Available online at www.derpharmachemica.com

ISSN 0975-413X
CODEN (USA): PCHHAX

Der Pharma Chemica, 2016, 8(9):74-79
(http://derpharmachemica.com/archive.html)

Characterization of Acyl Homoserine Lactone of pigment producing *Pseudomonas aeruginosa* SU-3

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ABSTRACT

Quorum Sensing (QS), a communication process is prevalent among most bacteria. Acyl Homoserine Lactone (AHL) is the most common QS molecule produced by Pseudomonas aeruginosa and Quorum sensing is believed to be responsible for biofilm formation, pigment production and antibiotic resistance in Pseudomonas aeruginosa. In this study, pigment producing and biofilm forming P.aeruginosa was isolated from air. Autoinducer molecule responsible for quorum sensing was extracted and characterised by HPLC and LC-MS analysis. The isolated autoinducer molecule was analysed for its role in pigment (pyocyanin) production and it was found to enhance the pigment production.

Keywords: QS- Quorum sensing, AHL- Acyl Homoserine Lactone, pyocyanin.

INTRODUCTION

Bacteria produce certain signalling molecules for communicating among themselves which is known as quorum sensing (QS). These QS signalling molecules are a way of interaction and integration between microorganisms which enable many mechanisms such as growth, biofilm formation[1], virulence[2,3], sporulation[4], pigment production[5], antibiotic resistance, symbiosis and increase the pathogenicity of the microorganism[6,7]. In gram negative bacteria quorum sensing takes place through autoinducer molecules mostly N-Acyl Homoserine Lactones (AHL) as signal molecules. These AHLs are produced by an enzyme which utilizes ademetionine (substrate) and ACP (acyl carrier protein), an intermediate obtained in fatty acid biosynthesis[8,9]. Quorum sensing mechanism regulates many genes, most of which are responsible for virulence factors, the pigment production in *Pseudomonas* sp being one of them[10].

Pseudomonas aeruginosa is known for its existence as a biofilm and is an excellent model for understanding the mechanism of quorum sensing. The pyocyanin production is positively maintained by quorum sensing systems[10]. The signalling systems found in *Pseudomonas aeruginosa* are las and rhl [11] where lasI gene produces N-(3-oxododecanoyl)-L-homoserine lactone (3OC12-HSL), a class of acetylated homoserine lactone (AHL) and rhl gene produces *N*-butyryl-L-homoserine lactone (C4-HSL). On a whole in *Pseudomonas aeruginosa* approximately 4% of the genes out of 6000 genes function by the mechanism of quorum sensing[12]. Pyocyanin is a terminal signalling molecule in the quorum sensing cascade of *P.aeruginosa* [13]. The rhlR is considered the regulator of the pyocyanin biosynthesis gene, which in turns requires the regulation by transcription factor lasR[14]. The transcriptional activator protein LasR and LasI, which guides the synthesis of autoinducer PAI-1, is composed of the las system. Whereas, the rhl system is composed of transcriptional activator protein rhlR and rhlI, that is responsible for the synthesis of autoinducer PAI-2 [15]. The rhl system, which mediates the synthesis of transcriptional factors rhlR and rhlI for the production of autoinducer molecule PAI-2, regulates the production of pyocyanin1. The phenazine

compound is an exotoxin engaging in generation of reactive oxygen species destroying bacterial and mammalian cells through its redox cycling [16]. This study was done to isolate AHL from *P.aeruginosa* and to characterize it. The influence of AHL in pigment production was also attempted.

MATERIALS AND METHODS

Isolation

Sterile nutrient agar plates were prepared and kept opened in open air for ten minutes. After exposure the plates were incubated at 37^0C for 24 hours. Green pigmented colony was chosen and transferred to nutrient agar slant. After incubation, slants were stored in refrigerator at 4^0C till further use.

