Research article

Inhibition of *Pseudomonas aeruginosa* biofilm formation and motilities by human serum paraoxonase (hPON1)

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Abstract: Human serum paraoxonase 1 (hPON1) which hydrolyzes *Pseudomonas aeruginosa* acyl homoserine lactone (AHL) signal molecules was used as antibiofilm agent. hPON1 was purified by using ammonium sulfate precipitation and specially designed hydrophobic interaction chromatography (Sepharose 4B-*L*-tyrosine-1-Naphthylamine) from the fresh human serum. As cell motility of swarming, swimming and twitching are proven instrumental in biofilm formation, we investigated whether or not hPON1 affected the *P. aeruginosa* motility. hPON1 was reduced the early stage of biofilm formation, mature biofilm and motilities. The early stage and old biofilm were decreased more than 50% by 1 mg ml^{-1} of hPON1 concentration within range of 0.1–10 mg ml^{-1}. Additionally, exopolymeric substance (EPS) of mature biofilm was indirectly decreased by hPON1. Inhibitory effect of hPON1 within range of 0.003–30 mg ml^{-1} on swarming and swimming motilities. But it resulted in highly inhibitory effects on twitching motility at concentration as low as 0.3 mg ml^{-1} concentration. This study proved that hPON1 alone can be safely used to inhibit/disrupt the mature biofilms and cell motility of *P. aeruginosa* and beholds much promise in clinical applications.

Keywords: biofilm; exopolysaccharide; hPON1; motility; *Pseudomonas aeruginosa*

1. Introduction

Pseudomonas aeruginosa is a Gram-negative, biofilm-forming bacterium that has been shown to exhibit quorum sensing behaviors using two distinct acylhomoserine lactone (AHL) based

pathways: the rhlI/rhlR pathway, which uses butyryl acyl homoserine lactone (C4-HSL), and the lasI/lasR pathway that uses 3-oxo-dodecanoyl homoserine lactone (3-oxo C12-HSL) [1,2]. *P. aeruginosa* biofilms are important issues in the pathogenesis of the bacterium in a wide variety of infections like ventilator-associated pneumonia and burn wound infections and also found in 10–20% of all hospital-acquired infections [3]. The established biofilms provides permeability barrier for antibiotics [4], making the treatment of *P. aeruginosa* infections intractable.

P. aeruginosa has three modes of surface motility: swarming [5,6], twitching [7], and swimming [8]. Twitching and swarming motilities are strongly linked with biofilm development and pathogenesis, and their mechanical components, the flagellum and type IV pili, have been demonstrated to be virulence factors [5,8]. The swarming, swimming and twitching are strongly related to pathology [9].

Twitching motility is a prerequisite for biofilm formation [10] and a surface-associated motility that involves the extension, tethering and retraction of the polar type IV pili [11]. According to Barrionuevo and Vullo [12], the presence of flagella allows bacterial cells to propagate through liquid or medium of very low viscosity. The formation of complex bacterial communities known as biofilms begins with the interaction of planktonic cells with a surface in response to appropriate environmental signals [13]. O'Toole and Kolter [13] have shown that nonswarming, nonswimming or nontwitching bacteria have reduced ability of biofilm formation.

The antimicrobial agents such as chemical biocides, detergents, and surfactants are the main strategies to control and prevent the formation of biofilms. Besides chemical treatment, several methods have been proposed to prevent and destroy biofilms including mechanical removal such as scraping, sonication, freezing, and thawing [14]. There is a need for methods that are capable of removing the biofilms and destroying the exopolymeric substance (EPS) as biofilm matrix. Moreover, because of the wide heterogeneity of EPS between bacterial species, a search for new enzymes and for understanding the role of EPS in biofilm cohesiveness is required to improve biofilm removal. Application of enzymes which remove biofilm by destroying the physical integrity of the EPS would be an attractive strategy for the control and removal of biofilms [15].

Paraoxonases (PONs) are enzymes originally described based on their ability to hydrolyze organophosphates. Since the initial descriptions of enzymatic activity of PONs, three members of the PON family have been described: PON1, PON2 and PON3 [16]. Recent studies have shown that all three PON proteins exhibit lactonase activities [17]. Interestingly human as well as mouse PONs are capable of hydrolyzing and thereby inactivating N-acyl-homoserine lactones, which are quorum-sensing signals of pathogenic bacteria such as *P. aeruginosa* [17,18,19]. Human paraoxonase (hPON1; EC 3.1.8.1) is a 45 kDa glycoprotein expressed primarily in the liver and found associated with HDL particles in the blood [20,21]. A previous study demonstrated inhibition of *P. aeruginosa* biofilm formation by PON1 in an *in vitro* biofilm model [18]. In this study, we investigated the effects of hPON1 on biofilm formation, mature biofilm and cell motilities of *P. aeruginosa* ATCC35032. hPON1 in various concentrations was tested with the aim of identifying the hPON1 that would exert effects on these physiological processes.

