Bioresource Technology 125 (2012) 119–126

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

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

Ecological roles and release patterns of acylated homoserine lactones in *Pseudomonas* sp. HF-1 and their implications in bacterial bioaugmentation

Mei-zhen Wang [a,b], Xin Zheng [a], Hong-zhen He [a], Dong-sheng Shen [a,b], Hua-jun Feng [a,b,*]

[a] College of Environmental Science and Engineering, Zhejiang Gongshang University, Hangzhou 310012, China
[b] Zhejiang Provincial Key Laboratory of Solid Waste Treatment and Recycling, Hangzhou 310012, China

HIGHLIGHTS

- This is the first report of release pattern of autoinducers in *Pseudomonas* sp. HF-1.
- Detailed autoinducer-threshold for specific quorum sensing was studied in HF-1.
- The modulation of biofilm formation based on quorum sensing was reported.
- Regulation of quorum sensing will facilitate construction of bioaugmented system.

ARTICLE INFO

Article history:
Received 24 June 2012
Received in revised form 20 August 2012
Accepted 23 August 2012
Available online 2 September 2012

Keywords:
Ecological role
Auto-inducer release
Quorum sensing
Pseudomonas sp. HF-1
Bioaugmentation

ABSTRACT

To enable development of a better bacterial bioaugmentation system for tobacco wastewater treatment, the roles and release patterns of acylated homoserine lactones (AHLs) in *Pseudomonas* sp. HF-1 were evaluated. Swarming was found to be induced by *N*-hexanoyl-homoserine lactone (C_6-HSL) and *N*-3-oxo-hexanoyl-homoserine lactone (3-oxo-C_6-HSL); the formation of extracellular polymeric substances (EPS) was induced by 3-oxo-C_6-HSL, C_6-HSL and *N*-3-oxo-octanoyl-homoserine lactone (3-oxo-C_8-HSL); and biofilm formation was induced by C_6-HSL and 3-oxo-C_8-HSL. When the culture conditions were 25 °C, pH 5–6, 3% inoculum, 1.5 g L^{-1} nicotine and 1% NaCl, the amount of AHLs released was sufficient for quorum sensing of swarming and EPS formation for strain HF-1, which was beneficial to the startup stage during bioaugmentation. When strain HF-1 was cultured at pH 8 in the presence of 1.2–1.8 g L^{-1} of nicotine and 1% NaCl, the threshold for quorum sensing of biofilm formation was reached and the bioaugmentation system showed an efficient performance.

1. Introduction

Quorum sensing, which is cell–cell communication among bacteria, has recently become an area of intense research. This process is accomplished through the exchange of extracellular signaling molecules called auto-inducers to regulate gene expression, which allows bacterial populations to enhance the effectiveness of community cooperation. These processes include virulence factor expression, antibiotic production, biofilm development and horizontal gene transfer (Chen et al., 2002).

Bioaugmentation is a technique in which a functional gene, strain or microbial consortium is introduced into an indigenous bacterial population (Teng et al., 2010). This method has been proposed as an effective strategy for the removal of pollutants (Paynet et al., 2011). The technical core of bioaugmentation is colonization, which means maintaining persistent survival rates and activities of the introduced bacteria in the remediation system (El Fantroussi and Agathos, 2005). However, many factors including predation, competition and sorption hamper colonization. During the process of bioaugmentation, the inoculated bacteria can release auto-inducers to stimulate quorum sensing in the remediation system, which can modulate community cooperation to enable successful bacterial colonization. However, little research has been conducted to the influence of quorum sensing on bacterial bioaugmentation (Jiang et al., 2006; Paliwal et al., 2012).

In our previous study, the nicotine-degrading bacterium *Pseudomonas* sp. HF-1 was bioaugmented in a sequencing batch reactor to treat tobacco wastewater. The bioaugmentation system showed high efficiency, with 100% nicotine degradation and more than

Abbreviations: AHLs, acylated homoserine lactones; C_6-HSL, *N*-hexanoyl-homoserine lactone; EPS, extracellular polymeric substances; β-Gal, β-Galactosidase; HPLC, high-performance liquid chromatography; ISM, inorganic salt medium; LB, Luria–Bertani; 3-oxo- C_6-HSL, *N*-3-oxo-hexanoyl-homoserine lactone; 3-oxo-C_8-HSL, *N*-3-oxo-octanoyl-homoserine lactone; SDS, sodium dodecyl sulfate.

