



Effects of pH, Temperature and Salinity on Extracellular Polymeric Substances of *Pseudomonasaeruginosa* Biofilm with N-(3-Oxooxtanoyl)-L-Homoserine Lactone Addition

Lijie Zhou, Siqing Xia^{*}, Zhiqiang Zhang, Biao Ye, Xiaoyin Xu, Zaoli Gu, Xin Wang

State Key Laboratory of Pollution Control and Resource Reuse, College of Environmental Science and Engineering, Tongji University, Shanghai 200092, China

ABSTRACT

Effects of pH, temperature and salinity on extracellular polymeric substances (EPS) of *Pseudomonasaeruginosa* (*P. aeruginosa*) biofilm with N-(3-oxooxtanoyl)-L-homoserine lactone (C₈-oxo-HSL, one of the common quorum sensing (QS) signal in environment) addition were studied. C₈-oxo-HSL addition increased polysaccharide concentration of biofilm EPS to promote the *P. aeruginosa* biofilm formation, but had little effects on protein of EPS. C₈-oxo-HSL addition should be carried out at pH 5-7 for *P. aeruginosa* biofilm formation. The alkali-resistance of *P. aeruginosa* biofilm formation was stronger than that of acid-resistance. Both polysaccharide and protein of EPS of *P. aeruginosa* biofilm reached the highest concentration at 20°C. C₈-oxo-HSL addition promoted the inducement of QS, which would reduce the demand of NaCl for both mass propagation and EPS release for *P. aeruginosa* biofilm.

Keywords: Extracellular polymeric substances; quorum sensing (QS); pH; temperature; salinity

1. INTRODUCTION

In the world of bacteria, quorum sensing (QS) was like “bacterial twitter” to describe how bacteria communicate with one another in an effort to make a coordinated attempt at altering gene expression with the population (Richards and Melander, 2009; Shrout and Nerenberg, 2012). In environmental bacteria (mainly Gram-negative bacteria), acyl homoserine lactone (AHL) has been considered as the major signal molecules in QS system to transfer message and induce bacterial behaviors. Lots of studies reported that biofilm formation was heavily controlled by QS pathways (An et al., 2006; Kolter and O’Toole, 1998; Sauer et al., 2002; Weibel et al., 2011). Additionally, QS also could shape biofilm development and its

organized structure according to the surrounding conditions (Jayaraman and Wood, 2008; Stoodley et al., 2002). QS control was reported to induce the secretion of extracellular polymeric substances (EPS) to control biofilm formation (Fuqua et al., 2001; Marketon et al., 2003). As one of the most significant attributes of bacterial biofilms, EPS worked as a barrier to defend the gathering microorganisms from microbicides and to provide an enclosed space for the biofilm formation (Richards and Melander, 2009). Therefore, many studies carried out lots of methods to inhibit QS for the control of EPS release and biofilm formation (An et al., 2006; Jiang et al., 2013; Kim et al., 2011). In addition, enhancement of EPS release for the promotion of biofilm formation with AHL addition was considered as a promising way in the application of wastewater

^{*}Corresponding to: siqingxia@gmail.com

treatment systems, especially for biofilm reactor (Xia et al., 2012).

Pseudomonas aeruginosa (*P. aeruginosa*) was a Gram-negative, aerobic, coccobacillus bacterium with unipolar motility. Although *P. aeruginosa* was an opportunistic pathogen of plants, animals and human being, it played a significant role in wastewater treatment. Many reports showed that *P. aeruginosa* was one of major parts in activated sludge community (Xia et al., 2009, 2010, 2012). *P. aeruginosa* also played a significant role in the biofilm formation of the immobilization of bacterium in wastewater treatment (Herzberg et al., 2009). However, both EPS release and biofilm formation of *P. aeruginosa* in the biofilm reactor for wastewater treatment needed a long time, which inhibited the performance of the biofilm reactor, especially at the early stage of wastewater treatment. Xia et al. (2012) suggested that the N-(3-oxooxetanoyl)-L-homoserine lactone (C₈-oxo-HSL, one of the common QS signal in environment) addition could promote the EPS release to enhance biofilm formation. However, effects of pH, temperature and salinity on EPS of *P. aeruginosa* biofilm were unclear. It might inhibit the performance of C₈-oxo-HSL addition for promotion of biofilm formation.

