The effect of *cymbopogon citratus* essential oil on community dynamics of biofilm-forming bacteria isolated from brine water of oil reservoir in South Sumatra

I A Purwasena*, P Aditiawati¹, I K Siwi¹ and N A Fauziyyah¹

¹School of Life Sciences and Technology, Institut Teknologi Bandung (ITB), Jalan Ganesha No.10 Bandung 40132 Indonesia

*E-mail: isty@sith.itb.ac.id

Abstract. Biofilm induces an electric potential difference on metal pipelines of oil and gas industries which result in accelerated pipeline corrosion. This study evaluated *Cymbopogon citratus* essential oil nanoparticle performance as an antimicrobial to prevent metal corrosion. This study aims to determine *C. citratus* essential oil nanoparticle’s minimum inhibitory concentration (MIC), minimum biofilm inhibitory concentration (MBIC), and minimum biofilm eradication concentration (MBEC), and its effect on bacterial community dynamics. *C. citratus* essential oil was encapsulated by ionic gelation. MIC, MBIC, and MBEC were determined by the microdilution method. Bacterial community dynamics were analyzed using Total Plate Count. Biofilm-forming bacteria were isolated from brine water of South Sumatra oil reservoir which identified as *Pseudomonas* sp. 1, *Pseudomonas* sp. 2, and *Pannonibacter pragmihetus* based on 16S rRNA sequence similarity analysis using NCBI-BLAST. *C. citratus* essential oil nanoparticle was successfully formed which was 174.1 nm in size, having MIC and MBIC value at 0.03%, and MBEC value at 0.06%. *Pseudomonas* sp. 1 was the most affected by essential oil nanoparticle exposure on planktonic phase while *P. pragmihetus* was the most affected on sessile phase. Based on this study, *C. citratus* essential oil nanoparticle has the potential to be an antimicrobial agent to prevent metal corrosion.

1. Introduction
The bacterial activity has a contribution to 50% of the corrosion processes in the pipeline system in the oil and gas industries [1]. Bacteria or other microorganisms activity that causes or accelerates corrosion process is called Microbial Induced Corrosion (MIC). Biofilm is one of the essential parts of MIC in metals. It creates different types of electrochemical potential in metal surface and consequently accelerates corrosion [1]. Biofilm may be very diverse. Within the biofilm, the interactions among different species or strain can create a cascade of biochemical reaction and induce corrosion. Different species bacteria in a biofilm may interact synergistically, resulting in more complex corrosion than corrosion from a single species [2].

Biofilm formation starts with a transition of planktonic bacteria (free-swimming) to its sessile form. Biofilm development consists of some stages. The first stage is an initial attachment which is reversible and followed by the irreversible attachment. In this stage, polymeric matrix substance is produced thus biofilm become mature. The last stage is biofilm dispersion which enables biofilm to spread and form a new colony in other surfaces.
Lemongrass (Cymbopogon citratus) essential oil is an active bio compound, which is known as an alternative to the synthetic biocide. Thirty percent of lemongrass essential oil is citrals, which have high hydrophobicity and short extension of carbon chains. Regarding it, lemongrass essential oil able to interact with lipid cell membranes of bacteria and affect the fluidity of bacterial external membranes, thus able to kill bacteria [3].

The essential oil has a low fluidity in water and easily evaporate and oxidized. As a result, the essential oil is difficult to apply as an antimicrobial compound for inhibiting corrosion-causing bacteria which grow in the water-rich phase. In order to increase the stability of essential oil, a nanoparticle technology by ionic gelation is performed.

This experiment is aimed to determine the minimum concentration of lemongrass essential oil to inhibit bacterial growth both in planktonic and sessile for as well as to eradicate the mature biofilm. The objective is also to analyze the dynamics of bacterial community which is isolated from brine water of an oil reservoir as a model to know the effect of lemongrass essential oil exposure to biofilm-forming bacteria. Furthermore, it is to determine the most affected bacterial species from lemongrass essential oil nanoparticle exposure to biofilm.

2. Materials and Methods

2.1. Bacterial community preparation

The bacterial community used in this study was isolated from brine water of oil reservoir at South Sumatra, Indonesia. In order to obtain mesophilic biofilm-forming bacteria, the isolated community was grown in a microtiter plate at 50°C for 24 hours by using Nutrient Broth as a growth medium. Pure culture of each isolate was examined and sent to Macrogen Inc. at South Korea to obtain its 16S rRNA DNA sequences. Sequence similarity analysis was carried out by using NCBI-Basic Local Alignment Search Tools with Gen Bank database (http://www.ncbi.nlm.nih.gov/BLAST/).