* Corresponding author at: College of Environmental Science and Engineering, Zhejiang Gongshang University, 198 Jiangong road, Hangzhou 310012, China. Tel./fax: +86 571 87397126.
E-mail address: fenghuajun@mail.zjgsu.edu.cn (H.-j. Feng).

0960-8524/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biortech.2012.08.116

84% chemical oxygen demand removal within 12 h (Wang et al., 2009). However, setup of the bioaugmentation system was not always successful owing to changes in the conditions of the inoculums and sources of activated sludge, which limited its application in practice. The controllability of bioaugmentation has also confused other researchers (Bouchez et al., 2000), which is one of the biggest problems facing in development of the bioaugmentation technique.

Biofilms are multi-cellular communities formed by bacteria (Geske et al., 2005). Switching from nomadic existence to biofilm communities significantly improves the ability of inoculated bacteria to colonize in their new environment (Lazar, 2011); accordingly, biofilm formation is beneficial for bacterial bioaugmentation. Extensive studies of biofilm development in various bacteria have led to a general five-stage developmental process: initial surface contact, irreversible attachment, matrix production and architecture development, maturation, and cell dispersion from the biofilm (Stoodley et al., 2002). According to Ouyang et al. (2012), flagella are considered to play important roles in seed cell attachment and extracellular polymeric substances (EPS) are vital for matrix formation. The entire process of biofilm formation, including swarming by flagella and the secretion of EPS, has been reported to occur via quorum sensing (Patriquin et al., 2008; Jiang and Liu, 2012). Thus, the theory of quorum sensing, especially as it relates to modulation of biofilm formation, has led to improved development of bacterial bioaugmentation systems for the treatment of tobacco wastewater.

Therefore, this study was conducted to determine the ecological roles of acylated homoserine lactones (AHLs), the main auto-inducers, on biofilm formation by *Pseudomonas* sp. HF-1. In addition, their releasing patterns under different culture conditions were evaluated. This is the first report of the modulation of biofilm formation in strain HF-1 based on the theory of quorum sensing. The results presented here will facilitate successful development of such bioaugmentation systems.

2. Methods

2.1. Bacterial strains

Pseudomonas sp. HF-1, a high efficiency nicotine-degrading strain, was isolated from tobacco waste-contaminated soil, which was conserved in our laboratory. Its sequence was deposited in the GenBank database under accession No. AY823996. Additionally, the quorum sensing reporter strains *Chromobacterium violaceum* CV026 (Double mini-Tn5 mutant from *C.violaceum* ATCC 31532) and *Agrobacterium tumefaciens* NTL4 (*A. tumefaciens* NT1 derivative carrying a *traG::lacZ* reporter fusion) were kindly provided by Professor Xu Yuquan on the School of Life Sciences & Biotechnology in Shanghai Jiao Tong University.

2.2. Culture media

Luria–Bertani (LB) broth and inorganic salt medium (ISM) were used in this study. ISM with nicotine as the sole source of carbon and nitrogen was used for strain HF-1 culture. This media was composed of 0.2 g K_2HPO_4, 0.8 g KH_2PO_4, 0.2 g $MgSO_4$, 0.1 g $CaSO_4 \cdot H_2O$, 0.0033 g $NaMoO_4$ and 0.005 g $FeSO_4 \cdot H_2O$ in one liter of distilled water. LB with 50 μg mL^{-1} of kanamycin was used for *C. violaceum* CV026 culture, while LB with 30 μg mL^{-1} of gentamicin was used for *A. tumefaciens* NTL4 culture.