This study aimed to investigate the effects of pH, temperature and salinity on EPS of *P. aeruginosa* biofilm with C₈-oxo-HSL addition. C₈-oxo-HSL addition of 0 mg/L, 10⁻⁷ mg/L and 10⁻⁴ mg/L was chosen in this study, because these concentrations of C₈-oxo-HSL showed typical effects on *P. aeruginosa* biofilm formation (Xia et al., 2012). *P. aeruginosa* biofilm formation with C₈-oxo-HSL addition of 0 mg/L, 10⁻⁷ mg/L and 10⁻⁴ mg/L was performed under variations of pH, temperature and salinity. Both polysaccharide and protein concentrations of EPS of *P. aeruginosa* were measured in each batch experiment.

2. MATERIALS AND METHODS

P. aeruginosa is a kind of common and representative bacteria in activated sludge and biofilm in wastewater treatment systems (Ridgeway et al., 1984; Xia et al., 2012). *P. aeruginosa* (China Center of Industrial Culture Collection, CICC, 23618) was carried out in this study as the single bacterial species, and it was isolated from the sewage biological treatment system for removing organic phosphorus. Xia et al. (2010) reported that N-(3-oxooxetanoyl)-L-homoserine lactone (C₈-oxo-HSL) could promote the EPS release of *P. aeruginosa* by QS system, and the critical concentration was 10⁻⁷ g/L. In this study, C₈-oxo-HSL (Sigma, O1764, CAS number: 147795-39-9) was dissolved gradually into methyl alcohol to prepare the stored C₈-oxo-HSL solution of 2×10⁻² and 2×10⁻⁵ g/L, and stored at 4 °C. Hollow-fiber polyvinylchloride plastic (PVC) UF-membrane (Li-tree Company, Suzhou, China) was used for the formation of *P. aeruginosa* biofilm on its surface. Its outside and the inner diameters were 1.50 mm and 0.85 mm, respectively.

Previous work (Xia et al., 2012) was reported that *P. aeruginosa* biofilm was formed and its growth rate was almost stable after 8 hours incubated (Xia et al., 2012). First, *P. aeruginosa* was incubated (37 °C, shaking at 160 r/min, 8 hr) to the late logarithmic growth phase in the 50 ml broth (peptone 5 g/L, beef extract 3 g/L, NaCl 5 g/L, pH 7.0). Then, 1 ml *P. aeruginosa* culture was inoculated with 1 ml broth (peptone 5 g/L, beef extract 3 g/L) in the 24-well round-bottom (Corning, USA), and the incubated condition was carried out as Table 1. Additionally, 1.5 cm sterilized UF membrane was put into the well. *P. aeruginosa* was incubated (160 r/min) with 0 g/L (control concentration), 10⁻⁷ g/L (10 μL addition of stored 2×10⁻⁵ g/L C₈-oxo-HSL solution) and 10⁻⁴ g/L (10 μL addition of stored 2×10⁻² g/L C₈-oxo-HSL solution) of C₈-oxo-HSL. The

concentration of C₈-oxo-HSL addition was selected according to previous work (Xia et al., 2012). After incubation, *P. aeruginosa* biofilm was adhered to the UF membrane. The UF membrane was washed by sterilized water for 3 times and the biofilm on the UF membrane was removed by an ultrasonic cleaner (15 min, 55 kHz) in 2 ml sterilized water. The water with biofilm was then measured for polysaccharide and protein as the representation of EPS. The polysaccharide was measured with the phenol-sulfuric acid method using glucose as the standard. The concentration of protein was analyzed by the Coomassie blue staining method using bovine serum albumin as the standard. The experiments were repeated six times for consistent result.

3. RESULTS AND DISCUSSION

3.1 Effects of pH on EPS of *P. aeruginosa* biofilm with C₈-oxo-HSL addition

As one of the major surrounding conditions, pH plays the significant role in bacteria growth and biofilm formation. Fig. 1 showed the polysaccharide and protein variations of EPS of *P. aeruginosa* biofilm with C₈-oxo-HSL addition.

