EFFECT OF GROWTH CONDITIONS ON BIOFILM FORMATION BY PHENOL-DEGRADING BACTERIA ISOLATED FROM POLLUTED AND NON-POLLUTED SOURCES

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ABSTRACT

Our previous research have isolated four phenol degrading bacteria. There are ATA6, DOK135, and DL120 which isolated from polluted source (hospital wastewater), also HP3 which isolated from non polluted source (peat soil). The purpose of this research is to analyze the effect of some environmental factors on the ability of four isolates to form biofilm. The environment factors were varied, such as growth medium, incubation temperature, and medium pH. Biofilm formation was measured using microtiter plate and crystal violet method, and the absorbance was read with microtiter auto reader at wavelenght 490 nm. The result showed that ATA6 was a strong biofilm former, DOK135 and HP3 were moderate biofilm former, and DL120 was a weak biofilm former. The results indicate that there is variation in the ability of selected isolates to form biofilm on various environmental factors. Generally, the isolates formed thicker biofilm in TSB medium which is a complex medium that provide more complete nutrient and formed biofilm optimally at 30^{o}C. ATA6 formed biofilm optimally at pH 7 and HP3 at pH 9, while pH treatment did not affect on isolates DOK135 and DL120 to form biofilm.

Key words: biofilm, environmental factors, microtiter

INTRODUCTION

The most common mode of bacterial growth in nature is biofilm. That is bacterial consortium which immobilized on a surface of liquid environment. Previous studies in biofilm usually were focused on biofilm that generate disadvantages, such as biofilm which related to a disease or can cause contamination of medical equipment (Møretrø, *et al.,* 2003; Allegrucci, *et al.*, 2006; Palmer *et al.,* 2006; Martinez and Arturo, 2007; Cerca and Jefferson, 2008; Pompilio *et al.*, 2008).

On the other hand, bacteria can develop biofilm which have some advantages, for example survival ability in hostile environment and disperse to form new colony (Martinez and Arturo, 2007). Another advantage is the ability to enhance degradation of recalcitrant compounds, because bacteria in biofilm will interact each other and complete the existing metabolic process (Andersson, 2009). Therefore, there is an extra concern on biofilm in degradation of contaminant. Study of biofilm in wastewater treatment has been done on biofilm formation in wastewater (Dumitru *et al.*, 2008), biofilm formation by chlorobenzoat-degrading bacteria (Yoshida *et al.,* 2009), and biofilm formation on some materials (Andersson *et al.*, 2008).

Phenol is one of hazardous aromatic pollutants that need to be concerned. It can be found in wastewater from chemical instalation, hospital (Lee *et al.,* 1997; Al-Thani *et al.,* 2007), industry of textile, dye, petroleum, and pharmaceutical (Tsai *et al.,* 2005; Prpich and Daugulis, 2005; Mailin and Firdausi, 2006). Phenol can be absorbed by skin contact, inhalation, and digestion. Phenol can cause some negative effects, such as chemical burn on the location contact with phenol, sistemic toxicity, reproductive toxicity, and can lead tumor (Anonymous, 2008).

Wastewater treatment is needed to reduce phenol and its derivatives in the environment. Wastewater treatment can be conducted by physical, chemical, or biological method. Biological treatment (bioremediation) offers an alternative which is more economical and more safe for the environment because of its low possibility to produce by-product (Al-Thani, *et.al,* 2007). Moreover, bioremediation can degrade pollutant completely or change the pollutant into less hazard compound (Mahiuddin *et al.,* 2012).

Bacteria are member of microbes that involve in biodegradation, including biodegradation of phenol. Phenol degrading bacteria can be isolated from industrial and municipal wastewater (Mailin dan Firdausi, 2006; Khuanmar *et al.*, 2007; Ying *et al.*, 2007; Movahedyan *et al.*, 2009; Chakraborty *et al.*, 2010), wastewater treatment plant of oil refineries (Ren *et* al, 2008), and soil in industrial area or which is contaminated by industrial wastewater (Tsai *et al.*, 2005; Al-Thani *et al.*, 2007; Agarry *et al.*, 2009; Mohite *et al.*, 2010). Phenol degrading bacteria can be also obtained from not-contaminated area, such as from sediment of ditch and river drainage (van Schie and Young, 1998) and soil (Amro dan Soheir, 2007; Wang YD, 2007).