2.   Material and Methods

2.1. Chemicals, bacterial strain and growth media

The materials used include sepharose 4B, *L*-tyrosine, 1-napthylamine, paraoxon, protein and polysaccharide assay reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals purchased from either Sigma-Aldrich and Merck (Darmstadt, Germany). *P. aeruginosa* ATCC35032 used in this study was maintained in Muller-Hinton Broth (MHB) and allowed to reach to stationary phase prior to inoculation for the biofilm assays. The purified hPON1 was prepared at different concentrations followed filter sterilization using 0.2 μm pore size filters.

2.2. Purification and enzyme activity assay

Human serum was isolated from fresh human blood. The blood samples were centrifuged at 1500 *g* for 15 min and the serum was collected and used for purification of hPON1 by ammonium sulfate precipitation The precipitation intervals for hPON1 were 60–80% [23]. Precipitated fractions were dissolved in 100 mM Tris–HCl pH 8.00 and subjected to hydrophobic interaction chromatography using a column with specially designed Sepharose 4B-*L*-tyrosine-1-napthylamine [22,23]. An incremental amount of gradient of ammonium sulfate from 0 to 1 M was used. Paraoxonase activity towards paraoxon was quantified spectrophotometrically by the method described by Gan et al. [24]. The protein samples containing the highest paraoxonase activity were combined and used for biofilm and motility assays. The purified hPON1 was stored in the presence of 2 mM $CaCl_2$ at +4 °C for no longer than a week, in order to maintain activity and controlled activity prior to inoculation into the each assay or each cultivation.

2.3. Determination of protein and exopolysaccharide concentration

The concentrations of purified protein samples and the protein content of EPS were determined by Lowry method using the wavelengh of absorbance at 600 nm with bovine serum albumin (BSA) as the standard [25]. For each purification, protein concentrations of hPON1 were determined as within range of 10–30 mg ml^{-1} within similar activity (data not shown). The amount of total exopolysaccharide from EPS was determined by phenol-sulfuric acid method using the wavelengh of absorbance at 490 nm with glucose as the standard [28].

2.4. Biofilm formation assay

The biofilm formation of *P. aeruginosa* ATCC35032 was determined by spectrophotometric and microscopic analyses using microtitre plate assay [26]. Briefly, each well of a microtitre plate was loaded with 100 μl of hPON1 within range of 0.1–10 mg ml^{-1} in MHB as well as a control well without enzyme. *P. aeruginosa* ATCC 35032 was cultured in MHB for 24 h (the stationary phase of bacterial strain) at 37 °C. The bacterial suspension was diluted to 1×10^6 CFU ml^{-1} in MHB and 100 μl of bacterial suspension was added to individual wells in 96-well microtiter plates. Plates were incubated up to 96 h at 35–37 °C. For mature biofilm assay, the media containing of hPON1 within range of 0.1–10 mg ml^{-1} in the wells were periodically (for each 24 hours of cultivation) replaced to fresh medium. After 24 h incubation, the wells were washed thrice with distilled water and allowed to air dry for 30 min. Furthermore, the wells were stained with 0.1% solution of crystal violet in water and incubated at room temperature for 30 min. The wells were washed with distilled water to remove excess stain again and then allowed to air dry. Finally, the wells were destained with

absolute ethanol and the absorbance was read at 590 nm in the spectrophotometer. After 96 h incubation, all experimental steps were done for mature biofilm samples. Results are means of 3 experiments using 3 independent samples.

For microscopic analysis, the early stage and mature biofilms-treated hPON1 were allowed to grow on 24-well polysystrene plates. The plates were incubated at 30 ^{o}C for 24 h in static condition. After incubation, the each well was stained by above mentioned steps. Stained samples were placed on slides and finally inspected by light microscopy at magnifications of ×100. The visible biofilms were documented with an attached digital camera.