At pH 5-7, the EPS polysaccharide concentration of *P. aeruginosa* biofilm increased with C₈-oxo-HSL addition advance. The increase of C₈-oxo-HSL addition caused a slight decrease of the protein concentration of EPS, and the protein concentration was highest at pH 7. The result indicated that C₈-oxo-HSL addition had a critical effect on the polysaccharide concentration of EPS of *P. aeruginosa* biofilm, especially at pH 5-7. However, C₈-oxo-HSL addition led to obvious EPS polysaccharide change but had little effects on protein concentration variation, although QS was the mechanism of protein release control (Dickschat, 2010). The reasons were as follow: EPS, consisting of polysaccharide, protein,

nucleic acids etc., had the function of the mediation of the initial attachment of cells to different substrata and protection against environmental stress and dehydration (Kokare et al., 2009; Vu et al., 2009). Vu et al. (2009) also reported that microbial extracellular polysaccharides worked as an integral part of bacterial biofilm. The biofilm formation and EPS production was controlled by the protein signal through the regulation of QS (Dickschat, 2010). Additionally, Psl polysaccharide played the dominant part in biofilm and the significant role in the attachment of *P. aeruginosa* to surface in microtiter trays and flow chambers during biofilm formation (Ma et al., 2009). Psl polysaccharide, which was regulated by QS, mediated cell-to-surface and cell-to-cell interaction to form mushroom-shaped structure for protection from the environmental stress or shock (Harmsen et al., 2010). Therefore, C₈-oxo-HSL (QS signal) addition should lead to the obvious EPS polysaccharides concentration change but have little effects on protein variation in the biofilm of *P. aeruginosa*.

Although the concentrations of polysaccharide and protein were different, the variation trends of their concentrations were similar. Both polysaccharide and protein concentrations decreased fast with pH dropping from 5 to 3. Low pH (high H⁺ concentration) would cause the destruction of small pH gradient balance across the cell membrane, which led to an intracellular accumulation of volatile fatty acid anions (Russell and Wilson, 1996). In addition, low pH also induced the bacterial DNA damage (Cotter and Hill, 2003). The lower concentrations decrease of both polysaccharide and protein was because of inactive bacterium due to low pH (3-5). However, the concentration variation trends of polysaccharide and protein with high pH (8-13) were gentler than those with low pH (3-5). It indicated that alkali-resistance of *P. aeruginosa* biofilm formation was stronger than that of acid-resistance. As Chopp et al.

(2003) reported, high pH (8-13, especially over 10.5) could increase the critical concentration for the inducement of QS. Therefore, high pH might inhibit the QS for biofilm formation, but not devitalize bacterium. C₈-oxo-HSL addition should be carried out at pH 5-7 for the promotion of *P. aeruginosa* biofilm formation.

Table 1 The conditions of each batch experiments

	pH batch experiments	Temperature batch experiments	Salinity batch experiments
pH value	3,5,7,9,11,13	7	7
Temperature (°C)	37	10,20,30,50,60	37
Salinity (g/L)	5	5	0,5,10,15,20,25,30