2.2. Preparation of C. citratus essential oil nanoparticles

The essential oil of C. citratus was obtained from CV. Pavetta Kurta Atsiri Subang with citral concentration of 33%. Nanoparticles were prepared according to the method from Hosseini et. al [4] with a few modifications. Chitosan solution (1% (w/v)) was prepared by agitating chitosan at 200 rpm in 50 ml aqueous 0.1 M acetic acid solution at room temperature (25°C) for 24 hours. The pH of chitosan solution was adjusted to 4.7 and filtered through Whatman 41, then 0.45 µm and 0.22 µm pore size filters. Tween 80 was added to the solution and stirred at 45°C for 1 hour by using magnetic stirrer at 1150 rpm. C. citratus essential oil with a concentration of 0.03%, 0.06%, and 0.12% was then added to chitosan solution during homogenization under an ice-bath condition with similar speed and time. 10 ml Tripolyphosphate 0.1% (TPP) was then added gradually into the solution. The stirring was continuously given. Ultrasonication was performed under an ice-bath condition for 4 min with 0.7 seconds on and 0.3 off. The stability test was performed through a series of centrifugation, heating-cooling cycle, first freeze-thaw, and second freeze-thaw. Nanoparticle diameter and distribution were analyzed by using Particle Size Analyzer (PSA).

2.3. Microdilution test

The test was performed according to Adukwu et. al [5]. The mixed culture of 10⁸ CFU/ml bacteria (1:1) was activated into Nutrient Broth at 50°C. Incubation time for each bacteria used in this experiment was determined by its growth curve. NB and cultures added to wells without nanoparticle was the positive control while NB and nanoparticles without cultures were the negative control. In order to obtain the minimum concentration of C. citratus essential oil to inhibit planktonic bacteria (MIC), microdilution test was performed. 20µL activated mixed culture was added to a sterile 96-well microtiter plate. 180µL nanoparticle in a concentration of 0.03%, 0.06%, and 0.12% then added into the wells. The reading of optical density (OD) using BIO-RAD Elisa Reader (OD595, pathlength correction 200µL, shaking time 5) was performed before and after incubation at 37°C for 24 hours. MIC was determined as the minimum
concentration at which OD value after incubation did not increase from its initial value (before incubation) [5].

In order to determine the minimum concentration of nanoparticle on inhibiting biofilm formation (MBIC), microdilution test was performed. 100µL of activated mix culture bacteria was inoculated into a 96-well microtiter plate. 100µL of each concentration of essential oil nanoparticle was then added. Incubation was performed for 48 hours. When incubation was done, the medium culture was discarded and wells were washed two times using PBS. Following it, heat fixation was performed at 70°C for 15 minutes. Wells were stained using 200µl crystal violet 0.1% (w/v) for 30 minutes at room temperature, then washed three times using PBS. The adhered biofilm then solubilized using ethanol 70% and the reading of OD was performed at 595 nm using BIO-RAD Elisa Reader. MBIC was determined as a minimum concentration of nanoparticle at which OD value equals its negative control [5].

To determine the minimum concentration of nanoparticle on eradicating mature biofilm (MBEC), microdilution test was also performed. The activated culture inoculation to a microtiter plate and incubation step was performed similar to the MBIC test, but the essential oil nanoparticle was not added prior to biofilm maturation. After biofilm maturation, the medium culture was discarded and wells were washed with PBS. Nanoparticle solutions with a concentration of 0.03%, 0.06%, and 0.1% were then added into the wells and incubated at 50°C. The staining of biofilm using crystal violet, washing step, and the reading of OD was done similar to MBIC test. MBEC was determined as a minimum concentration of nanoparticle at which OD value equals its negative control with eradication value ≥ 50% [5].

2.4. Analysis of Bacterial Community Dynamics

The community dynamics due to the exposure of C. citratus essential oil nanoparticles on both planktonic and sessile form of bacteria were analyzed using Total Plate Count (TPC) method. Nanoparticle concentration used in this analysis was based on MIC, MBIC, and MBEC value which were determined before. The bacterial community sample from planktonic phase was taken every 4 hours for 24 hours from the microtiter plate. From the sessile phase, the bacterial community was taken every 8 hours for 24 hours. The bacterial community from the mature biofilm was taken after incubation every 4 hours for 24 hours. The sampling of the bacterial community in the sessile phase and mature biofilm were carried out by harvesting the biofilm cells from the microtiter plate with PBS. The samples were then inoculated in Nutrient Agar by pour method and then incubated at 50°C. The observation of TPC result was done after 24 hours of incubation.

