INVESTIGATION OF *N*-ACYL HOMOSERINE LACTONE-BASED QUORUM-SENSING SYSTEM AND ALIGINATE LYASE ACTIVITY IN MARINE BACTERIAL SPECIES OF *GRIMONTIA MARINA* AS01 AND *ALTEROMONAS MACLEODII* AS02

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ABSTRACT: Production, detection, and reaction to external signaling molecules are essential steps in quorum sensing (QS) process. Through the use of QS, bacterial communities may synchronize their responses to shifts in the density and diversity of their vicinal neighbors. QS also play an important role in regulating enzmyatic activities among marine bacteria. The aim of the present study was to detect and identify *N*- acyl homoserine lactones (AHLs) based QS signaling molecules and the possible influence on alginate lyase in marine bacterial isolates of *Grimontia marina* AS01 and *Alteromonas macleodi* AS02. Marine water samples were collected from Arabian Sea, Karchi Pakistan, following the standard collection methods. Bacterial strains were isolated and pure cultured using Zobell 2216 marine medium. Molecular identification was achieved based on 16S rRNA gene analysis. Screening for AHLs was achieved using *Agrobacterium tumefaciens* A136 as a biosensor. Based on 16S rRNA analysis, the bacterial strains were identified as *Grimontia marina* strain AS01 (OP143768) and *Alteromonas macleodii* strain AS02 (OP143769). Cross-feeding bioassay revealed the positive reactions for the production of AHLs. Reversed phase-TLC analysis showed the identification of C6-HSL produced by *G. marina* AS01 and 3OXO-C6-HSL by *A. macleodii* AS02 strain. Moreover, QS inhibitor AiiA protein reduced the production of alginate lyase in *A. macleodi* AS02, while no effect was observed in *G. marina* AS01. These results substantiate the involvement of QS system in regulating alginate lyase activity in *A. macleodii* AS02. QS in marine bacteria may involve in hydrolysis of complex organic matter in marine environment.

Key word: Arabian sea, Acyl homoserine lactone, Bioassay, Reporter strain and Quorum-sensing

INTRODUCTION

Quorum sensing (QS) is a mechanism of communication among the bacterial communities (S. Wang et al., 2020). Bacteria may secrete several types of chemicals as QS signaling molecules facilitating communication among individual bacterial species leading to coordinating group performance (Jatt et al., 2015). Bacterial species have ability to sense changes in cellular density of self or even other bacterial community in their surroundings via QS signaling molecules (Jatt, 2021). When signaling molecules reach at threshold concentration, bacterial cells may coordinate expression of certain genes to carry out regulatory mechanism and biological functions. The bacterial species have distinct chemical signaling molecules and may produce more than a single signaling molecule. Among various kinds of QS signaling molecules, *N*-acyl homoserine lactones (AHLs) are found as main conversation signaling molecules in Gram-negative bacteria, and the modified oligopeptides (AIPs) are known as QS signaling molecules found in Gram-positive bacteria. There are certain other non-species-specific signaling molecules called as auto-inducer-2 (AI2), which are found in Gram-negative as well as in Gram-positive bacterial groups and are employed for intraspecific and interspecific conversation system (M. Wang et al., 2020).

The Ocean is considered as the robust reservoir for biogeochemical cycling mainly carbon cycle, which involved in mitigating the impacts of global warming (Hmelo et al., 2011; Jatt et al., 2015). The bacterial communities in Ocean may involve in transformation of nutrients through different biogeochemical cycles (Hmelo et al., 2011). Marine bacteria are the major source of organisms involved in hydrolysis of complex organic compounds into smaller organic compounds and provide nutrient source to other marine organisms. Marine bacteria produce hydrolytic enzymes in surrounding environment. These extracellular hydrolytic enzymes are considered as the main components widely utilized in degradation of