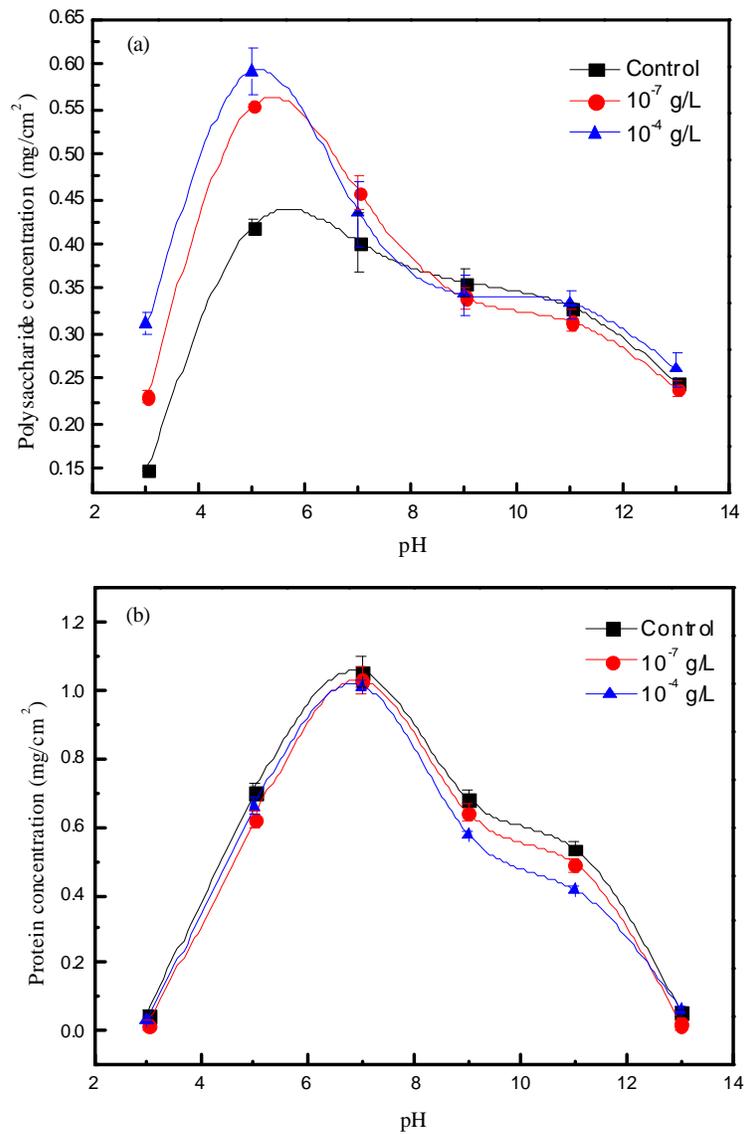


Figure 1 Effects of pH on (a) polysaccharide and (b) protein of *P. aeruginosa* biofilm with C₈-oxo-HSL addition

3.2 Effects of temperature on EPS of *P. aeruginosa* biofilm with C₈-oxo-HSL addition

Fig. 2 presents the polysaccharide and protein variations of EPS of *P. aeruginosa* biofilm under different temperature. Both polysaccharide and protein of EPS of *P. aeruginosa* biofilm reached the highest concentration at 20°C. According to China Center of Industrial Culture Collection, *P. aeruginosa* had the fastest growth rate at 37°C. It indicated that *P. aeruginosa* biofilm could be formed at low temperature, because *P. aeruginosa* accumulated as biofilm was better for prevention from the environmental stress. The community of microorganism in biofilm is well organized and cooperated, and EPS (especially polysaccharide and protein) work as the backbone of the biofilm and cover bacteria as shelter and homeostasis to bacteria residing in biofilm to reduce the direct environmental stress on bacteria (Kokare et al., 2009; Vu et al., 2009). EPS not only has the potential to physical prevent the access of environmental stress, like the shock of pH and metal, by acting as anion exchanger (Kokare et al., 2009), but also work as thermal barrier for bacteria (Goller and Romeo, 2008; Pollanen et al., 2013). Fig. 2 also presented that the *P. aeruginosa* biofilm formation was extremely mitigated at 10°C, which indicated the low temperature inhibited QS to mitigate the biofilm formation. This was because the activity of *P. aeruginosa* was reduced and the enzymes were also retarded due to low temperature. According to Fig. 2 (a), the polysaccharide concentration of EPS of *P. aeruginosa* biofilm decreased with the increase of temperature from 20 to 60°C. According to the CICC introduction, the growth temperature of *P. aeruginosa* was 25-42°C and the *P. aeruginosa* began to be inactive or die at 50°C. Therefore, the protein concentration of EPS suddenly increased at 50°C. At 60°C, the low

polysaccharide and protein concentrations of EPS were due to the death or inactivation of bacterium and the extinction of EPS. However, the biofilm formation of *Burholderiapseudomallei* is faster at 30°C than 37°C due to QS system (Ramli et al., 2012). The temperature had no effect on the QS and biofilm formation of *Vibrio vulnificus* (McDougald et al., 2006) and *Favia sp.* (Golberg et al., 2013). Thus, effects of temperature on the QS and biofilm formation might depend on the class of bacteria.

