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OPTIMIZATION OF GROWTH CONDITIONS AND PURIFICATION OF QUORUM SENSING SIGNAL MOLECULES PRODUCED BY *PSEUDOMONAS AERUGINOSA*

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ABSTRACT

Expression of virulence factors and biofilm formation in *P.aeruginosa* is associated with production of quorum sensing signal molecules (QSSMs) belonging to the class of acyl homoserine lactones (AHLs). Besides regulating virulence factors, these molecules also interact with eukaryotic cells and can modulate immune response. In most of the studies, synthetic QSSMs have been employed as therapeutic agents. Although 98-99% homology exist between synthetic and natural AHLs but the biological response against either may in fact be different in natural host. In the present study, under optimized growth conditions there is increase in the production of natural AHLs. Extracted AHLs were detected using C18 reverse phase analytical thin layer chromatography (RP TLC) by employing *Agrobacterium tumefaciens* as biosensor strain. Preparative TLC assay was successfully performed to purify the 3oxo-C12-HSL and 3-oxo-C10-HSL. This study provides easy and simple method for purification of natural AHLs under optimized conditions, hence these molecules can be employed for future research involving control of infections associated with *P.aeruginosa.*

KEY WORDS: **Quorum Sensing; AHLs; TLC; *P. aeruginosa***

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INTRODUCTION

Pseudomonas aeruginosa, an extremely versatile opportunistic pathogen is responsible for a number of infections of eyes [1], burns, wounds [2], respiratory [3] and urinary tract [4]. Quorum sensing (QS) plays a very important role in the pathogenesis of *Pseudomonas aeruginosa* infections. Recent research in quorum-sensing signal molecules (QSSM) has demonstrated that acyl homoserine lactones (AHLs) regulate the production of virulence factors, antibiotic resistance, and biofilm development in *P. aeruginosa* [5,6,7]. Quorum sensing gene network consist of two distinctive and interdependent systems, the *las*I-*las*R and *rhl*I-*rhl*R [6]. *las*I-*las*R system regulate the production of *N*-(3-oxododecanoyl) homoserine lactone (3O-C12-HSL) and *rhl*I-*rhl*R system regulate the production of *N*-butyryl homoserine lactone (C4-HSL). These molecules also interact with eukaryotic cells and modulate immune responses [8, 9]. QSSM have been characterized as important virulence factors [10] and can be employed as candidates for providing protection against infections caused by *P.aeruginosa*. More recently, Miyari *et al.,*[11] have shown synthetic 3-oxododecanoyl-L-homoserine as a promising potential vaccine agent for blocking bacterial quorum-sensing system in respiratory tract infection. Although various studies have reported 98–99% homology between synthetic acyl homoserine lactones (AHLs) and natural AHLs but the biological response against synthetic AHLs and natural AHLs may in-fact be different in natural hosts due to slight difference in structure. This has been depicted in study from our lab where it was demonstrated that natural AHLs were found to be more immunosuppressive than that of synthetic AHLs.[9, 12, 13] The chiral configuration of synthetic AHLs may also be different from natural AHLs. For example, all natural acyl-HSLs are (S) stereoisomer while synthetic AHLs are (R) stereoisomer. [12, 13] In most of the studies, only synthetic AHLs have been employed to evaluate their biological activities. Natural AHLs have not yet been employed in such studies since these molecules are produced in very low amount during culture and extraction with ultra sensitive methods such as HPLC becomes quite expensive.[11, 13-14] Moreover; natural AHLs have been observed to be more biologically active than synthetic AHLs.[9, 13] Simultaneously, their expression is also sensitive to natural environmental conditions.[15] Since it has been observed that natural AHLs are more biologically active and their extraction with HPLC from culture is costly. Therefore, keeping this in view, present study was undertaken to optimize the environmental growth conditions for optimal production of natural AHLs and to purify AHLs using a simple, easy, rapid, cheap and sensitive preparative TLC assay.

MATERIALS AND METHODS

Bacterial strains

Pseudomonas aeruginosa PAO1 standard strain was maintained as nutrient agar stabs kept at 4^0C. *E. coli* MG4 was used as reporter strain for the detection and quantification of AHLs and was maintained on Luria Bertani agar slants containing ampicillin (100µg/ml). *Agrobacterium tumefaciens* A136 was used for the detection of AHLs in preparative TLC and was maintained on LB agar with spectinomycin (50µg/ml) and tetracycline (4.5 µg /ml).

Cross feeding assay for AHL detection

Luria agar plates covered with 40 µl of X-Gal (20 mg/ml) were streaked 1 cm apart with reporter strains *E. coli* MG4 and culture to be tested. AHLs produced and diffused through the agar results in appearance of blue colour in the reporter strain.