complex compounds. In marine ecosystem, heterotrophic bacteria are highly active in transformation and remineralization of large organic particles via certain extracellular enzymes. Enzymatic activities in marine bacteria consists of various environmental factors, micro-environment, ecosystem and predominantly by QS systems. Previous studies have reported QS system in large numbers of bacterial species, which is utilized to regulate expression of certain genes for important biological activities. For instance, *Agrobacterium tumefaciens* has been reported to use QS system to organize conjugational transmission of Ti plasmids (Fuqua & Winans, 1994; Jones et al., 1993), *Erwinia carotovora* use QS system to regulate induction of virulent extracellular enzymes (Beck von Bodman & Farrand, 1995), *Pantoea stewartii* regulate capsular polysaccharides production through AHL based QS system (Ploug et al., 1999). Marine bacterium *Pantoea ananatis* has been reported to regulate extracellular phosphatase enzyme (Jatt et al., 2015). QS system in bacteria has been reported to regulate gene expression patterns to control wide range of products globally. Marine bacteria via QS signaling molecules regulate specific genes responsible for various biological functions, e.g., antibiosis, production of bioluminescence, development of biofilm and virulence (Dobretsov et al., 2009). QS system was first reported in *Vibrio fischeri* a marine bacterium associated with light organ of Sepiolid squid, in which light bioluminescence was regulated by QS system (Eberhard et al., 1981; Gram et al., 2002).

Marine bacterial isolates such as *Grimontia marina* and *Alteromonas macleodii* are proteobacterial species. *G. marina* is a Gamma-proteobacteria placed under *Vibrionales* order and *Vibrionaceae* family (Mahmoud et al., 2021). *A. macleodii* is also a Gamma-proteobacterial species, placed under order of *Alteromonadales* and family *Alteromonadaceae* (Mehta et al., 2014). Proteobacteria is known as the largest phylum consisting of Gram-negative bacterial species. Proteobacterial group consists of six different classes and few of them are represented by the Greek letters start from α (alpha) to ε (epsilon) and remaining are named as Oligoflexia and Acidithiobacillia. Proteobacterial group has been reported in numerous environments and the major role is found in biogeochemical cycling (Nair et al., 2017). A metagenomics study which was carried out in sediments of the Arabic Sea, Proteobacteria was recognized as a predominant phylum with 62.8% of the library (Stincone & Brandelli, 2020). Mainly, among the other bacterial groups, the Proteobacterial group is found at highest levels in Ocean environment (Kai et al., 2017; Wu et al., 2013).

The present study was carried out to detect and identify chemical signaling molecules in *G. marina* strain AS01 and *A. macleodii* strain AS02 and to investigate influence of AHLs and QS inhibitor AiiA protein on alginate lyase production. The present study represents AHL mediated QS signals and its role in alginate lyase activity in marine bacteria.

MATERIALS AND METHODS

Collection of marine samples: Marine water samples were collected from the Arabian Sea, Karachi, Pakistan. Sterile wide-mouth glass bottles were used for the collection of surface marine water samples at the depth of 1-2 m. Marine water samples were obtained from three different stations, e.g., HKC, SKC and AKC. Marine water samples were processed within 3 h of the collection time, if delayed the samples were kept at 4^0C until processed.

Isolation and identification of marine bacteria: Marine water samples were processed to isolate bacteria using Zobell marine 2216 medium (Jatt et al., 2015). Bacteria were grown at 28 ^{0}C for 24-48 h. The obtained colonies with unique colonial characteristics were further transferred several times to fresh culture media to obtain pure culture colonies. After preliminary identification based on biochemical tests, the pure cultured strains were further identified based on 16S rRNA gene sequencing. Briefly, the extraction of DNA from marine bacterial isolates was carried out using a commercial kit as per manufacturer's instruction (Thermo Fisher). The primers used for amplification of 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. Gene sequences were carried out by Macrogen Inc. (Korea). The obtained results of DNA sequences were blasted against the NCBI database of GenBank (www.ncbi.nlm.nih.gov). MEGA, version 7.0 was used to construct a phylogenetic tree based on neighbor joining method.