Based on the above explanation, biofilm has important role in wastewater treatment because there is an interaction between bacteria in degradation of wastewater toxic compound. On the other side, phenol degaradation technologies still need to be improved.

Some environmental factors, such as nutrition and temperature can influence biofilm formation (Goller dan Romeo, 2008). The number and type of nutrient in medium

can affect the rate of biofilm formation. Phosphat is one of important nutrient that supports cell to adhere each other. High temperature in range of culture growth can increase cellular growth rate, production of extra polymeric substrates (EPS), and attachment to a surface (Qureshi *et al.*, 2005). In some cases, low concentration of oxygen will generate oxidized metabolit, such as organic acid which will be used by other bacterial species in biofilm. Low concentration of oxygen also support the existence of micro-aerophil bacteria. Similar condition also happened when bacteria incubated in various pH (Anonim, 2010).

This study aimed to analyze the effect of environmental factors on biofilm formation ability of phenol degrading bacteria which isolated from hospital wastewater and peat soil. The environment factors were varied, such as medium, incubation temperature, and medium pH.

MATERIALS AND METHODS

Bacteria Isolates

The phenol degrading bacteria which used in our study were isolated from some hospital wastewater in Daerah Istimewa Yogyakarta Province and from peat soil of palm oil area at Desa Asam Jawa, Kecamatan Air Batu, Labuhan Batu, Riau.

Growth Medium and Chemical Reagentss

Ramsay medium (NH_4NO_3 2 g; KH_2PO_4 0,5 g; K_2HPO_4 1 g; $MgSO_4.7H_2O$ 0,5 g; $CaCl_2.2H_2O$ 0,01 g; KCl 0,1 g; aquades 1 L) with 300 ppm phenol and 0,5% glucose was used for qualitative test of biofilm formation and Trypticase Soy Broth (TSB) medium for quantitative biofilm formation test. Chemical reagents for biofilm test were 0,1% crystal violet and PBS (Phosphate Buffer Saline) pH 7,3.

Effect of Various Medium on Biofilm Formation

Assessment of biofilm formation ability was modified from Mathur et al (2006) and Merrit et al (2011) methods. Qualitative test was conducted with tube method (TM) and quantitative test using microtiter plate. Qualitative test was done by inoculating the isolates in Ramsay Broth medium which was added with 300 ppm of phenol and 0,5% glucose ; also in TSB medium. Each inoculum was incubated for 48 hours at 37^{o}C. The tubes were decanted and washed with PBS then dried. Dried tubes were stained with 0,1% crystal violet. Excess staine was removed and tubes were washed with destilated water. Tubes then dried in inverted position and observed for biofilm formation.

Quantitative assessment of biofilm formation was conducted by inoculating 10^6 CFU/mL of each isolate in TSB medium for 24 hours at 125 rpm and room temperature. Then, 100µL sample of each isolate was inoculated in four wells of microtiter plate. Microtiter plate was covered and incubated for 48 and 72 hours at room temperature. After incubation, medium in microtiter plate was decanted and washed with aquades. Then, 125 µL of crystal violet solution 0,1% was added in each well and stand for 10 minutes at room temperature. Crystal violet was removed, the plate was washed with aquades and the plate was air dried in inverted position for 1-2 weeks.

Quantitative analysis was conducted by adding 200 µL of PBS in each well and stand for 10-15 minutes at room temperature. Content of each well was briefly mixed by pipetting, and removed 125 µL of that solution into new microtiter plate. Next, that crystal violet solution was read with microtiter auto reader (Bio-Rad Mode 680) at wavelenght 490 nm. Classification of biofilm formation ability was based on Møretrø et al. (2003). The isolate was classified as a weak biofilm former when $A_{490} < 0,20$; as a medium or moderate biofilm former if $0,20 \leq A_{490} \leq 1,0$; and as strong biofilm former if $A_{490} > 1,0$.

Effect of Temperature on Biofilm Formation

Isolates (10^6 CFU/mL) were inoculated in TSB medium and incubated at various temperature (25, 30, and 35^{o}C) for 1, 2, 3, 4, 5, 6, and 7 days. The assessment of biofilm formation was done as quantitative test.