2.5. EPS production in presence of hPON1

The EPS of mature biofilm was extracted as per Nithya et al. [27]. The content of the EPS was chemically analyzed. Briefly, the precultured cells were cultivated within 1, 5 and 10 mg ml^{-1} of hPON1 at 96 h and the supernatant was collected from culture medium by centrifugation at 8.000 g for 5 min. Cells collected by centrifugation were washed twice with 0.9% NaCl and then dried at 70 ^{o}C until the cell weight remained constant. Cell biomass was determined by weighing. The EPS on mature biofilm without enzyme treatment served as control. To analyze, the exopolysaccharide content of supernatant was determined by the phenol-sulfuric acid method described by Dubois et al. [28] with glucose as the standard (1 mg ml^{-1}) and the protein content of supernatant was estimated by Lowry method [25] with bovine serum albumin (200 μg ml^{-1}) as the standard.

2.6. Motility assays

The swarm, swim, and twitch media were prepared to assess the corresponding type of *P. aeruginosa* motility. The swarm medium consisted of 8 g l^{-1} nutrient broth and 0.5% (w:v) agar supplemented with D-glucose (5 g l^{-1}, filter sterilized and added separately). The swim and twitch media consisted of Luria-Bertani (LB) broth supplemented with 0.3% and 1.0% (w:v) agar, respectively [29]. The agar media were air dried for 15 min before use. The 100 μl of hPON1 within range of 0.003–30 mg ml^{-1} was spreaded over the surface of the each agar medium. An equal amount of deionized water without hPON1 was agar medium that served as a control.

For the swarming and swimming assays, the bacterial cells were gently inoculated using a toothpick at the center of the agar surface, and the plates were incubated at 30 ^{o}C for 16–24 h. For the twitching motility assay, the bacterial cells were stabbed into the bottom of a petri dish containing the above agar medium using a toothpick and incubated at 37 ^{o}C for 20 h [30]. The movement of the colony on the interface between the agar medium were observed and halo diameters were measured.

3. Results
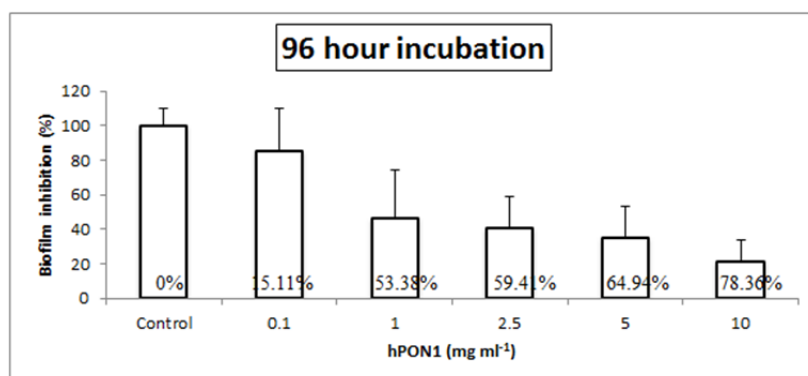
3.1. Antibiofilm effect of hPON1

The hPON1 was evaluated for antibiofilm activity against *P. aeruginosa* ATCC35032. The hPON1 was found to be an effective antibiofilm agent. The quantification analysis (Figure 1) and light microscopic images (Figure 2) confirmed the efficiency of hPON1 in removing/decreasing of

the biofilm. The hPON1 revealed remarkable ability to disrupt the early stage of biofilm formation and mature biofilms (Figure 1). As per the quantification analysis, the maximum inhibition by hPON1 was observed at 10 mg ml^{-1} of hPON1 caused 87.95% and 78.36% repression of the early stage (24 hour incubation) and the late stage (96 hour incubation) of biofilm, respectively. The inhibited biofilm is 12.05% for early stage of biofilm and 21.4% for mature biofilm when compared with the control biofilm (Figure 1). While the 0.1 mg ml^{-1} of hPON1 at 96 h incubation had no significantly effect on mature biofilm, the same concentration of hPON1 significantly inhibited biofilm formation at 24 h incubation.

As seen in Figure 2, it was clearly found that the hPON1 enzyme-treated biofilm architectures of the early and late stage of biofilm were looser than control biofilm. These observations proved the real potential of hPON1 in disturbing the biofilm architecture of *P. aeruginosa* ATCC35032.
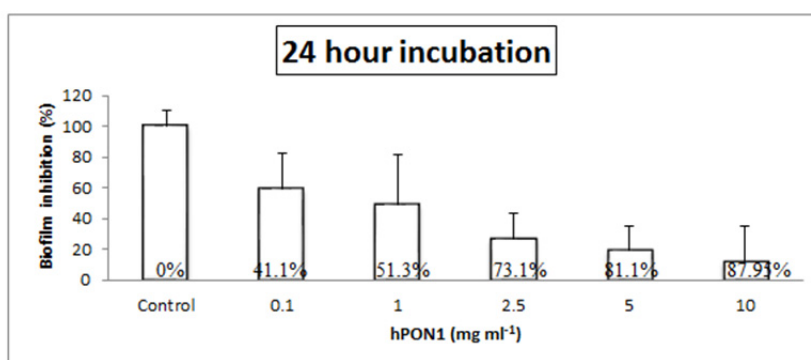
(a)