Optimization of environmental growth conditions for QSSM production:

Inoculum preparation

100 µl of the 6 hour old culture of *P.aeruginosa* PAO1 (used as reference strain for the extraction of AHLs) was used to inoculate 100ml of luria broth and was grown for 18-20 hours at 37^0C under shaking

conditions (150 rpm) with optical density adjusted to 0.4 at 540 nm.

a) ***Presence of Glucose:*** Sterile M9 medium supplemented with different concentration of glucose (0.1%, 0.2%, 0.3%, 0.4% and 0.5%) were inoculated and incubated at 37^0C for 24 hrs.

b) ***Presence of Lactose*****:** Sterile M9 medium supplemented with different concentration of lactose (0.5%, 1.0%, and 2.0%) were inoculated and incubated at 37^0C for 24 hrs.

c) ***Iron:*** Sterile M9 medium with (50μM, 100μM, 150μM and 200μM) or without Iron supplementation were inoculated and incubated at 37^0C for 24 hrs.

d) ***Variable pH conditions:*** Sterile M9 medium with pH adjusted to 6.0, 7.0, and 8.0 (using 1N NaOH and 0.1 N HCl) were inoculated and incubated at 37^0C for 24 hrs.

e) ***Incubation temperature:*** Sterile M9 medium inoculated and incubated at different incubation temperatures viz.: 37 and 42º C for overnight respectively.

f) ***Aerobic/Anaerobic conditions:*** Sterile M9 medium inoculated and incubated at aerobic and anaerobic conditions at 37º C for overnight respectively.

Extraction of AHLs

Pseudomonas aeruginosa PAO1 was grown overnight under optimized environmental growth conditions for 14-16 hours at 37^0C. Overnight grown culture supernatants (10ml) were extracted twice with equal volume of acidified ethyl acetate. Pooled extracts were dried over anhydrous magnesium sulphate and were evaporated to dryness. Residues were resuspended in 50-100μl of HPLC grade ethyl acetate.

AHLs quantification

Culture supernatant was extracted from overnight grown culture for β-galactosidase activity. Reporter culture was diluted 1:1 in Z buffer and assayed for β-galactosidase activity by using *o*-nitrophenyl-D-galactopyranoside (ONPG) as a substrate as described by Miller.

Analytical thin layer chromatography (TLC)

To evaluate the profiles of AHLs, TLC was carried out according to the method of Shaw *et al*.,[16]. Briefly, 4μl of sample was applied to the silica gel C18RP TLC plates (Merck, Germany). The chromatograms were developed with methanol: water (60:40 v/v). Once the solvent front migrated to within 2cm of the top, plates were air dried. Plates were then overlaid with a thin film of agar seeded with biosensor strain *A. tumifaciens* A136 supplemented with X-Gal (65μg/ml). All experiments were done in triplicates. AHLs were identified by comparing the retention factor of synthetic standard AHLs and test AHL spots.

Preparative TLC

Briefly, 100μl sample was spotted onto a reporter TLC plate (C18RP Silica gel plates, Merck, Germany) along with a set of standards. The C18 matrix in the regions corresponding to the compound to be analyzed were scraped off, combined, and extracted three times with 1–2ml volumes of HPLC-grade ethyl acetate. The combined extracts were clarified by centrifugation and passed through a fine sintered glass filter. The filtrates were dried and the residue was re-dissolved in HPLC-grade ethyl acetate.

Statistical Analysis

All the experiments were repeated three times to reproduce the results and level of significance was calculated using student *t* test by Graphpad Prism 11.05 software.

RESULTS AND DISCUSSION

Pseudomonas aeruginosa possesses the metabolic versatility. It is resistant to high concentration of salts, dyes, weak antiseptics and common antibiotics. These natural properties of bacterium undoubtedly contribute to its ecological success as an opportunistic pathogen. They also help to explain the ubiquitous nature of the organism

and its prominence as nosocomial pathogen.[17] Interaction of a pathogen with the host environmental conditions may alter the magnitude of expression of QSSM. The present study aimed to determine the effect of environment growth conditions for maximum production of AHL molecules and further to purify these molecules.