Effect of Various pH on Biofilm Formation

Isolates (10^6 CFU/mL) were inoculated in TSB medium at various pH (5,7, and 9) and incubated for 1, 2, 3, 4, 5, 6, and 7 days. The assessment of biofilm formation was done as quantitative test.

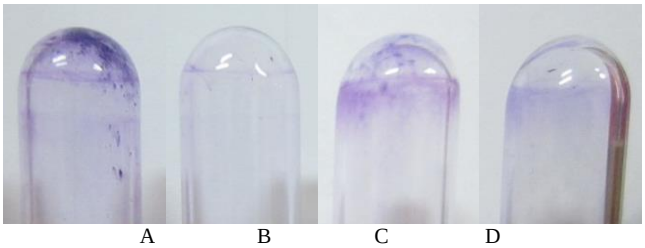
RESULT

Biofilm Formation in Ramsay Phenol-Glucose and TSB Medium

The TM test showed that the isolates performed various biofilm development ability, whether in Ramsay phenol-glucose medium (fig. 1) as well as in TSB medium (fig. 2). The ability to form biofilm can be tested by adding crystal violet solution. Polysaccharides in biofilm will bind crystal violet. The intensity of violet color was representing the biofilm thickness. In TM test, biofilm formation was shown by violet ring on tube wall. The violet ring was thicker in TSB medium which meant that biofilm formation was more optimal in TSB medium than in Ramsay phenol-glucose medium. Therefore, TSB medium was used for further quantitive test of biofilm formation. It can be seen from figure 3 that the isolates formed biofilm with various violet color intensity after 48 and 72 hours of incubation.

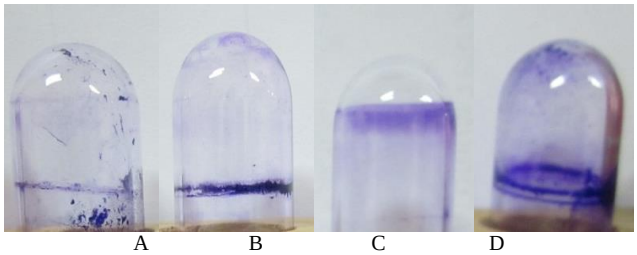
Crystal violet solution in microtiter plate then was measured using microplate auto reader at wavelength of 490 nm (table 1). Based on Møretrø et al (2003), isolate ATA6 can be classified into strong biofilm former. DOK135 and HP3 were classified as medium or moderate biofilm former, and DL120 as weak biofilm former. It is shown in table 1 that HP3 perform the increasing of biofilm formation after 72 hours incubation, while the other three isolates perform declining biofilm formation. However, the biofilm formation dynamic did not change the classification

of biofilm formation ability. Based on the dynamic in biofilm formation at two incubation time, it can be assumed that there was a fluctuation of biofilm formation.



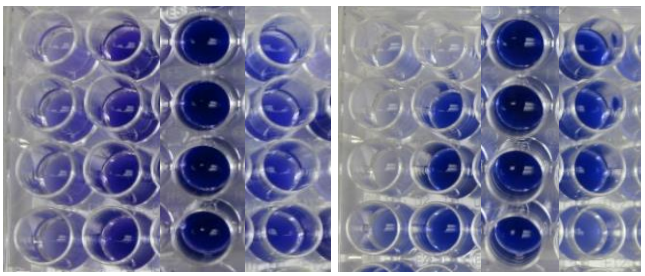
A B C D

Figure 1. Result of qualitative assesment of biofilm formation using test tube method in Ramsay phenol-glucose (300 ppm-0,5%) medium. All isolates shown thin biofilm (represent by the intensity of violet color). A) DL120, B) DOK135, C) ATA6, and D) HP3



A B C D

Figure 2. Result of qualitative assesment of biofilm formation using test tube method in TSB medium. All isolates shown thicker biofilm than if the isolates was grown in Ramsay phenol-glucose (300 ppm-0,5%) medium (represent by the intensity of violet color). A) DL120, B) DOK135, C) ATA6, and D) HP3



A B C D A B C D

Figure 3. Biofilm formation in microtiter plate after 48 (left) and 72 (right) hours of incubation. A) DL120, B) DOK135, C) ATA6, and D) HP3