Screening of bacterial isolates for signaling production: *A. tumefaciens* A136 was applied as a reporter strain to screen bacterial species for the production of chemical signaling molecules particularly AHLs through cross-feeding agar plate bioassay (Jatt et al., 2015) . Briefly, test bacterial strain was grown along with reporter strain at a distance of 1 cm on LB agar (Oxide^{TM}) medium (Morgan-Sagastume et al., 2005) . While, 0.5% of X-gal was used as a chromogenic substance. The bacterial strains were grown for 24-48 h at 28 ^{0}C. The cross-feeding bioassay agar plates were observed for the blue coloration after transcription of β-galactosidase enzyme by A136 biosensor in presence of AHL signaling molecules. A known AHL negative bacterial strain was used as a negative control.

Extraction of AHL signaling molecules: Marine bacterial isolates were grown in Zobell 2216 broth (200

mL) for 24-48 h at 28 ^{0}C with shaking (200 rpm). Supernatants were obtained by centrifugation of cell suspension at 12,000 rpm with 4 ^{0}C. Supernatants were used for the extraction of AHLs. Extraction was obtained by mixing supernatants with equal volume of ethyl acetate as per method described by Hmelo et al. (Hmelo et al., 2011). Briefly, the extract (organic phase) was processed to obtain dried material at 30^{0}C using rotatory evaporator. The dried material was dissolved with 0.5-1 mLmethanol and stored at -20^{0}C until further use.

TLC analysis: The identification of AHL-signaling molecules produced by marine bacterial strains *G. marina* AS01 and *A. macleodii* AS02 was performed using reversed phase TLC analysis. TLC plate was developed as described by Jatt et al. (Jatt et al., 2015). In brief, 1µL of the ethyl extract was applied on TLC plate using capillary tubes along with specific AHL standards. TLC plate was developed with methanol-millipore water (60:40 v/v) (Zhang et al., 2020). Subsequently, the plate was air-dried and overlaid with semi solid LB medium (0.5% agar) supplemented with reporter strain A136 and 0.5% of X-gal along with specific antibiotics. RP-TLC plate was incubated at 28^{0}C for 24 h. The plate was observed for the production of purple blue spots. Mainly, C6-HSL, 3OXO-C6-HSL and C8-HSL were used as AHL standards.

Screening for the production of alginate lyase and QS inhibition: Marine bacterial isolates of *G. Marina* AS01 and *A. Macleodi* AS02 were screened for production of alginate lyase enzyme by growing them in nutrient agar plates supplemented with 0.5% sodium alginate. After 2-3 days of incubation at 28 ^{0}C, the plates were added with approximately 5.0 mL of Lugol's iodine solution. Clear zones produced around the bacterial colonies indicated the positive reactions for alginate lyase activity (Li et al., 2011).

The enzymatic assay was obtained as per method described by Somogi (Somogyi, 1926). One unit of enzyme was set as the amount of enzyme required for the production of reducing sugar (glucose) per min, and the lyase activity was confirmed by measuring absorbance at 235 nm.

The effect of AHL based QS system on alginate lyase activity in marine isolates was achieved by disruption of AHL signaling molecules through addition of QS inhibitor AiiA protein in growth media. The inhibition of AHL based QS system was confirmed by A136 reporter strain. The results of enzymatic activity of the strains with AHL QS system (control) were compared with enzymatic activity of the strains without AHL QS system.

RESULTS

Isolation and identification of marine bacteria: The two marine bacterial strains named as AS01 and AS02 were isolated and pure cultured from the marine samples which were collected from Arabian Sea, Karachi, Pakistan. After basic identification based on Gram's staining and biochemical tests, the strains were further identified based 16S rRNA gene analysis. The NCBI-blast results of the 16S rRNA gene sequences revealed the identification of these two marine bacterial strains as *Grimontia marina* strain AS01 with NCBI-GenBank accession number OP143768, and *Alteromonas macleodii* strain AS02 with accession number OP143769. Both bacterial strains belonged to the class of *Gammaproteobacteria*.