(b)

Figure 1. Inhibitory effect of hPON1 on the early stage of biofilm formation (a) and mature biofilm (b) by *P. aeruginosa* ATCC35032. The average ± SD is shown in each column. The ratio of biofilm absorbance/planktonic absorbance was calculated, and this value was used to calculate the early stage of biofilm formation and mature biofilm on the y axis. x axis represents the concentration of hPON1 used in the wells. Bars represent means ± SEM (*n* = 3 independent experiments).
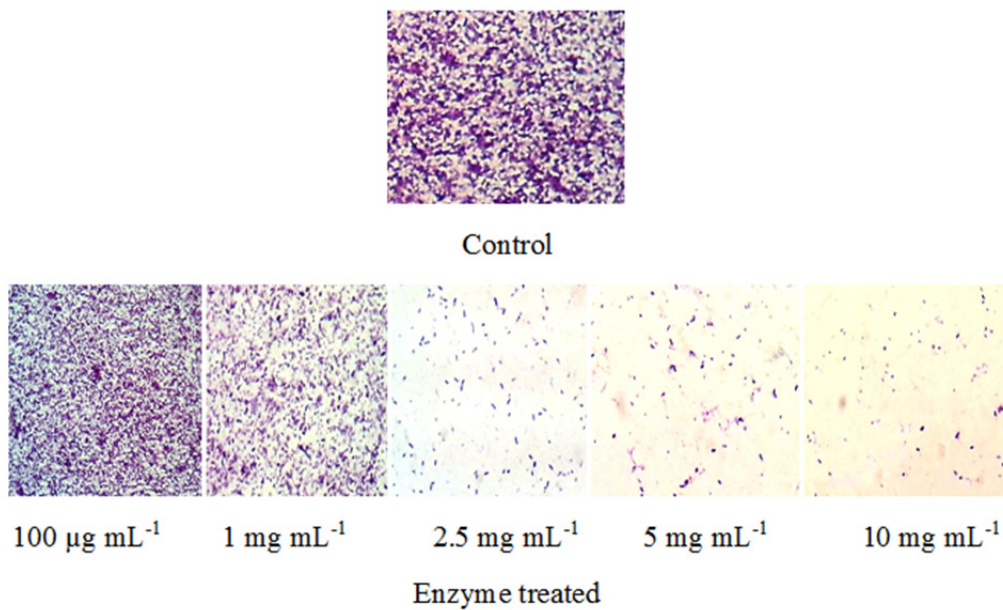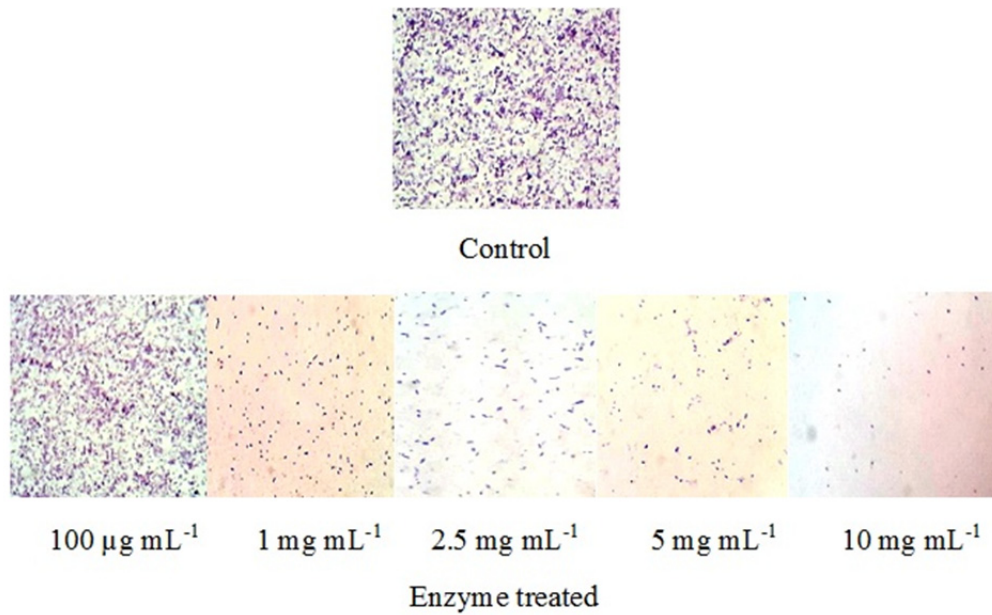
(a)

(b)

Figure 2. Microscope observation of the early stage of biofilm formation (a) and mature biofilm (b) inhibition in presence of hPON1. Removal of the early stage of biofilm formation and mature biofilm of *P. aeruginosa* ATCC35032 was shown on glass cover slip under a light microscope at a magnification of ×100. Control: no enzyme.