Optimization of environmental growth conditions

P.aeruginosa PAO1 is known to produce AHL molecules. These quorum sensing signal molecules act as virulence factors per se and have been suggested as futuristic immunoprophylatic targets for control of infections caused by *P.aeruginosa.*[11] Naturally produced AHLs should be employed for such purpose but these are produced in low quantity. Therefore, we tried to optimize environmental growth conditions for increased AHL production (Table-1). Sugar plays an important role in the growth of *P.aeruginosa*. It has been proposed, that at very high concentrations of glucose, catabolite repression takes over which is mediated by cAMP and CRP (cAMP receptor protein)[18]. Under this mechanism, glucose affects the expression of genes located throughout the genome including those involved in biofilm formation and production of AHL molecules. In line with these studies, different concentrations of glucose ranging from 0.1-0.5% were used and results of the present study showed that the amount of AHL produced by PAO1 in M9 medium supplemented with 0.3% glucose was significantly ($p<0.001$) more (128.4 MU) than that formed in very high and very low concentration of glucose. *P.aeruginosa* is lactose fermenting bacteria so besides glucose, concentration of lactose may also influence its growth during infection. Sharma *et al.,*[19] showed enhanced production of siderophore in the presence of lactose thus helping in the establishment of infection. In the present study the amount of quorum sensing molecules (AHLs) produced in presence of different concentration of lactose ranging from 0.5-2.0% was investigated. Maximum AHL production was observed with 0.5% lactose i.e. 134.9 MU and inhibition of AHL production was observed with 2% lactose. Besides glucose and lactose iron also plays a key regulatory role during infection. In recent study by Musk *et al.,*[20], effect of iron concentration on mature biofilm formation was observed. Bollinger *et al.,*[21] also linked iron stress response to quorum sensing circuitry. These workers showed overall effect of iron on *LasI* expression in stationary phase cells of *P.aeruginosa* indicating a clear link between quorum sensing genes involved in iron regulation in *P.aeruginosa*. In the present study, a defined medium (M9 medium) was used to create iron varying conditions by using different concentrations of $FeCl_3$ ranging from 0 to 200 µM. Production of AHL molecules by *P.aeruginosa* under these conditions was assessed. Highest production of AHL (134.9MU) was observed at a concentration of 150µM of iron. Lowest AHL production was observed in absence of iron and 50µM of iron.

Other parameters like pH of the body fluids, metabolic intermediates, the level of final catabolic products and body temperature also has an important role in pathogenesis of infection. Optimum pH is necessary for the growth of *P.aeruginosa.* Variable pH range exists under diseased inflammatory conditions which may play an important role in expression of certain factors. Body temperature varies due to physical activity, environmental conditions and infection. All these mentioned conditions are encountered by pathogens during infection and the regulation of their biological function gets significantly influenced. Yates *et al.,*[14] have shown that AHLs showed highest activity at a temperature similar to that found in physiological conditions in mammalian tissue fluid that is, 37^0C. In line with these studies, we have also assessed AHL production under variable environmental conditions like temperature, pH, aerobic and anaerobic conditions. It was seen that PAO1 produced significantly higher ($p<0.001$) amounts (124.12MU) of AHL at 37^0C incubation temperature as compared to other temperatures of incubation (30^0C, 42^0C).

Results obtained in present study also showed significantly ($p<0.001$) higher production of AHL molecules (136.49MU) at pH 7.0 as compared to acidic or alkaline pH ranges. Besides temperature and pH, Oxygen also plays a key role in pathogen growth. We evaluated the effect of aerobic and anaerobic conditions for the production of AHL molecules. Under aerobic condition, PAO1 showed highest AHL production (141.05MU) and low AHL production was observed in anaerobic conditions (76.0MU). These results indicated that the anaerobic conditions are not suitable for quorum sensing signal molecule production and may interfere with quorum sensing system. When *P.aeruginosa* was grown under the above mentioned optimized growth conditions, significantly increased AHL production (178.67MU Fig.1a, b) was observed. All the conditions synergistically enhanced AHL production.

Table 1

Effect of environmental growth conditions on AHL production by P.aeruginosa. Each value represents the mean value of 3 independent experiments± standard deviation

S.No.	Growth conditions		AHL production (MU)
1.	Glucose (%)	0.1	119.00 ±1.1
		0.2	122.10 ±1.5
		0.3	128.40 ±1.4
		0.4	123.10 ±2.1
		0.5	117.10 ±1.6
2.	Lactose (%)	0.5	134.90 ± 1.4
		1.0	116.80 ± 2.5
		2.0	126.90 ± 2.4
3.	Iron (µM)	0	104.20 ±2.8
		50	113.62 ±1.9
		100	130.90 ±1.9
		150	134.90 ±1.4
		200	118.90 ±1.8
4.	pH	5.0	127.40 ±1.2
		6.0	130.70 ± 0.7
		7.0	136.49 ± 1.4
		8.0	130.10 ±1.5
5.	Temperature (^{0}C)	37.0	124.12 ± 1.4
		42.0	120.45 ±1.2
6.	Oxygen (O_2)	Presence of O_2	141.05 ± 1.4
		Absence of O_2	76.00 ± 1.4