Identification of Microbes

Isolated organism was identified by performing routine biochemical tests and Gram's staining reaction. DNA was isolated [17] and amplified using universal primers 27F AGAGTTTGATCMTGGCTCAG and 1492R TACGGYTACCTTGTTACGACTT. 1400 - 1500 bp amplified products were subjected for sequencing. Unincorporated primers and dNTPs were removed from PCR products using Montage PCR Clean up kit (Millipore). The PCR product was sequenced using the 518F/800R primers (CCAGCAGCCGCGGTAATACG/ TACCAGGGTATCTAATCC). Sequencing reactions were performed using an ABI PRISM BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems)®.

Disc Diffusion Method for drug resistance pattern

Disc diffusion test was performed for *Pseudomonas aeruginosa* with the following antibiotic discs obtained from HimediaTM: Ceftazidime, Co-Trimoxazole, Amoxycillin, Impenem, Penicillin-G, Nalidixic Acid, Ticarcillin, Nofloxacin, Piperacillin, Cefuroxime, Sparfloxacin, Penicillin-C and Meropenem.

Pyocyanin pigment extraction

Pigment extraction was performed following the method of Essar et al [18] with certain modifications. Overnight culture of *P.aeruginosa* was standardized to OD value 1.0 at 600nm before inoculating into 20ml nutrient broth. After inoculation and incubation, the culture was centrifuged at 10000rpm for 10 minutes. 20ml of supernatant was mixed with 12 ml of chloroform and vortexed. The sample was spun at 10000rpm for 10 minutes which resulted in two phases in which chloroform along with pyocyanin sinks to the bottom. Solvent phase was mixed with 0.2N HCL till the colour changed from blue-green to pink. The solvent phase was centrifuged again at 10000rpm for 2 minutes. The resulting pink layer allowed to dry and weighed. Absorbance was read at 520 nm. Pyocyanin was quantified by following equation [18,19].

$$\text{Concentration of pyocyanin } (\mu g/ml) = OD520 \times 17.072$$

Growth vs. Pigment production

Growth of *P.aeruginosa* was analyzed by turbidometry method at 570nm and the growth curve was plotted against pigment concentration at various time intervals (16h, 24h, 40h, 48h, 64h, 72h, 88h, and 96h). In another set, to study the influence of antibiotic in pigment production, the organism was allowed to grow with sub-inhibitory concentration of ampicillin (1µg, 2µg and 4µg) anda graph was plotted between growth and pigment concentration for various time intervals- 16h, 24h, 40h, 48h, 64h, 72h, 88h, and 96h.

Extraction of autoinducer molecules

One liter of cell supernatants in the stationary phase cultures were extracted by two equal volumes of ethyl acetate acidified with 0.5% acetic acid for three times. The solvent was removed by rotary evaporation (40–45°C) and the residue was resuspended in 1 ml 20% ACN. Thus obtained homoserine lactone samples were stored at -20°C till analysis [20].

Characterization of autoinducer by HPLC analysis

The samples were then applied to a C8 reverse-phase preparative HPLC column (250 mm × 8 mm). Then the samples were eluted with an isocratic mobile phase of 70% (v/v) Acetonitrile in water [21] at a flow rate of 2 ml/min for a run time of 50-70 minutes. Samples were then monitored at 210 nm by a PDA (photo diode-array) detector.

Characterization of autoinducer by LC-MS analysis

LC-MS/MS analysis was performed by using Agilent 6400 Series Triple Quadrapole. The samples were eluted with an isocratic mobile phase of Acetonitrile: 5mM Ammonium formate (70:30).

Activity of AHL on Pigment Production

Acyl Homoserine Lactones (AHL) obtained by above procedure was mixed with 50% acetonitrile in the proportion of 1:1 and supplemented to the culture in varying concentrations - 10µl, 20µl and 40µl. 50% ACN was checked for sensitivity against the organism, it did not show any inhibition against the organism. Hence, ACN was used to dissolve the crude AHL into the culture medium. The pigment production for different time intervals- 16h, 24h, 40h, 48h, 64h, 72h, 88h, and 96h was plotted.