3.2. Effect of hPON1 on EPS production

The effect of hPON1 on EPS production was analyzed by isolating of the EPS from *P. aeruginosa* ATCC35032 mature biofilm treated with the hPON1. The exopolysaccharide and protein content of EPS were estimated in nontreated and treated samples. Here, it was evident that the exopolysaccharide and protein contents treated with hPON1 were indirectly reduced. When it was compared the cell biomass (mg of cell dry weight per ml of culture) of EPS samples nontreated and treated with hPON1, the use of hPON1 resulted in a reduction on cell dry weights in presence of enzyme.

As can be seen in Table 1, the control strains contained 279.6 µg ml^{-1} of protein and 1914 µg ml^{-1} of exopolysaccharide. After treatment with 10 mg ml^{-1} of purified hPON1, the protein and exopolysaccharide content were reduced to 163.4 µg ml^{-1} and 902 µg ml^{-1}, respectively. The 10 mg ml^{-1} of hPON1 indirectly caused 41% and 53% reduction of proteins and exopolysaccharides in EPS by AHL hydrolysing capability of enzyme.

Table 1. Analysis of total exopolysaccharide and protein content of control nontreated and treated with purified hPON1 of EPS. Error bars represent SD of three replicates. The experiment was performed on the glass slides on which the mature biofilm formed. EPS was isolated from *P. aeruginosa* ATCC35032 mature biofilm treated with different concentration of hPON1 for 96 h. The EPS without hPON1 treatment served as control. Cell dry weights of samples were determined. After incubation, the protein and exopolysaccharide content of supernatant were spectrophotometrically determined by using BSA and glucose standard, respectively.

| hPON1 (mg ml^{-1})	Cell dry weight (mg ml^{-1})	Protein content (µg ml^{-1}) in EPS	Exopolysaccharide content (µg ml^{-1}) in EPS
0 (Control)	22.76 ± 0.028	279.6 ± 0.05	1914 ± 0.1
1	20.32 ± 0.11	245.6 ± 5.7	1268 ± 7.1
5	17.57 ± 0.23	189.9 ± 2.6	987.3 ± 2.9
10	15.68 ± 0.21	163.4 ± 0.02	902 ± 0.08