Based on the obtained 16S rRNA sequences, a phylogeny tree was constructed for the strain AS01 and AS02 by neighbor joining method. AS01 strain showed the relatedness to gene sequences of the genus *Grimontia*, and AS02 strain indicated relatedness to the genus *Alteromonas* of the class *Gammaproteobacteria* (Fig. 1).

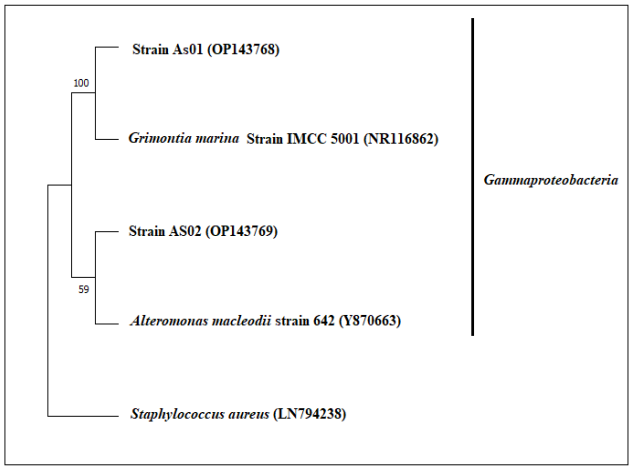
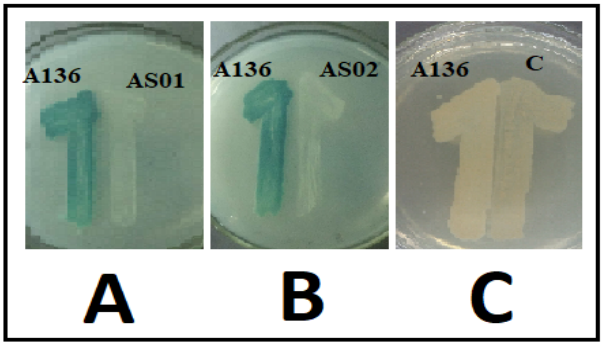


Figure 1. A phylogenetic tree was constructed using neighbor joining method of the marine isolates of *G. marina* strain AS01 and *A. macleodii* strain AS02 based on gene sequences blasted at NCBI GenBank database. Staphylococcus aureus (LN794238) was used as outgroup.

Screening of bacterial isolates for AHL signaling production: Screening of the *G. marina* AS01 and *A. macleodii* AS02 marine bacterial strains for AHL production using cross-feeding agar plate bioassay revealed the positive reactions for both bacterial strains. A clear purple blue coloration was observed in agar plate bioassay with A136 reporter strain in LB-agar medium. Purple blue coloration was due to the transcription of β-galactosidase enzyme by A136 biosensor in the presence of AHL signaling molecules. Moreover, a negative control

consists of known AHL negative bacterial strain indicated no production of AHL signaling molecules (Fig. 2).

Figure 2. Results of cross-feeding bioassay using A136 as a reporter strain. Plate "A" shows the production of AHL



signaling molecules by G. marina strain AS01, Plate "B" indicates AHLs produced by A. macleodii strain AS02 and Plate "C" represents negative control.

Identification of AHL signaling molecules: Reversed phase TLC plate was used for the identification of AHL signaling molecules. RP-TLC analysis revealed a single AHL molecule produced by each AS01 and AS02 bacterial strain. Clear spots of AHL molecules produced by marine isolates were identified as C6-HSL signaling molecule in *G. marina* AS01 and 3OXO-C6-HSL in *A. macleodii* AS02 based on size and *Rf*-value matched with AHL standards (Fig. 3).