Statistical analysis

All the tests were done in triplicates. Mean value was taken and standard error was calculated. The results were given illustrated as Mean± standard error.

RESULTS AND DISCUSSION

Isolation and Identification of Organism

The organism found to produce bluish-green pigment on cetrimide agar and nutrient agar was isolated and their 16srRNA was sequenced. The organism was identified as *Pseudomonas aeruginosa* SU-3. The 16srRNA sequence was submitted and GENBANK accession number is GU395986.

Disc Diffusion Method for drug resistance pattern

Drug resistant patterns of *Pseudomonas aeruginosa* SU-3 is listed in Table 1. It was observed that organism shows resistance to a variety of antibiotics. Thus it can be concluded as a multi-drug resistant organism. The drug resistance has become chromosomally encoded, which made *P. aeruginosa* as one of greatest therapeutic challenges [22].

Growth vs. Pigment

The growth of the organism increased at 48th hour and decreased eventually, while the pigment production was constantly increasing till the 96th hour. Pigment production was found to be higher in death phase (Figure 1). Pyocyanin is normally secreted at the exponential phase which induces the quorum sensing and pigment production is the terminal signalling molecule in the *P. aeruginosa* quorum sensing network [23]. Biofilm formation was influenced by sub-inhibitory concentration of ampicillin, but it was not promoting the pigment production (Figure 1).

HPLC analysis of autoinducer molecules

AHLs isolated in this study showed retention times of 3.054 and 4.659 which is assigned for C4-HSL and C5-HSL respectively (Figure 2), whereas Lade et al [24] assigned retention time of 3.224 for C4-HSL and 6.296 for C6-HSL. *Pseudomonas aeruginosa* synthesizes *N*-(3-oxododecanoyl)-L-homoserine lactone (3OC12-HSL), which acts as a signaling molecule , regulates virulence factors and biofilm formation [25].

LC-MS analysis of autoinducer molecule

The autoinducer molecule was subjected to LC-MS ESI-MS analysis and the ESI-MS Spectrum is given in Figure 3. In our study the m/z we obtained are as follows and the AHLs are also listed - m/z 180 and 197.9 = C4-HSL, m/z 221 = C5-HSL, m/z 254.2 = C10-HSL, m/z 218 = 3OH C6-HSL and m/z 270 =3O C10-HSL. Khan et al [26] assigned m/z for the AHL isolated from *Sinorhizobium meliloti* are as follows - m/z 172 and 190 = C 4 -HSL; m/z 202 = 3-OH-C 5 -HSL; m/z 220 = C 5 -HSL with a H_2O adduct; m/z 216 = 3OH C 6 -HSL; m/z 228 = C -HSL; m/z 270 = 3O C 10-HSL and m/z 298 = 3O C 12 –HSL. Mass-to-Charge (m/z) ratio of 254.2000 was nearly similar to that of 3-OH-C10-HSL synthetic molecule [27].

Activity of AHL on Pigment Production

It is observed that different concentrations of AHL to promote the production of pyocyanin pigment, even influence of acetonitrile alone on pigment production was also checked as AHL was dissolved in it (Figure 4). Though the 10µl concentration produced low pigment, the pigment production was higher in the case of 20µl and 40µl concentrations. Thus indicates role of AHL on pyocyanin pigment production in *P.aeruginosa*. Phenazine, a precursor of pyocyanin production starts with the synthesis of AHL during exponential phase. This is followed by secondary QS molecule during late exponential phase which controls the expression of *phzA-G* operons resulting in production of phenazine-1-carboxylic acid (PCA). PCA is then modified to produce primarily pyocyanin (modification encoded by *phzM*) [28-30].