Means ± SD
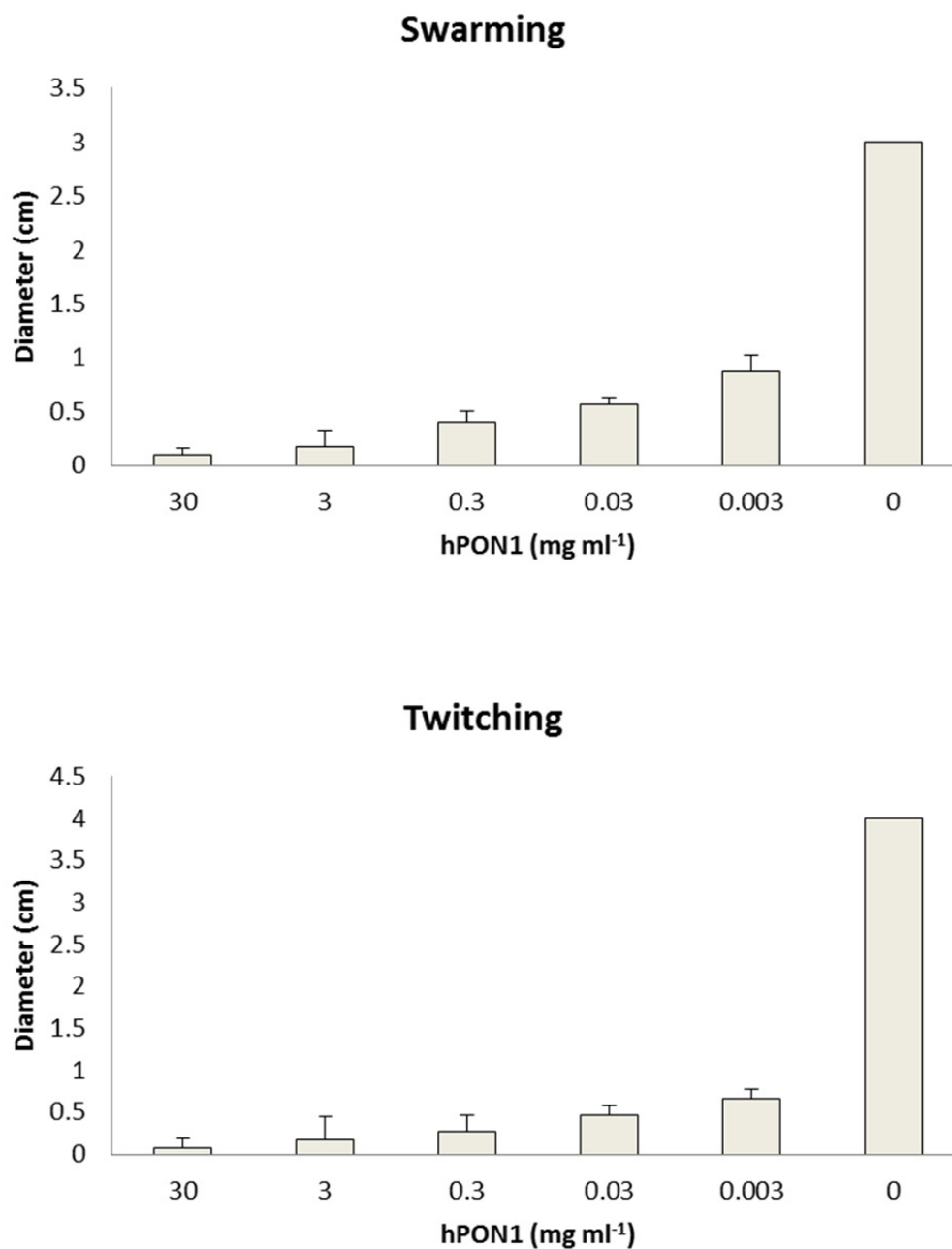
3.3. Inhibition of P. aeruginosa motilities

P. aeruginosa is known to exhibit movement on surfaces by three types of motilities: swimming, swarming and twitching. Figure 3 showed the typical colony spreading patterns of these motilities of *P. aeruginosa* ATCC35032 in presence of hPON1 within range of 0.003–30 mg ml^{-1}. The twitching was strongly inhibited by hPON1, into the agar medium. As shown in Figure 3a(A), *P. aeruginosa* twitching was repressed in a dose-dependent manner by hPON1 and was almost completely inhibited at 30 mg ml^{-1} of hPON1.

Furthermore, the hPON1 showed a significant inhibitory effect on swimming and swarming motilities (Figure 3a (B and C)). The averages and SDs (n = 3) of the swimming and swarming diameters after 20 h of incubation in the absence and presence of 30 mg ml^{-1} of hPON1 were lesser than 0.5 cm (Figure 3b).