3. Results and Discussion

Three mesophilic biofilm-forming bacterial strains were successfully isolated and cultured in Nutrient Agar. Microscopic identification result showed that colonies of Isolate A were crème, circular, and cells were rods with Gram-negative type. Colonies of Isolate C were crème, serrated, and cells were rods with Gram-negative type. Colonies of isolate E were circular, translucent, white, and cells were coccus with Gram negative-type.

The result from nucleotide-nucleotide BLAST program showed that the sequence of 16S rRNA gene of Isolate A has 98% similarity to Pseudomonas sp. 1, Isolate C has 99% similarity to Pseudomonas sp. 2, and Isolate E has 100% similarity to Pannonibacter phragmitetus. Based on Striebich [6] and Wang [7], P. aeruginosa and P. phragmitetus have the capability to degrade hydrocarbon as a carbon source. It supports the result that the three isolated bacteria may exist in brine water of an oil reservoir. The result of the stability test showed that various concentrations of C. citratus essential oil nanoparticle solution did not exhibit any turbidity change nor precipitation. It indicates that the size of particles in the solution after the test was similar to what it was before [8] and no break of bonds between chitosan and TPP were found, thus confirming that the release of essential oil from its encapsulant has not occurred. Particle Size Analyzer result revealed that the nanoparticles possessed an average diameter of 174.1 nm and 0.283 for PI.
Figure 1 shows the result of microdilution test for MIC determination. Positive value from ΔOD of control treatment in the MIC test indicated an increase of growth of planktonic cells and confirms that there was no inhibition in the control treatment. *C. citratus* essential oil nanoparticle solution with the concentrations of 0.03%, 0.06%, and 0.12% has effectively inhibited the growth of planktonic bacteria. It was shown by the negative value of ΔOD in all concentrations. Higher concentration *C. citratus* essential oil nanoparticle gave higher inhibition for bacterial planktonic growth. At a concentration of 0.03%, the growth of planktonic bacteria was already inhibited. According to this result, it was determined that the MIC value of *C. citratus* essential oil nanoparticle is 0.03%.

The essential oil nanoparticle was encapsulated by chitosan. It causes the mucoadhesive property of nanoparticle in regards to the polycation which interacted with the anionic part of the bacterial outer membrane. When nanoparticle successfully attaches to the bacterial outer membrane, diffusion of the essential oil will occur. Citral component of *C. citratus* essential oil is lipophilic. It may disturb the stability of bacterial outer membrane as well as bacterial peptidoglycan and disrupt the fluidity of the bacterial cell membrane. It may cause the disappearance of ions, disrupts protein motive force mechanism, and the lysis of bacterial cells [3].

In the MBIC determination, it was shown that the biofilm formation by a mixed culture of bacteria was inhibited due to the presence of *C. citratus* essential oil nanoparticle (Figure 2). The higher concentration of the nanoparticle solution also gave higher inhibition for biofilm formation. The result showed that the nanoparticle solution with a concentration of 0.03% inhibited 14.3% of biofilm formation, 0.06% nanoparticle solution gave 23% of biofilm inhibition, while 0.12% nanoparticle solution gave the highest biofilm inhibition up to 31%. Based on the result, it was determined that the MBIC of *C. citratus* essential oil nanoparticle is 0.03%.
Figure 2. The absorbance of biofilm after 48 hours of incubation in microtiter plate with *C. citratus* essential oil nanoparticle with various concentrations and without nanoparticle solution (control treatment) for MBIC determination.

It was found that the MBIC and MIC values of *C. citratus* essential oil nanoparticle were similar. This is due to the ability of *C. citratus* essential oil nanoparticle to simultaneously decrease the number of planktonic bacteria that can attach to a surface to form a biofilm, due to the detrimental effect of the essential oil nanoparticle to planktonic bacteria. Not only disturb the stability of the cell membrane, but the citral of *C. citratus* essential oil also breaks the bonding of lipopolysaccharide in a motor protein of bacterial flagella which are important for bacterial movement. This disturbance will affect the motion of flagella and influence the movement of bacteria towards a surface [3].