Table 1 DRUG RESISTANCE PATTERN OF *Pseudomonas aeruginosa*

S.No	Antibiotics	Results
1.	Nofloxacin	Sensitive
2.	Ceftazidime	Resistant
3.	Co-Trimoxazole	Resistant
4.	Amoxycillin	Resistant
5.	Impenem	Sensitive
6.	Penicillin-G	Resistant
7.	Nalidixic Acid	Resistant
8.	Ticarcillin	Resistant
9.	Nofloxacin	Sensitive
10.	Piperacillin	Resistant
11.	Cefuroxime	Resistant
12.	Sparfloxacin	Sensitive
13.	Penicillin-C	Resistant
14.	Meropenem	Resistant

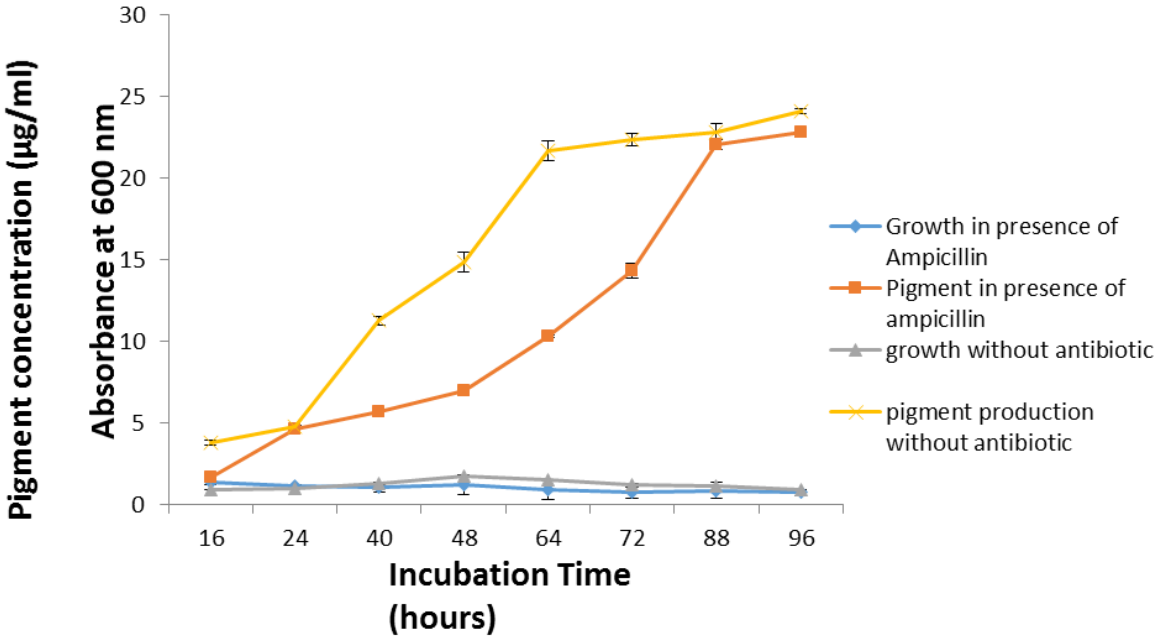

Figure 1 Growth vs. Pigment production in absence and presence of ampicillin

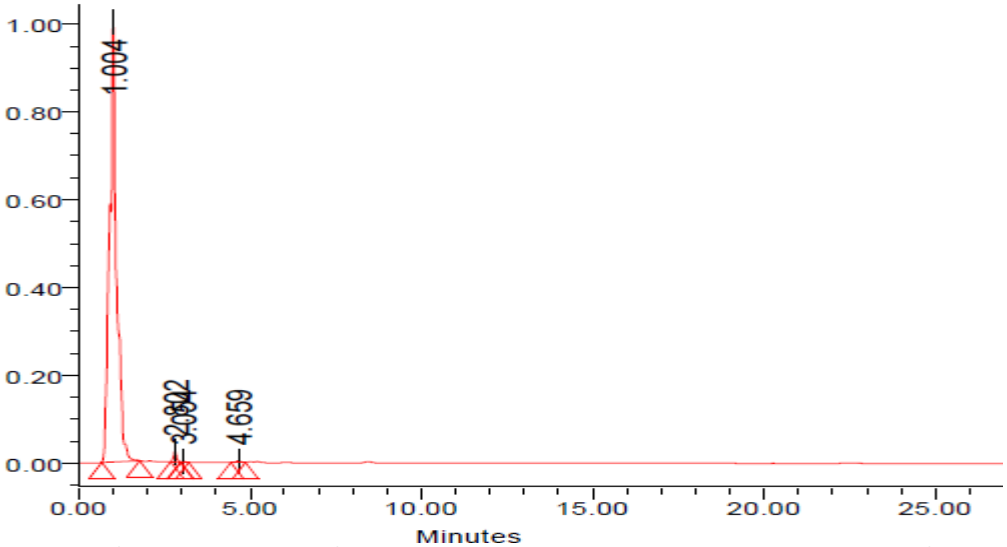

Figure 2 HPLC analysis of autoinducer extracted from *P.aeruginosa*. Chromatogram showing the retention times of AHLs with mixture of C4-HSL and C5-HSL