(a)
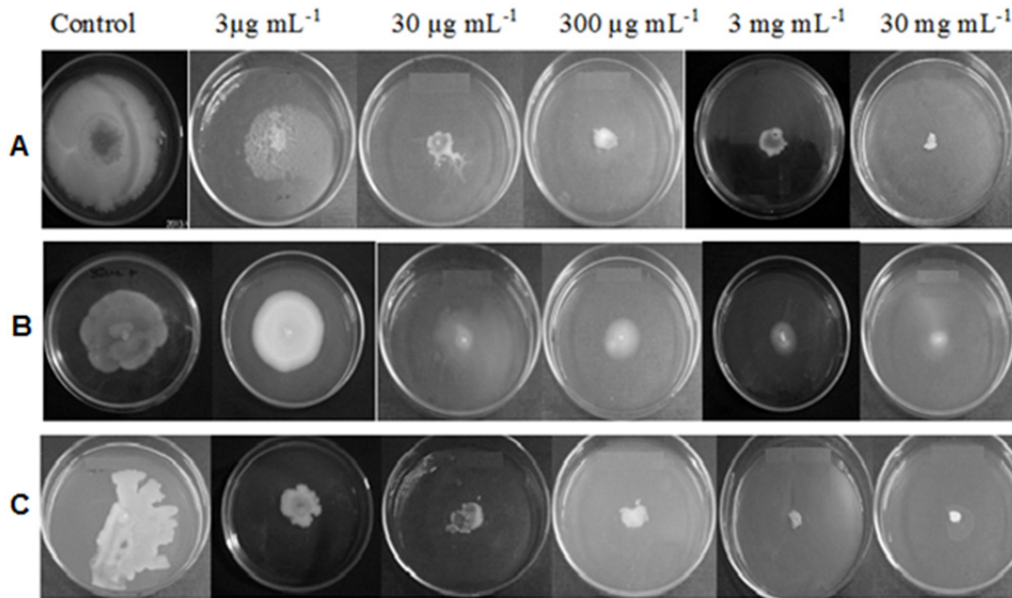
Swarming
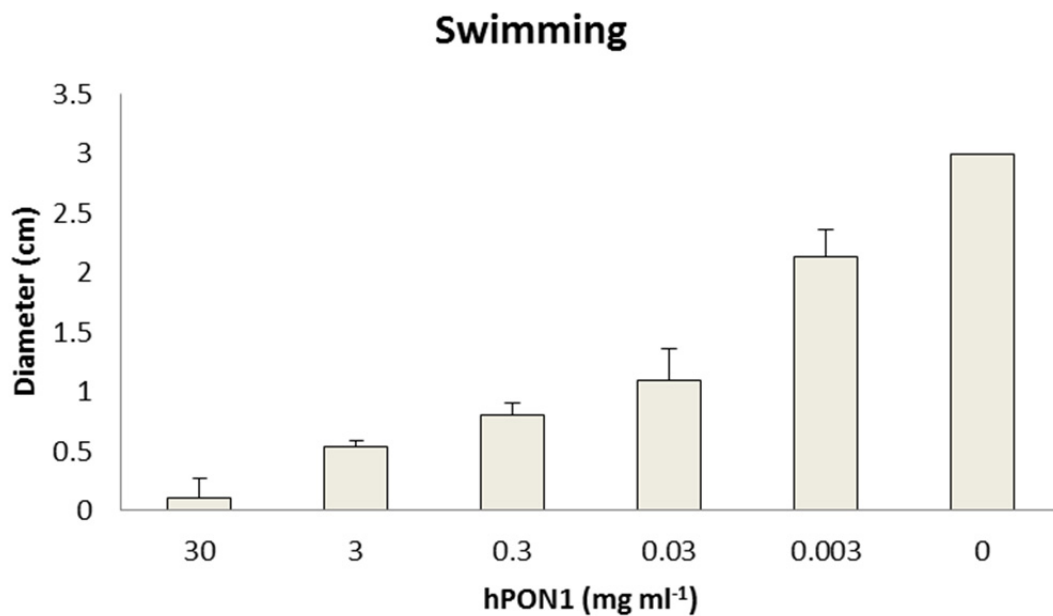
Twitching

(b)

Figure 3. Representative images of *P. aeruginosa* swarming (A), swimming (B) and twitching (C) motility under control conditions and in the presence of different concentrations of hPON1 (a). Dose-dependent inhibition zone diameters (cm) of *P. aeruginosa* swarming, swimming and twitching motilities in the presence of 0.003–30 mg ml^{-1} of hPON1. hPON1. Error bars represent SD of two replicates. A colony of the overnight culture of *P. aeruginosa* ATCC35032 was added to each motility agar within range of 0–30 mg ml^{-1} of hPON1 and incubated as mentioned Materials and methods section (b).

4. Discussion

It was reported that mammalian cells release enzymes called paraoxonases 1 (extracted from human and murine sera) that have lactonase activity, played an important role against biofilm formation of *P. aeruginosa* utilized AHLs in the form of (QS) signals to promote biofilm formation and to regulate several virulence factors [17,18,31,32,33]. It was known that hPON1 hydrolyzed the homoserine lactone ring of AHL signals to indirectly inhibit QS-controlled biofilms and virulence factors of *P. aeruginosa* [17,18,19,23]. In a study, N-acyl-homoserine lactones (AHLs) were detected on bacterial supernatants of late exponential and stationary phase culture broths [34]. It was reported that signal molecules were produced maximally when cultures reach the late stationary phase of growth [35,36]. So that stationary growth phase time of *P. aeruginosa* ATCC35032 were determined as 24 h (data not shown) and the stationary phase cells were inoculated to biofilm and motility assays' media.

In the present study, hPON1 as lactonase treatment was significantly reduced the early stage biofilm formation. Recent studies verified that *P. aeruginosa* biofilm formation was inhibited by paraoxonases [17,18,19,23]. *P. aeruginosa* is capable of undergoing transient phenotypic changes that allow the bacteria to increase their antibiotic resistance. Hence, the mature biofilms are difficult to eradicate than the planktonic bacteria. Surprisingly, the mature biofilm was firstly inhibited by purified serum PON1. Additionally, analysis of the biofilm samples using light microscope indicated disruption of biofilm architecture after treatment with hPON1. It has been observed that the hPON1 as an antimicrobial enzyme hinders biofilm formation of *P. aeruginosa*, this enzyme could potentially be used as a safe and effective antibiofilm agent.