Detection and Purification of QSSM

Next step was to detect AHL molecules from the ethyl acetate extract of cell free culture supernatant obtained from *P.aeruginosa* grown under optimized growth conditions. C-18 reverse phase TLC plates were used for this purpose. Samples were chromatographed on TLC plates, developed with methanol/water (60:40, vol/vol). The AHL molecules were detected by overlaying the plate with biosensor strain *A. tumefaciens* 136. This strain does not produce its own signal molecule, but it can induce the *traG*::*lacZ* reporter when supplied with an exogenous active AHL. Locations to which an active compound migrated on the plate yielded a blue zone resulting from hydrolysis of the X-Gal in the medium by the β-galactosidase expressed from the *traG*::*lacZ* reporter fusion. *A. tumefaciens* detects AHLs with acyl chain lengths from 6 to 12 carbons. It detected four standard AHL molecules 3oxo-C6-HSL, 3oxo-C8-HSL C6, 3oxo-C10-HSL and 3oxo-C12-HSL in concentrated ethyl acetate extract (Fig. 2 lane b). Synthetic AHL standards (Fig.2 lane a) C4-HSL, C6-HSL, oxo-C6-HSL, C8-HSL, C10-HSL and C12-HSL were also run simultaneously. The assay is sensitive generally requiring very less amount of extracts (10µl) for detection.

Relative Retention Factor (RF) was calculated and compared with that of standards. Unavailability of rapid and simple purification technique is a major problem to work with natural AHLs. Highly sophisticated semi-preparative high performance liquid chromatography (HPLC) is the only technique available for the purification of the AHL molecule from mixture of AHLs. It requires highly skilled and trained manpower. We were able to successfully perform preparative TLC assay to purify 3-oxo-C10-HSL (Fig. 2 lane c) and 3oxo-C12-HSL (Fig. 2 lane d) by scrapping of AHLs from corresponding region and extracting AHLs with ethyl acetate followed by centrifugation. This study highlights the increased production of natural AHLs under optimized conditions and purification of specific AHLs using preparative TLC. Various authors have reported that synthetic AHLs have different effect on biological systems than that of natural AHLs. So it is suggested that to simulate the *in vivo* conditions, natural AHLs should have been used instead of synthetic one.[8-9, 13]

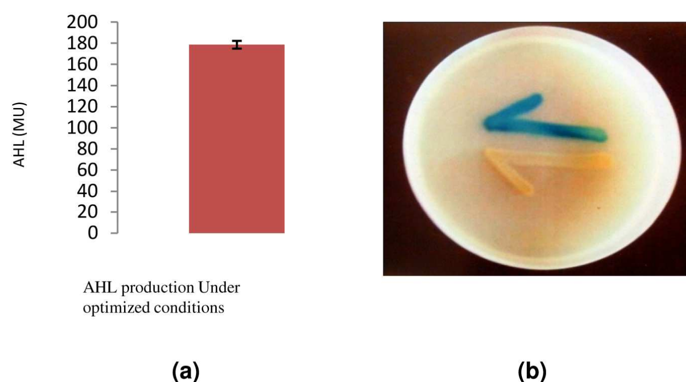

Figure 1

(a) AHL production of 178.67MU by P.aeruginosa strain PAO1 under optimized growth conditions of glucose (0.3%), lactose (0.5%), iron (150µM), pH (7.0), temperature (37^{0}C) and presence of oxygen. (b) Photograph showing the production of quorum sensing molecules by Pseudomonas aeruginosa PAO1 using E.coli MG4 as reporter strain for cross feeding assay.

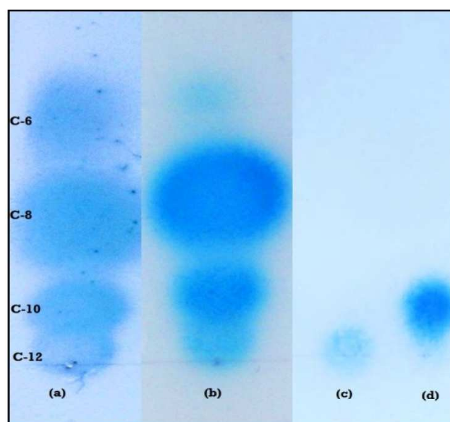

Figure 2

TLC of AHLs: Samples were chromatographed on C18 reversed-phase thin-layer plates, developed with methanol/water (60:40, vol/vol), and the spots were visualized with the A. tumefaciens A136 reporter strain. (a) Mixture of Synthetic AHL (3oxo-C12-HSL, 3-oxo-C10-HSL, 3-oxo-C8-HSL, 3-oxo-C6-HSL as standard (b) Concentrated Culture supernatant Ethyl acetate extract (c) Purified 3oxo-C12-HSL (d) Purified 3-oxo-C10-HSL.

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CONCLUSION

In conclusion, results of the present study indicate that under optimized condition there was enhanced production of AHL molecules. Further by employing preparative Thin Layer Chromatography Assay these AHLs can be successfully purified form the AHL mixture. This approach can further be exploited for enhanced AHL production and purification of natural AHLs from culture supernatants of any quorum sensing producing bacterium.

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