Chemical Engineering Journal 302 (2016) 172–186

Contents lists available at ScienceDirect

Chemical Engineering Journal

journal homepage: www.elsevier.com/locate/cej

Effect of synthetic N-acylhomoserine lactones on cell–cell interactions in marine *Pseudomonas* and biofilm mediated degradation of polycyclic aromatic hydrocarbons

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HIGHLIGHTS

- PAHs degrading marine *Pseudomonas* spp. were isolated after selective enrichment.
- Supplementation of AHLs in the growth medium enhanced the biofilm formation.
- 3OC12-HSL showed the pronounced effect in degradation of phenanthrene and pyrene.
- Catechol pathway for PAHs degradation was followed by the marine bacteria.

ARTICLE INFO

Article history:
Received 23 February 2016
Received in revised form 8 May 2016
Accepted 9 May 2016
Available online 10 May 2016

Keywords:
Acyl homoserine lactones
Pseudomonas
Biofilm
Auto aggregation
PAHs
Biodegradation

ABSTRACT

Effect of exogenous N-acyl homoserine lactones (AHLs) on biofilm growth, cell surface hydrophobicity, auto-aggregation and polycyclic aromatic hydrocarbons (PAHs) degradation potential of two marine *Pseudomonas* isolates (*Pseudomonas pseudoalcaligenes* NP103 and *Pseudomonas aeruginosa* N6P6) were evaluated in the present study. Increased biofilm growth, auto-aggregation and swarming motility was observed in the presence of exogenous AHLs (3OC8-HSL and 3OC12-HSL) resulting in enhanced phenanthrene and pyrene degradation. *P. pseudoalcaligenes* NP103 biofilm was able to degrade up to 79% of phenanthrene and 49% pyrene in 7 d whereas 85.6% phenanthrene and 47.56% pyrene degradation was achieved using *P. aeruginosa* N6P6 biofilm. 3OC8-HSL significantly ($P < 0.05$; Tukey's HSD test) potentiated the phenanthrene and pyrene degradation by *P. pseudoalcaligenes* NP103 biofilm (89% and 65.5%), whereas the phenanthrene and pyrene degradation potential of *P. aeruginosa* N6P6 biofilm increased significantly ($P < 0.05$; Tukey's HSD test) in presence of 3OC12-HSL (97.4% and 54.39%). Furthermore, the degradation achieved by both the isolates in presence of tannic acid, a quorum sensing inhibitor (QSI), was highest in presence of 3OC12-HSL suggesting the most pronounced effect of long chain AHL in degradation of phenanthrene and pyrene. Both the isolates followed catechol pathway for PAHs degradation. The findings suggest that AHL can significantly affect the biodegradation performance specifically when bacteria are present in abundant numbers in biofilms.

1. Introduction

Quorum sensing (QS) is a cell–cell communication process in bacteria via signaling molecules known as autoinducers (AIs) [1]. It enables bacteria to respond in a collective manner as a function of population density. QS phenomena has been extensively studied in Gram-positive and Gram-negative bacteria, in which QS are regulated via autoinducing peptides (AIPs) and acyl homoserine lactones (AHLs) respectively. Apart from that, many miscellaneous autoinducers such as autoinducer-2, diffusion signaling factors, *Pseudomonas* quinolone signaling and α-hdroxyketones have been reported in bacteria [2]. Autoinducers help bacteria in intercellular communication resulting in differential behavioral response to cell density [3]. The expression of QS relevant gene(s) is mediated by binding of autoinducer to its cell surface receptor. Thus, autoinducers are unambiguous in diverse bacterial groups. AHLs, in particular, have gained much attention for QS and related gene expression [4,5]. The representatives of AHL producing bacteria are abundant in the environment and most of them are under *Proteobacteria*. It covers approximately 7% of genera, containing known AHL producing representatives [6]. AIs mediate interactions between different

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http://dx.doi.org/10.1016/j.cej.2016.05.042
1385-8947/

species of bacteria and between bacteria and higher organisms, either through the phenotypes they regulate or directly through their chemical behavior [7].

The distinct practice of QS is an effective response carried out in a group [3]. QS is responsible for the regulation of certain fundamental features in the bacterial life cycle. The key process reported to be controlled by QS regulator circuit includes swarming motility, biofilm development and exopolysaccharide (EPS) synthesis. These are also important from environmental perspectives [1,8–10]. However, the involvement of AHL (indigenous or exogenous) in remediation of organic pollutants and cell surface dependent phenomenon such as surface hydrophobicity and aggregation is not well documented. Understanding the involvement of AHLs in biological activities and their connection to bacterial community structure may provide an opportunity to manipulate the composition and function of bacterial consortia for bioremediation of organic pollutants. Moreover, application of signaling molecules for environmental restoration also increases the usage of QS for biotechnological application [5]. In the present work, the effect of exogenous AHLs on biofilm growth and biofilm mediated degradation of polycyclic aromatic hydrocarbons (PAHs) such as phenanthrene and pyrene has been studied.

PAHs are the most lethal organic contaminants ubiquitously present in coastal areas. PAHs are widely spread in the environment and are of great concern because of their persistent nature. As per environment protection agency (EPA), the maximum contaminant level (MCL) of PAHs is 0.0002 mg/l in the environment. PAHs (phenanthrene and pyrene) are highly toxic to aquatic organisms and their toxicity profile is often exaggerated by metabolism and photo-oxidation in the presence of ultraviolet light. Soils heavily contaminated with PAHs often exert toxic effects on terrestrial invertebrates. Exposure to PAHs adversely affects the reproductive system, overall development and immunity [11].