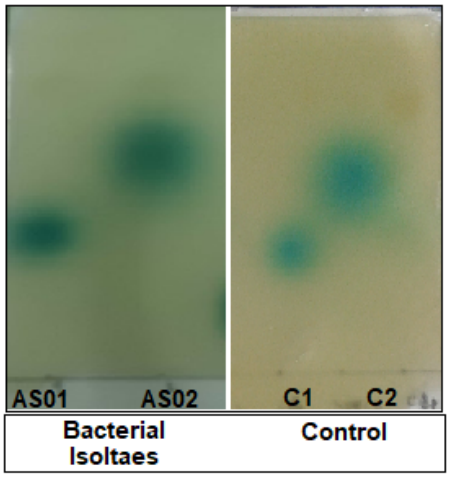
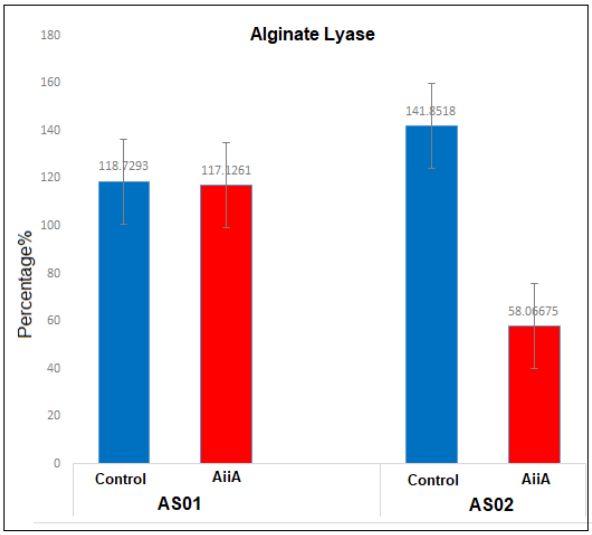


Figure 3. Results of the identification of AHLs by RP-TLC analysis. "AS01" lane shows the separation of AHL produced by *G. marina* strain AS01, "AS02" lane indicates AHL produced by *A. macleodii* strain AS02. Whereas, C1 shows AHL standard of C6-HSL and C2 indicates AHL standard of 3OXO-C6-HSL.

Production of alginate lyase and effect of QS inhibitor protein (AiiA): Screening of marine isolates of AS01 and AS02 showed the positive reactions for production of extracellular alginate lyase. Enzymatic assay with addition of QS inhibitor protein (AiiA) in growth medium revealed no effect on alginate lyase activity in *G. marina* AS01. However, AiiA protein reduced the production of alginate lyase in marine isolate of *A. macleodii* AS02 (58.066 U/mL) as compared to enzymatic activity (141.851 U/mL) obtained on the growth medium as control without disruption of AHL based QS system (Fig. 4).

Figure 4. Results of the influence of QS inhibitor showed no effect on alginate lyase activity in *G. marina* AS01. However, AiiA adversely reduced the production of



alginate lyase in *A. macleodii* AS02 (58.066 U/ml) as compared to enzymatic activity in control (141.851 U/mL).

DISCUSSION

Marine bacterial communities are found as abundant and diverse microbial communities throughout the Oceans. Most of these bacterial species form biofilms and live in microenvironments attached with particulate organic matters. Marine bacteria may use QS system to speed up transformation and remineralization of the complex organic matter in marine environments. Quorum sensing (QS) is known as a conversation mechanism used by bacterial species to work for a coordinated performance particularly play critical role in nutrient cycling including carbon cycle. QS is a language of communication among bacterial communities via certain signaling molecules. This study was carried out to investigate the detection and identification of AHL based QS signaling molecules in *G. marina* AS01 and *A. macleodii* AS02 marine bacterial species isolated from the coastal areas of Arabian Sea, Karachi, Pakistan, and to investigate the influence of QS system and QS inhibitor AiiA protein on alginate lyase activity. The results of this study revealed the positive

reactions in agar plate bioassay by *G. marina* AS01 and *A. macleodii* AS02 marine isolates in presence of *A.tumefaciens* A136 biosensor strain. A136 bacterial strain is a mutant strain and itself cannot produce AHL signaling molecules, however, can sense these signaling molecules produced by adjacent bacteria and activate the transcription of β-galactosidase enzyme in presence of chromogenic substance X-gal, which is converted into purple blue coloration after hydrolysis by β-galactosidase. The identification of AHLs produced by marine bacterial isolates was confirmed by reversed phase TLC. TLC results showed the identification of C6-HSL signaling molecule produced by *G. marina* AS01and 3OXO-C6-HSL was produced by *A. macleodii* AS02. Mainly, several types of QS biosensors have been reported and detect both short and long chain signaling molecules. It is speculated that these marine strains may also produce some other types of QS signaling molecules and could not be recognized by the A136 biosensor bacterial strain used in this study.