Table 1. Result of quantitative assessment of biofilm formation using microplate auto reader at 490 nm after 48 and 72 hours of incubation

No	Isolate	Source	OD Biofilm (λ = 490 nm)	
			48 hours	72 hours
1	ATA6	Hospital wastewater	1.034±0.061	0.906±0.135
2	DOK135	Hospital wastewater	0.534±0.056	0.357±0.240
3	HP3	Peat soil	0.248±0,021	0.410±0,190
4	DL120	Hospital wastewater	0.190±0.028	0.090±0.024

Effect of temperature on biofilm formation

Each isolate shown different response to various temperature (fig. 4). Generally, 4 selected isolates shown optimum biofilm formation at 30°C, even though each isolate performed various biofilm thickness and pattern. Isolate ATA6 performed similar pattern on biofilm formation at three different temperatures, however ATA6 just formed medium biofilm at 25°C and at 35°C. It formed strong biofilm after 7 days of incubation. Isolate HP3, DOK135, and DL120 were not strong biofilm former, but they performed better biofilm formation at 30°C.

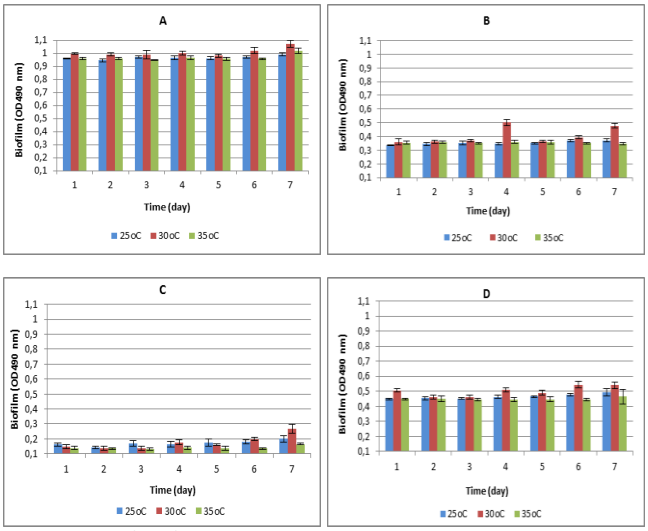


Figure 4. Biofilm formation by 4 selected isolates at various temperature. A) ATA6, B) HP3, C) DL120, and D) DOK135

Effect of various pH on biofilm formation

Graph in figure 5 and 6 shows that ATA6 isolate developed biofilm optimally at pH 7, whereas the other three isolates developed biofilm optimally at pH 9. Each isolate showed similar pattern dynamic of biofilm formation at three pH levels. Therefore, it can be assumed that various pH levels has no effect to dynamic pattern of biofilm formation, even each isolate still formed thicker biofilm at their optimum pH.

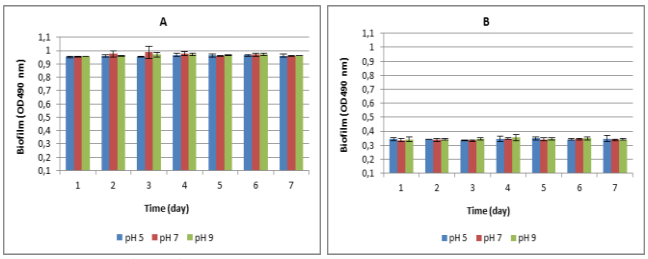


Figure 5. Biofilm formation by 4 selected isolates at various pH. A) ATA6and B) HP3

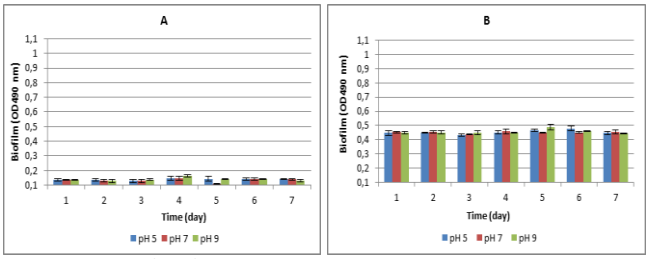


Figure 6. Biofilm formation by 4 selected isolates at various pH. A) DL120 and B) DOK135