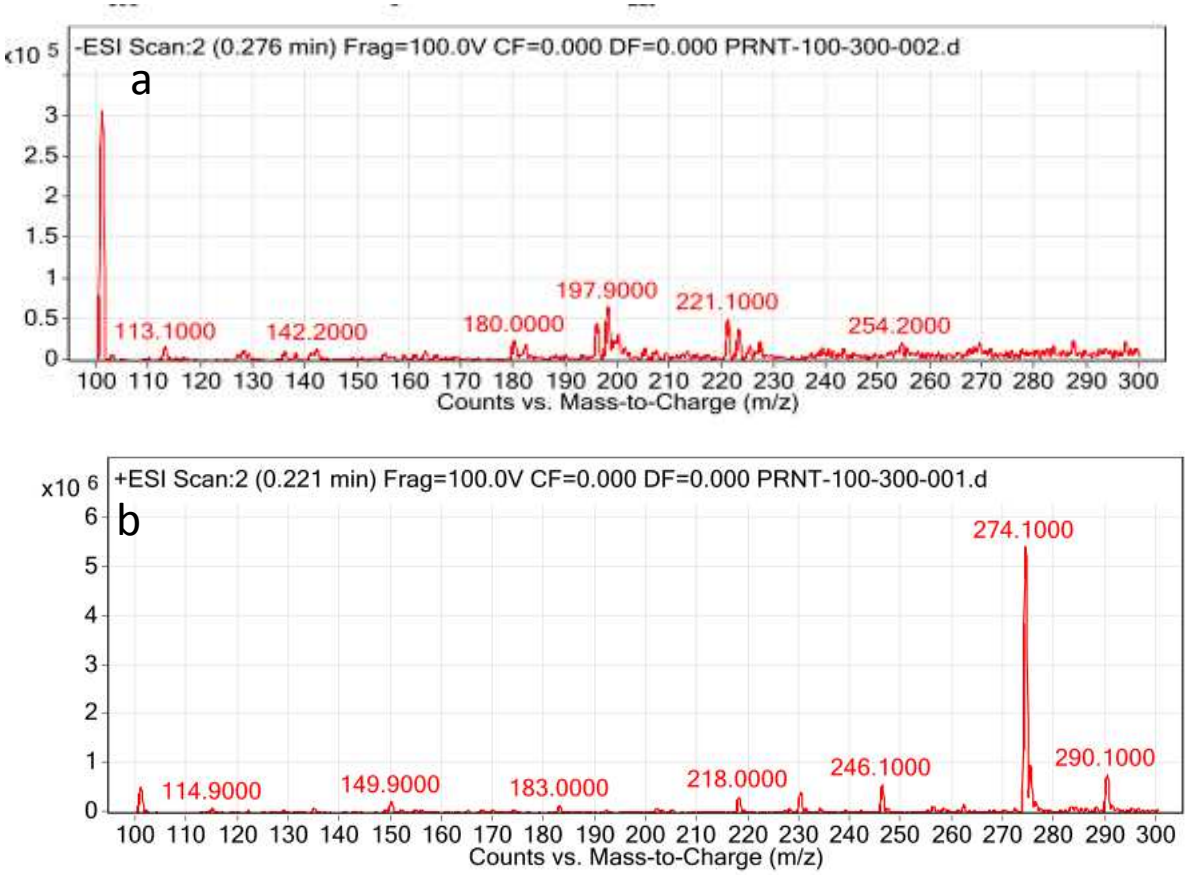

Figure 3 LC-MS/MS Spectrum of Autoinducer Extracted From *P.aeruginosa*

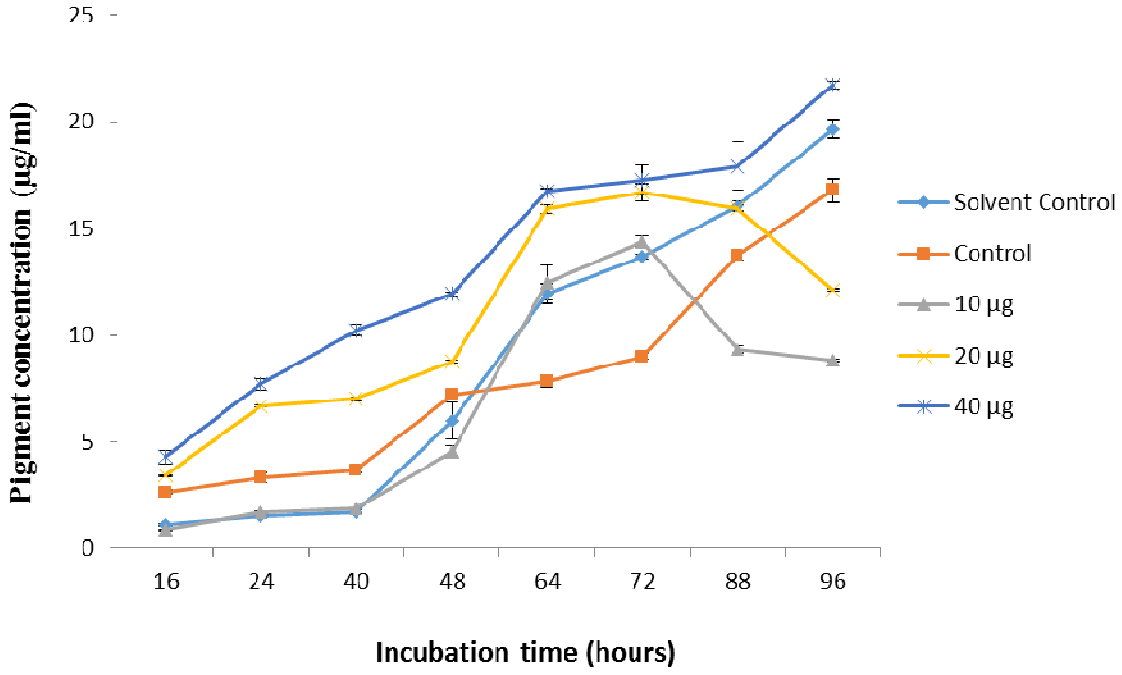

Figure 4 Activity of extracted AHL on Pigment Production

CONCLUSION

P.aeruginosa SU-3 was isolated from air. The autoinducer molecule produced was characterized by HPLC and LC-MS analysis. The isolated autoinducer was found to influence the production of pyocyanin pigment.

Acknowledgement
We are grateful to Sathyabama University for giving us support. We are also thanking Mr.Antony Rio Joseph, Mr.Rejin Prasad and Mr.Venkatraman for rendering great support throughout this work.

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