3.3 Effects of salinity on EPS of *P. aeruginosa* biofilm with C₈-oxo-HSL addition

The mechanism of biofilm formation controlled by QS was first found from marine bacterium (Dobretsov et al., 2011; Miller and Bassler, 2001). Many studies indicated that AHLs production in QS system, especially for marine bacteria, was dependent on salinity (but the mechanism was still unclear) (Bjelland et al., 2012; Ramli et al., 2012; Tavio et al., 2012). Therefore, salinity was considered as one of significant factors for QS (Cai et al., 2013). Fig. 3 showed that both polysaccharide and protein concentrations variations of EPS of *P. aeruginosa* biofilm with C₈-oxo-HSL addition under different salinity.

The protein concentration of EPS was almost similar in the salinity experiment. It was consistent with the results of Fig.1 (b) and Fig.2 (b). C₈-oxo-HSL addition did not induce the variation of protein concentration of EPS. The protein might be a signal in the *P. aeruginosa* biofilm formation, and it needed a further study. Additionally, the variation trend of polysaccharide concentration had a left shift with C₈-oxo-HSL addition in the range of 0-20 mg NaCl/L, which suggested that NaCl was the important factor during bacteria growth. In the natural condition, QS was induced through

the mass propagation of bacteria to release and promote AHL to reach the critical concentration. C₈-oxo-HSL addition facilitated AHL to reach the critical concentration, and bacteria required less NaCl for mass propagation. With over 20 mg/L of NaCl concentration, the polysaccharide concentration trends with variation C₈-oxo-HSL addition were similar. With high NaCl concentration, bacteria growth

was controlled by the osmotic pressure. The highest polysaccharide concentration at 25 mg/L was because of the breach of bacterium membrane due to high osmotic pressure. The decrease of polysaccharide concentration with over 25 mg NaCl/L was due to that the environmental condition overlapped the salinity-resistance of bacteria, which inhibited the bacteria growth.

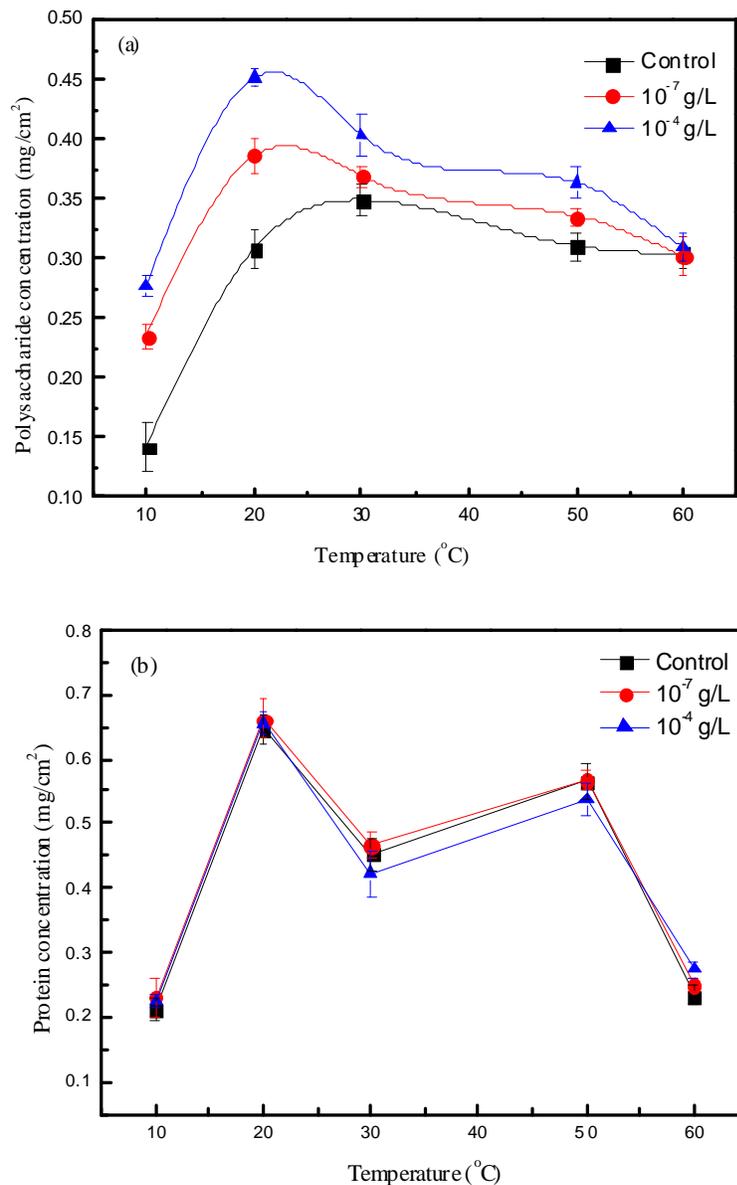


Figure 2 Effects of temperature on (a) polysaccharide and (b) protein of *P. aeruginosa* biofilm with C₈-oxo-HSL addition