DISCUSSION

Our previous work has isolated 4 aerobic phenol-degrading bacteria from hospital wastewater (ATA6, DOK135, and DL120) and peat soil (HP3). DOK135 and DL120 are gram negative bacteria; ATA6 and HP3 are gram positive bacteria. Those isolates performed high ability in degrading 300ppm phenol which is around 79-96.35% of reduction for 96 hours of incubation. Hospital wastewater potentially contain phenol because phenol is basic compound for synthesis of drugs, antiseptic, and disinfectant active compound (Lee, *et al.*, 1997; Andrianto, 2002; Jolibois *et al.*, 2002). Another source of phenol degrading bacteria is peat soil. Peat soil in Indonesia and other tropical areas contain higher lignin than peat soil from temperate areas. Tropical peat soil is formed from trees, lignin from trees then degraded to humus compounds and phenolic acids. Moreover, peat soil also contain high carbon (Barchia, 2006; Agus dan Subiksa, 2008) which can support the growth and abundance of phenol degrading bacteria in peat soil.

Wastewater was chosen as source of phenol degrading bacteria because it ~~is~~ was expected that isolates will easily adapted in phenolic wastewater. Based on Vogel dan Michael (2002), bioaugmentation limitation is encountered from low survival ability of microbes which used in the treatment. Hence, wastewater indigenous bacteria usage is expected able to diminish that limitation. Peat soil also used as source of phenol degrading bacteria because peat soil contain high amount of phenol, therefore high performance phenol degrading bacteria can be obtained from it.

Bacteria can use contaminant in wastewater as source of carbon and energy for growth, as nutrient for growth sustainability, or as electron acceptor in respiration process (Anonim, 2010). One of that contaminant is phenolic compound which is known as toxic compound and can hamper microbial growth, yet its extensive distribution in environment which makes some microbes can utilize that compound as carbon and energy source (Abdullah-Al-Mahin et al., 2011).

Microbes generally live by forming biofilm. Bacteria that live in biofilm community can perform some complex metabolic process (Davey and O'toole, 2000). That ability make the bacteria have important role in environment because the bacteria can be used for bioremediation of hazardous waste, both in industry or clinical waste, also can be used as biobarrier to protect soil and soil water from contamination (Parsek and Fuqua, 2004).

Immobilized microbes exhibit better effectiveness in phenolic wastewater treatment and produce less sludge, whereas the usage of free cell in activated sludge will give rise to another problem e.g. solid waste production (Ying *et al*, 2007). Other advantages of biofilm are protect bacteria from environmental stress and support bacteria cell in planktonic state, metabolic collaboration among microbes in biofilm can increase the degradation rate of phenolic compound (Davey dan O'toole, 2000; Flemming et al., 2000; Melchior et al., 2006; Andersson, 2009). Parsek and Fuqua (2004) also stated that biofilms have an important role in wastewater treatment which contain nitrogen and phosphorus, as well as lowering the COD or BOD.

The result of this study showed that isolates from hospital wastewater (ATA6 and DOK135) performed better ability of biofilm formation than isolate from peat soil (HP3). That difference ability to form biofilms is suspected was influenced by different environmental conditions of sources of each isolates. Irie and Parsek (2008) also Andersson (2009) mentioned that biofilm is known as common mode of microbial growth on liquid surface in nature, clinical, and industrial environment. Thus the presence of water will affect biofilm formation. Andersson (2009) added that production of extracellular matrix is needed in biofilm formation which is generally biofilm matrix consist of 97% water, 2-5% microbial cell, 3-6% extracellular polymeric substances (EPS) and ions.

The ability to form biofilm is also influenced by several environmental factors, such as nutrient, temperature, and pH. The result of qualitative test of biofilm formation showed that all isolates formed thicker biofilm when they were grown in TSB medium. TSB medium is a complex medium that provide more complete nutrient for microbial growth, rather than Ramsay medium. Even Ramsay medium was added with glucose as simple carbon source, but the isolates only formed thin biofilm. Therefore, the next quantitative test of biofilm formation was done using TSB medium.