2.3. Bioassay for AHLs in strain HF-1

2.3.1. Extraction of auto-inducers

Cell-free supernatants were prepared by centrifugation at 18 000 *g* for 5 min. Next, 100 mL of cell-free supernatants were extracted with an equivalent volume of ethyl acetate. The mixture was then shaken vigorously, after which the phases were allowed to separate (Ravn et al., 2001). The entire extraction process was repeated three times, after which the combined ethyl acetate fractions were evaporated to dryness and dissolved in 2 mL of 50% acetonitrile for subsequent analysis.

2.3.2. Bioassay for AHLs release

The release of AHLs by strain HF-1 during different growth phases (8, 10, 12, 14 and 16 h) was estimated based on the quorum sensing functions of reporter strains. To accomplish this, 1% (v/v) logarithmic phase reporter strain CV026 was inoculated into 5 mL of LB media with 50 μg mL^{-1} of kanamycin while reporter strain NTL4 into 5 mL of LB media with 30 μg mL^{-1} of gentamicin, after which 50 μL of AHLs extracted from strain HF-1 in different growth phases were added to the media, and the samples were then incubated at 30 °C and 130 rpm for 12 h. The violacein production by strain CV026 was then used to indicate the presence of short-chain AHLs (Vílchez et al., 2010), while the inducement of β-galactosidase by strain NTL4 was used for long-chain AHLs (Ponnusamy et al., 2009).

2.3.2.1. Violacein content assay. To estimate the violacein content, 1 mL of strain CV026 culture containing strain HF-1 AHLs was transferred into a 10 mL centrifuge tube. The cells were then lysed by adding an equal volume of 10% (w/v) sodium dodecyl sulfate (SDS), mixed with a vortex for 30 s and then incubated at 37 °C for 5 min. Violacein was quantitatively extracted from the cell lysate by adding 4.5 mL of water-saturated butanol and mixing with a vortex for 30 s. The organic phase was separated by centrifugation at 18 000 *g* for 5 min. The upper phase, butanol containing violacein, was read at 585 nm, while the population density of the cells was measured based on the absorbance at 600 nm. The violacein unit was then calculated as $(A_{585}/A_{600}) \times 1000$, according to Blosser and Gray (2000).

2.3.2.2. β-Galactosidase (β-Gal) activity assay. To estimate β-Gal activities, 100 μL of the strain NTL4 culture containing strain HF-1 AHLs was transferred into a 2 mL micro-centrifuge tube and then mixed with 900 μL of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, pH7.0). The cells were then lysed by adding 100 μL of $CHCl_3$ and 50 μL of 1% (w/v) SDS, mixing with a vortex for 10 s and incubation at 37 °C for 3 min. Next, 200 μL of the substrate solution (4 mg mL^{-1} o-nitrophenyl- β-D-galactopyranoside) was added to each tube, and the time of addition was noted. After sufficient color development, the reaction was stopped with 500 μL of stop solution (1 M Na_2CO_3) and the stop time was noted. The tubes were then centrifuged at 22 000 *g* for 5 min to remove the cell fractions. Next, the mixture was analyzed at 420 nm and the population density of cells was detected at 600 nm. Finally, the Miller unit was calculated as $[A_{420}/(A_{600} \times 0.1\ mL \times \text{reaction time})] \times 1000$, as described by Ponnusamy et al. (2010).

2.4. Evaluation on the ecological roles of AHLs in biofilm formation of strain HF-1

The ecological roles of AHLs in biofilm formation of strain HF-1 were evaluated by AHLs adding treatments. To accomplish this, strain HF-1 in the logarithmic phase was collected by centrifugation at 22 000 *g* for 5 min. Subsequently, the cells were washed with 0.85% (w/v) NaCl twice to remove auto-inducers and the OD_{600} was set at approximately 0.6 using ISM. Finally, the washed cells were mixed with *N*-3-oxo-hexanoyl-homoserine lactone (3-oxo-C_6-HSL), *N*-hexanoyl-homoserine lactone (C_6-HSL) and *N*-3-oxo-octanoyl-homoserine lactone (3-oxo-C_8-HSL), respectively.