In general, 16S rRNA gene analysis was used to identify bacterial isolates and the obtained sequences were blasted with NCBI GenBank database. Genus *Grimontia* is a Gram-negative, rod shaped, aerobic and a motile member of the family of *Vibrionaceae* of the class Gammaproteobacteria. The genus *Alteromonas* is also a Gram-negative with rod-shaped morphology, motile and aerobic in nature belongs to *Alteromonadacea* family of the class *Gammaproteobacteria*. Approximately 70% of the earth crust consists of marine environment with different habitats. Marine bacteria are of great importance in producing secondary metabolites and produce broad range of hydrolytic enzymes involved in transformation and remineralization of organic materials (Al-Saari et al., 2015). Marine microbes are the important source for carbon cycle by playing role organic matter remineralization (Zhang et al., 2018). The class of *Gammaroteobacteria* has been found as the best bacterial class among the marine bacterial groups as the members of this class play great role in up taking and remineralization of various nutrient components such as carbon, phosphorus, and nitrogen components.

Screening of the marine isolates for the production of extracellular alginate lyase showed positive results for the production of alginate lyase by both of the AS01 and AS02 bacterial strains. The enzymatic assay with addition of QS inhibitor protein highly reduced the alginate lyase activity in *A. macleodii* AS02, while no effect found on enzymatic activity in *G. marina* AS01. Since, *A. macleodii* AS02 is a QS positive marine bacterial strain and showed the enhanced production of alginate lyase when grown as control without disruption of QS system (141.851 U/mL). However, after inhibition of AHL based QS system by AiiA inhibitor protein, the enzymatic activity was reduced (58.066 U/ml). These results speculate that AHL based QS system may involve in regulation of extracellular alginate lyase activity in *A. macleodii* AS02 bacterial strain isolated from Arabian Sea, Karachi, Pakistan. However, further study based on gene level may confirm the specific AHL regulatory genes involved in regulation of the extracellular alginate lyase. QS is a system of communication through which bacteria form a threshold density and thus marine bacteria with QS system may involve in degradation of large complex organic and inorganic materials by producing various extracellular enzymes including alginate lyase. In this study, marine bacterial isolates showed the production of AHL signaling molecules and may utilize these AHL signaling molecules for transformation and remineralization of organic compounds in marine environment. Previously, AH mediated QS system has been reported in marine bacteria from different marine habitats in distinct geographical regions worldwide (Gram et al., 2002; Hmelo et al., 2011; Jatt et al., 2015), however till now no such a study has been carried in Arabian Sea, Karachi, Pakistan. This study will be a milestone to propose further studies regarding QS and their major role in other marine bacterial species in Arabian Sea, Karachi, Pakistan.

Conclusions: The present study has revealed the production of AHL-based QS signaling molecules produced by *G. marina* AS01 and *A. macleodii* AS02 isolated from Arabian Sea, Karachi, Pakistan. Moreover, the results revealed the involvement of AHL based QS system and enhanced the production of extracellular alginate lyase activity in *A. macleodii* AS02, while QS inhibitor AiiA protein extremely reduced the production of alginate lyase. Marine bacteria have a great value in transformation and remineralization of complex organic materials. These bacteria via QS signaling molecules may also play a crucial role in biogeochemical cycling particularly carbon cycle. The increase in temperature globally may pose a serious threat for living organisms including human beings, in this situation marine microbes with QS system may play a great role to overcome this threat of global warming.

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CONFLICT OF INTEREST

The authors declare no conflict of interest to report regarding this study.

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