Loo et al. (2000) reported that maltose, mannose, sucrose, fructose, galactose, lactose, and maltotriose were alternative carbon sources which can be used to support bacteria growth or biofilm formation. Similar study which were done by Anderssson et al. (2008) showed that various carbon sources and nutrient supply influenced biofilm growth. The carbon sources can influence quorum sensing (QS) signals, regulating swarming motility which has a strong effect on biofilm formation, also affect biosynthesis of other signaling substances, cell-surface appendages, and EPS. Based on Irie and Parsek (2008), there are some mechanisms of quorum sensing or intercellular signaling. Gram negative bacteria use acyl homoserine lactone QS system, while gram positive bacteria can use peptide.

Quorum sensing has important roles in controlling some processes in biofilm formation, such as in the

production of exopolysaccharide and bacterial motility (Irie and Parsek, 2008). When cells are in biofilm matrix, production of motility appendages on cell surface is being repressed, therefore cells become immobile (Andersson, 2009). Based on the results of this study, it ~~is~~ seemed that cell motility can influence the bacteria ability to form biofilm. Isolate ATA6 was known as non-motile cell while the other three isolates were known as motile cell, therefore ATA6 could form thicker biofilm than other isolates. In addition, isolate ATA6 and HP3 were known as gram positive bacteria, while DOK135 and DL120 were gram negative bacteria. Data in table 1 shows that gram characteristic did not determine the ability of the isolate to form biofilm.

The result of this investigation showed that four isolates performed different biofilm formation in response to a wide range temperature, although treatment of these temperature variation do not change the category of each isolates ability to form biofilm. The order of the ability to form biofilms on the three treatment temperature can be written as follows: ATA6> DOK135> HP3> DL120. Additionally, ATA6, HP3, and DL120 which were incubated at 30^{o}C still showed the increasing of biofilm formation until the 7th day of incubation. Thus, it can be presumed that three isolates are able to form thicker biofilms if the incubation period is extended. The isolates was categorized as weak or moderate biofilm-forming probably could be categorized to the above categories. According to the statement of Stoodley et al (2002) which said that biofilm formation is a slow process and often takes several days to reach a mature biofilm structure.

Loo et al. (2000) mentioned that generally biofilm formation was more sensitive to pH changes than bacterial growth. The four isolates in this study presented various biofilm formation in response to the various pH. Isolate ATA6 formed biofilm optimally at pH 7, while the other isolates indicated optimum pH for biofilm formation is pH 9. All of the isolates were able to form biofilm on various pH, even the thickness of biofilm was different at each pH level. Perhaps biofilm gave a benefit for the isolates which is resistance to environmental stresses such as changes in environmental pH. Based on Martinez dan Casadevall (2007), biofilm provide protection against changes in environmental conditions due to the interaction between cells and exopolymer matrix on biofilm acts as a layer that protects from environmental stress.

Temperature and pH can influence biofilm formation by stimulating polysaccharides bind to solid materials. Changes in temperature and pH can also affect the maturation of biofilm (Martinez dan Casadevall, 2007). Previous study also showed that seasonal changes can affect the rate of attachment and biofilm formation by bacteria on some surfaces in sea water system (Fera et al., 1989). The dynamic of biofilm formation into planktonic condition is a response to environmetal conditions. The process of biofilm formation involves coordination and regulation of genetic information, which will determine the enzymatic and structural elements which necessary for the formation and release of biofilm (Goller and Romeo, 2008; Andersson, 2009).

In summary, four phenol degrading bacteria from contaminated source (hospital wastewater) and non contaminated source (peat soil) were able to form biofilm and potential for bioremediation. The results indicated a variation of selected isolates biofilm formation ability on various environmental factors. Generally, the isolates formed thicker biofilm if they grow in complex medium that provide more complete nutrient. The isolates could form biofilm optimally at 30^{o}C, while pH treatment did not affect on biofilm formation ability. Moreover, culture time affected on biofilm formation which can be seen from the fluctuation of formed biofilm. Whereas, the study conducted by Andersson et al. (2008) showed that incubation time only affected biofilm formation in one bacteria isolate. Further studies are needed to investigate the effect of some environmental factors combination and interaction among the isolates with indigenous bacteria in contaminated area to the ability of biofilm formation and pollutant degradation. Therefore, comprehensive information will available in developing effective bioremediation.

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