *Staphylococcus aureus* (LN794238) was used as an outgroup.

Screening and identification of AHL signaling molecules: Cross-feeding agar plate bioassay for the *V. alginolyticus* strain AS05 showed highly positive reactions for the production of AHL based QS signaling molecules with a clear indication of violacein pigment production with purple color by *Chromobacterium violaceum* CV026 biosensor strain (Fig. 2). While, a known AHL negative bacterial strain used as negative control showed no production of AHL molecules. Moreover, the Identification of AHLs produced by AS05 bacterial strain through RP-TLC analysis showed the production of two different spots which were identified as C6-HSL and C8-HSL on basis of size and *Rf*-value matching with AHL controls (Fig. 3).

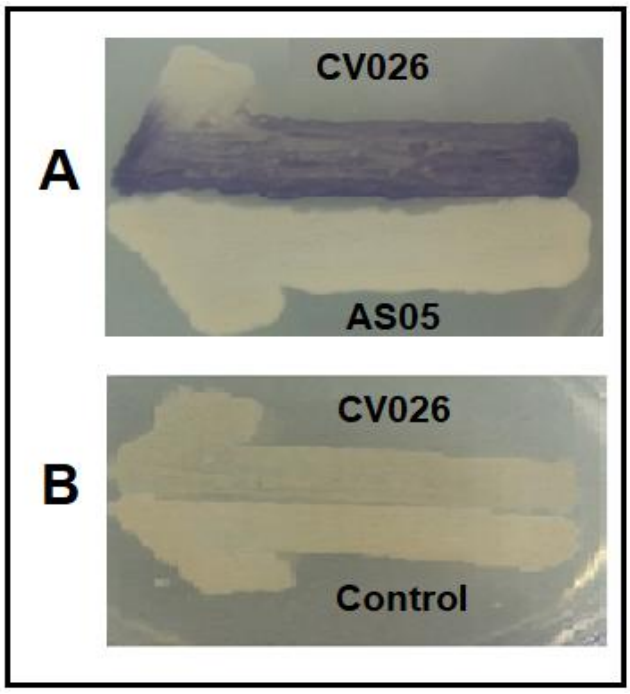

Figure 2. Results of AHLs production by agar plate bioassay. "A" shows positive results for AHL production by *V. alginolyticus* AS05 detected of CV026 biosensor. While "B" indicates negative control with no any change in color.

Discussion.

Marine environments, including aquatic and coastal environments, are highly prevalent of marine bacteria with the highest levels of *Vibrio* species (Wang *et al*., 2019). Marine-originated Vibrios are of great importance due to their fast growth rate, highly motile, halophilic and enhanced extracellular enzymatic activities (Zhang *et al*., 2018; Rizzo *et al*., 2016). Marine *Vibrio* species may use a density-dependent mechanism called quorum sensing (QS) to play a vital role in various remineralizing types of large organic complexes. QS is a conversation system found in a broad range of bacterial species. It is involved in regulating specific genes to carry out physiological and biological functions. Though QS has been studied well in several *Vibrio* species, however, the type of QS signaling molecules and its role in *V. alginolyticus* is unclear. This study was carried to detect

and identify the AHL-based QS signalling molecules produced by the marine bacterium *V. alginolyticus* AS05.

V. alginolyticus AS05 was isolated from marine water collected from the Arabian Sea, Karachi, Pakistan. Principally, marine medium Zobell-2216 was used for isolation and pure culturing of AS05 bacterial strain. The Identification was achieved based on Gram's staining and biochemical tests, followed by molecular Identification based on 16S rRNA analysis. The obtained 16S gene sequences were blasted and submitted NCB-GenBank with accession number OQ130030. Moreover, screening of AS05 bacterial strain for producing AHLs produced highly positive reactions producing violacein pigment by CV026 biosensor strain. Since biosensor CV026 can not produce its own AHL molecules, however, this bacterium can sense and express certain genes to regulate the production of violacein pigment only in the presence of the external source of AHL molecules. The discovery of QS as a communication system resulted in recognition of several types of biosensors particularly used for detecting AHL signalling molecules produced by Gram-negative bacteria. Among the biosensors, *Chromobacterium violaceum* CV026 (CviI mutant of *chromobacterium* strain ATCC 31532) is the most important biosensor strain used widely to detect AHL signalling molecules (Harrison & Soby, 2020). *C. violaceum* species was first reported in 1981 (Gills & Logan, 2015).

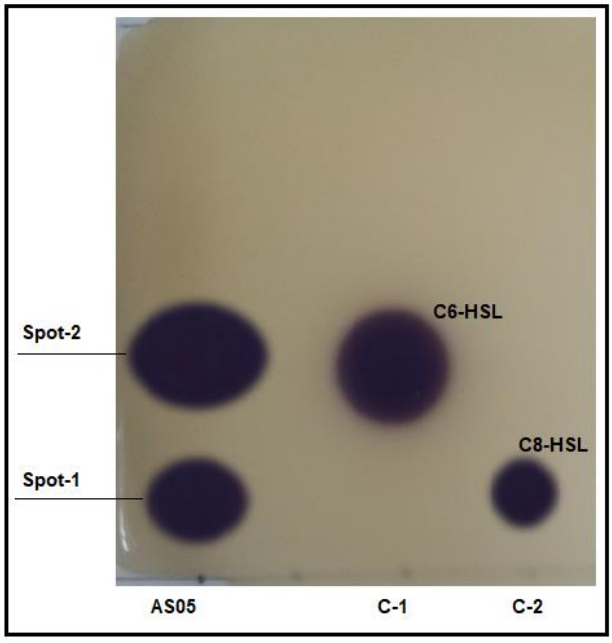

Figure 3. Results of TLC analysis, AS05 bacterial strain shows production of two different spots (Spot-1 & Spot-2). While, C-1 indicates AHL control-C-6HSL and C-2 AHL control of C-8HSL.

TLC analysis for the Identification of AHLs produced by *V. alginolyticus* AS05 strain revealed the Identification of two types of AHL signalling molecules such as C6-HSL and C8-HSL. Several bacterial species may produce more than one type AHL molecule (Jatt *et al*., 2015). This study also reveals two types of AHL molecules produced by AS05 bacterial strain. Moreover, it is also speculated that *V. alginolyticus* strain AS05 isolated from marine water may produce more than two AHL types and could not be detected by the currently applied biosensor strain. This could be possible to use more biosensor bacterial strains to confirm if this marine strain produces other types of AHLs.

Generally, marine-origin bacterial species are the most important in producing large numbers of extracellular enzymes and play a vital role in biological cycling, particularly the carbon cycle, through the remineralization of complex organic compounds in marine environments. Moreover, the production of different QS signalling molecules in marine bacteria may enhance the production of extracellular enzymes and other secondary metabolites.

Conclusion: This study has revealed the detection and Identification of two types of AHL-based QS signalling molecules such as C6-HSL and C8-HSL by *V. alginolyticus* strain AS05 isolated from marine water of Arabian Sea, Karachi, Pakistan. Few *V. alginolyticus* strains have been known as opportunistic pathogens in fish and other marine organisms. However, most of the strains of marine origin are non-pathogenic and may play a crucial role in the remineralization and transformation of organic compounds and may influence biological cycling, particularly the carbon cycle.

FUNDING STATEMENT

The authors declare they have no conflicts of interest to report regarding the present study.

CONFLICT OF INTEREST

The Authors declare that they have no conflicts of interest to report regarding the present study.

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