Volatilization, photo-oxidation, chemical oxidation, bioaccumulation and adsorption to soil particles are the possible fates of PAHs in the environment. Among these conventional techniques, photocatalytic degradation is mostly used which is titanium dioxide (TiO_2) based [12]. TiO_2 application is practically limited in bioremediation of PAHs because of its low adsorbability for PAHs and the difficulty of separating it from treated waste water, whereas, microbial transformation and degradation are environmentally benign strategy to address PAHs contaminated sites. Therefore, bioremediation is regarded as the best method over chemical and various physical methods for the detoxification and removal of PAHs from the environment. Several metabolic pathways of PAHs biodegradation by bacteria have been reported [13]. Bacterial PAHs degradation mostly, follows phthalate route or catechol route [14]. However, most of the recent studies documented the formation of 2-hydroxy-1-naphthoic acid, leading to formation of catechol in the degradation of PAHs [15].

However, a major constraint in the degradation of high molecular weight PAHs is their low availability to bacteria due to their high hydrophobicity. AHLs might enhance the solubility of PAHs and bring them in the vicinity of bacterial cells for efficient degradation [16]. Hence, in the present study, the role of exogenous AHLs in degradation of phenanthrene and pyrene has been examined by two ways. In the first way, the isolates were allowed to form biofilm spontaneously in the presence of exogenous AHLs and in the second way, tannic acid, a quorum sensing inhibitor (QSI), were applied along with exogenous AHLs to block its internal QS system. The metabolic pathway of PAHs assimilation by the biofilm forming marine bacteria was elucidated. The process involving cell-to-cell interactions such as cell surface hydrophobicity and auto-aggregation were also studied to bring out information on the enhanced degradation of polycyclic aromatic hydrocarbons by biofilm forming marine bacteria.

2. Materials and methods

2.1. Marine bacterial strains and screening for AHLs

Pseudomonas pseudoalcaligenes NP103 and *Pseudomonas aeruginosa* N6P6 used in the present study were isolated from marine water sample collected from Odisha coast, India after selective enrichment with phenanthrene and pyrene. Biofilm screening and molecular identification of the isolates were done following Mangwani et al. [17]. 16S rRNA gene sequences of both the isolates have been submitted to NCBI GenBank under the accession number JX273778 and KJ461700 respectively. The biofilm forming isolates have also been submitted to the Belgian Coordinated Collections of Microorganisms (BCCM) under the deposition number LMG 28190 and LMG 28185 respectively.

Screening for the long chain AHL was done by T-streaking method on Luria Bertani (LB) agar (Hi-Media, India) plates supplemented with 40 $\mu g\ ml^{-1}$ X-gal. Test isolate was perpendicularly streaked to the *Agrobacterium tumefaciens* NTL4 (ATCC@ BAA-2240™) and the plate was incubated at 30 °C for 24–48 h. The T-streak with blue color *A. tumefaciens* BAA 2240 streak line was considered AHL positive [18,19]. Screening for short chain AHL was done using *Chromobacterium violaceum* CV026 by T streaking method. Appearance of purple pigment by *C. violaceum* CV026 was considered positive for short chain AHL production [20].

2.2. Effect of AHLs on swarming motility

Swarming motility, a QS regulated phenomenon, was assayed using LB medium having 0.5% agar and 1% glucose (swarming agar). 15 ml swarming agar was poured and was allowed to dry. The solidified swarming agar plate was overlaid with 5 ml of swarming agar supplemented with different concentrations of exogenous AHLs i.e. N-butyryl-L-homoserine lactone (C4-HSL), N-octanoyl-L-homoserine lactone (C8-HSL), N-(3-oxooctanoyl)-L-homoserine lactone (3OC8-HSL) and N-3-oxo-dodecanoyl-L-homoserine lactone (3OC12-HSL) were obtained from (Sigma Aldrich, India). 1 $mg\ ml^{-1}$ stock of all the AHLs was prepared in DMSO. Different concentrations of AHLs (1 $\mu g\ ml^{-1}$, 2 $\mu g\ ml^{-1}$ and 3 $\mu g\ ml^{-1}$) in LB medium was used for analyzing their effect on swarming motility. Overnight grown culture of *P. pseudoalcaligenes* NP103 and *P. aeruginosa* N6P6 (5 μL) was inoculated in the center of the AHL supplemented swarm agar and control plate (LB swarm plate) and incubated for 16 h at 37 °C [21].

2.3. Planktonic and biofilm growth in presence of synthetic AHLs

Planktonic cell growth in the presence of exogenous AHLs was monitored using UV–visible spectrophotometer (A_{600}). The AHLs were used at a final concentration of 2 $\mu g\ ml^{-1}$ in LB medium. The isolate was grown in LB broth overnight and diluted to 1:100 in LB broth. 3 ml of the diluted LB broth was transferred to 6-well plate with a glass slide (2 cm × 2 cm). It was incubated at 37 °C under static condition. AHL was added to a final concentration of 2 $\mu g\ ml^{-1}$. After 48 h, slide with biofilm growth was washed with sterilized distilled water twice and stained with 0.02% aqueous solution of acridine orange for 5 min [22]. After 5 min, the slide was washed twice with distilled water and observed under 20X objective using Fluorescence microscope (Olympus, Japan). Fluorescence microscope images were analyzed using IMAGE J 1.46 [23].

2.4. Effect of AHL on auto-aggregation and cell surface hydrophobicity

The bacterial culture was grown overnight (in LB broth). The cell mass was collected by centrifugation of 50 ml broth culture.