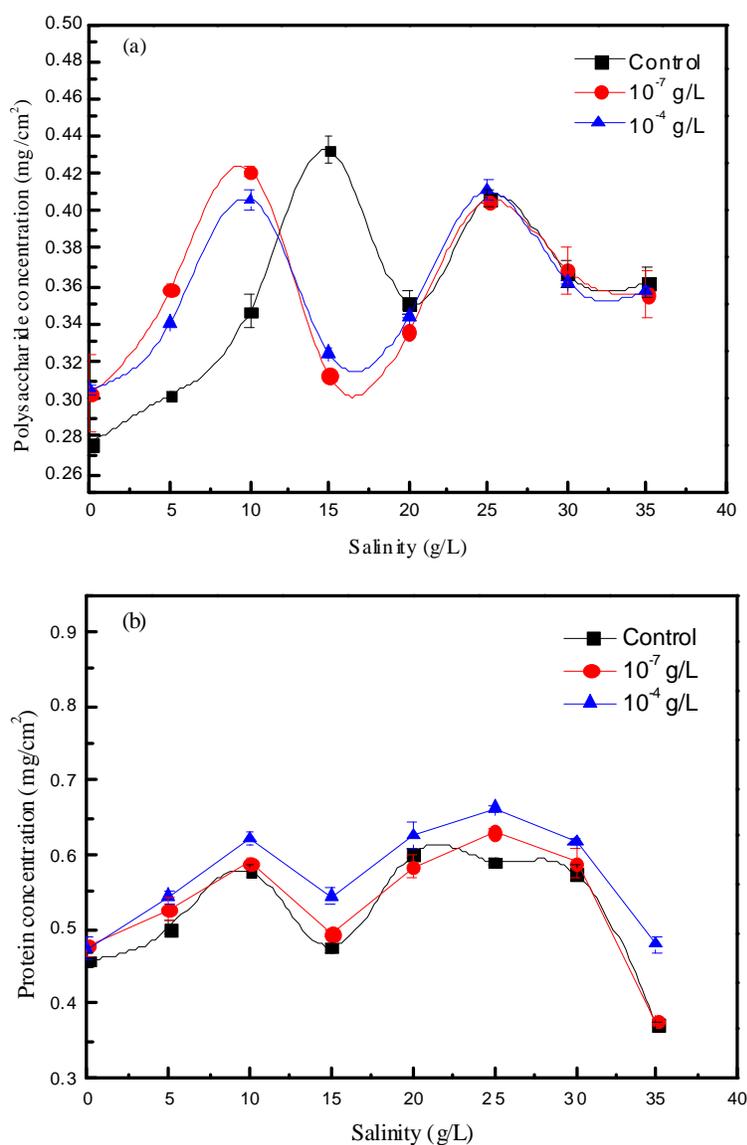


Figure 3 Effects of salinity on (a) polysaccharide and (b) protein of *P. aeruginosa* biofilm with C_8 -oxo-HSL addition

CONCLUSIONS

The effects of pH, temperature and salinity on EPS of *P. aeruginosa* biofilm with C_8 -oxo-HSL addition were evaluated in this study. C_8 -oxo-HSL addition mainly enhanced the *P. aeruginosa* biofilm formation and EPS release through the increase of polysaccharide concentration. C_8 -oxo-HSL addition had little effects on protein of EPS. C_8 -oxo-HSL addition should be carried out at pH 5-7 for *P.*

aeruginosa biofilm formation. The alkali-resistance of *P. aeruginosa* biofilm formation was stronger than that of acid-resistance. Both polysaccharide and protein of EPS of *P. aeruginosa* biofilm reached the highest concentration at 20°C. The C_8 -oxo-HSL addition reduced the effect of salinity on EPS of *P. aeruginosa* biofilm through promoting AHL to reach the critical concentration.

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