The production of EPS leads to alterations in biofilm architecture that correlate with an increased resistance of the biofilm to biocides such as chlorine and hence their persistence [37]. So the EPS must be disrupted to achieve permanent biofilm removal. The EPS production in mature biofilm was indirectly decreased by enzyme. Cell concentration of nontreated EPS was found more than samples in presence of hPON1. In some studies, it was observed that exopolysaccarides are the main constituents of the EPS while some studies found proteins to dominate [38]. In this study exopolysaccarides were also found to be dominant (1914 µg ml^{-1}) rather than protein (279.6 µg ml^{-1}). The exopolysaccaride and protein content of EPS produced in presence of hPON1 were indirectly reduced. hPON1 can not be acted as protease or amylase to degrade of EPS contents. During EPS production, it can be reduced by AHL molecules which are responsible for production of EPS matrix. The alginates which regulate by AHL-related genes are the major polysaccaride of *P. aeruginosa* EPS matrix [39]. The large variety of alginates required for bacterial adherence and stability of biofilm structure. It indicated that inhibition of biofilm formation by hPON1 was also related with reduction of EPS content such as polysaccarides in presence of hPON1.

Because there is an inverse relationship between bacterial motility and biofilm formation [13,40,41], we investigated the effect of hPON1 on *P. aeruginosa* motility. Importantly, an indirect link between biofilm formation and QS has been reported, through the control of swarming, swimming and twitching motilities. The swarming motility, a form of organized surface translocation, depends on extensive flagellation and cell to cell contact; regulated by the *hl* ystem, is implicated in early stages of *P. Aeruginosa* iofilm establishment [43]. n general, both biofilm formation and swarming are considered to require various cellular processes, including the production of flagella and surface polysaccharides. Therefore, it is possible that hPON1 coordinately

regulates the production of these surface materials [44]. In previous studies, it was found that a BCFA, anteiso-C15:0, inhibited swimming, swarming, colony wetness, and biofilm formation in *P. aeruginosa* [44]. In another study, the swarming motility of *P. aeruginosa* was blocked by cranberry proanthocyanidins and other tannin-containing materials [45]. There was also a direct inhibitory effect of salicylic acid on swarming, swimming, and twitching motility in both PAO1 and PA14 lab strains [46]. Additionally, researchers showed that heterologous expression of aiiA (AiiA lactonase gene) in *P. aeruginosa* PAO1 completely prevented the accumulation of the *rhl*1 generated AHLs. This strongly reduced AHL content correlated with a markedly decreased expression and production of several virulence factors and cytotoxic compounds such as elastase, rhamnolipids, hydrogencyanide and pyocyanin, and strongly reduced swarming [47]. In this study, a normal swarming movement was not observed in almost cells grown on swarm plates with different concentrations of hPON1 under the light microscope; this finding supports that flagella biosynthesis and function were severely impaired in the presence of hPON1. Although the inhibition of swimming motility was not as drastic as that of swarming motility, the hPON1 might have some effects on flagella-related processes, namely, flagella biosynthesis, rotation, and chemotaxis, which may lead to a decrease in swimming and swarming activities. Twitching motility, a flagella-independent form of bacterial translocation, occurs by successive extension and retraction of polar type IV pili [48]. Known to be regulated by the *hl* stem, twitching motility is required for the assembly of a monolayer of *P. Aeruginosa* ells into microcolonies and plays a role in the early stages of biofilm formation on abiotic surfaces by using type IV pili [13]. Work by Pamp and Tolker-Nielsen showed that Type IV *pili* otility is facilitated by rhamnolipid [49] and rhamnolipid production can be reduced by hPON1 [33]. So that, twitching motility was the most affected by the addition of hPON1; this result indicated that twitching inhibition by addition of hPON1 could be caused by defects in pili-related functions.

5. Conclusion

hPON1 as lactonase treatment indirectly reduced the early stage of biofilm formation, mature biofilm, EPS as biofilm matrix and cell motility including swarming, swimming and twitching which are controlled by *las* and *rhl* QS system by signal interference effect of hPON1. The findings from this study would contribute to future investigation on the potential use of enzymes like hPON1 for treatment or controlling of microbial diseases associated with uncontrolled mature biofilm.

Conflict of Interest

All authors declare no conflicts of interest in this paper.

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