In the MBEC test, it was shown that the mature biofilm by mixed culture was successfully eradicated by the exposure of *C. citratus* essential oil nanoparticle with various concentrations (Figure 3). The higher concentration of *C. citratus* essential oil nanoparticle also gave higher eradication activity to mature biofilm. Biofilm eradication from *C. citratus* essential oil nanoparticle with concentrations of 0.03%, 0.06%, and 0.12%, were 12.7%, 39%, and 57% respectively. MBEC is identified as the minimum concentration of nanoparticle to eradicate the mature biofilm $\geq 50\%$ [9]. Fifty percent value was chosen as a minimum eradication value in order to prevent the regrowth of an eradicated biofilm. According to the result, it was known that the MBEC value of *C. citratus* essential oil nanoparticle is 0.12%.
MBEC value was higher than MBIC and MIC. This is due to the higher resistance of mature biofilm to antimicrobial compounds up to 100-1000 times higher than its planktonic form. Biofilm extracellular polymeric substance (EPS) protects bacteria within the biofilm by retarding the transport of antimicrobial compounds into the cell, as well as producing enzymes which able to degrade those antimicrobials [9]. Although nanoparticle form of C. citratus essential oil in this study can improve the diffusion ability of essential oil as an antimicrobial compound into the Biofilm, higher concentration was needed to overcome the resistance mechanism of bacteria within the biofilm which was enveloped by EPS.

Table 1 reveals the community dynamics of Isolate A, Isolate B, and Isolate C on its planktonic and sessile form after exposure of C. citratus essential oil nanoparticle using MIC, MBIC, and MBEC value and each respective microdilution test scheme. In planktonic phase, the exposure of nanoparticle solution with MIC value significantly reduce the number of cells of Isolate A, followed by Isolate C and Isolate E. It was suspected that the resistance of Isolate A to the nanoparticle isn’t as strong as the others. Bacterial resistance to antimicrobials in the planktonic form can be influenced by the thickness of the outer membrane and periplasmic space that are varied greatly between each species or strain type of bacteria. Efflux system of the outer membrane has the capability to pump the antimicrobials out of the cell. In addition, the periplasmic space contains several hydrolytic enzymes that may degrade antimicrobial compounds [10].
Biofilm inhibition treatment using MBIC value reveals a decrease in the log number of cells of all three isolates used to form biofilms. After 48 hours of incubation, Isolate C was the most affected by the exposure of *C. citratus* essential oil nanoparticle, followed by Isolate A and Isolate E. It was predicted that during the biofilm formation, Isolate C resided in the outermost layer of biofilm where cells are more vulnerable to nanoparticle exposure.

Biofilm eradication treatment using MBEC value also results in a considerable decrement of the log number of cells of all isolates. Isolate A was eradicated up to 37.59%. Slightly higher, Isolate C was successfully eradicated up to 38.72%. Meanwhile, Isolate E was eradicated up to 18.46%. From the result, it can be concluded that after 24 hours of nanoparticle exposure to a mature biofilm, Isolate C was the most affected by the *C. citratus* essential oil nanoparticle with the concentration of 0.12%. It was predicted that the Isolate C still resided in the outermost layer after biofilm maturation and therefore experienced a higher exposure of nanoparticle than the other isolates.

From the bacterial community analysis, it was found that Isolate E was the most unaffected by *C. citratus* essential oil nanoparticle exposure. It might indicate that Isolate E resided in the most inner layer of biofilm, therefore, received a lower exposure of nanoparticle than the other isolates. It can also be assumed that Isolate E is an anaerobic facultative bacteria which can live in a place where the concentration of oxygen is low, such as the inside of biofilm [11].

Based on this study, *C. citratus* essential oil nanoparticle has the potential to be an antimicrobial agent for biofilm-forming bacteria which may cause corrosion in oil and gas pipelines. Further study can be done to develop *C. citratus* essential oil nanoparticle to become an antimicrobial agent to prevent metal corrosion.

**Acknowledgement**

The authors would like to express appreciation to ITB for the financial support through ITB research and development scheme which made this research possible.

**References**

[1] Little B J and Lee J S 2007 *Microbiologically Influenced Corrosion* (New Jersey: John Wiley and Sons) p 1.

[2] Donlan R M 2002 *Emerg. Infect. Dis.* **8** 881.

[3] Pedro A S, Santo I E, Silva C V, Detoni C and Albuquerque E 2013 The use of nanotechnology as an approach for essential oil-based formulations with antimicrobial activity *Microbial Pathogens and Strategies for Contacting Them: Science, Technology, and Education* vol 2 ed Mendez-Vilas A (Spain: Formatex) p 1364.

[4] Hosseini S F, Zandi M, Rezai M and Farahmandghavi F 2013 *Carbohydr. Polym.* **95** 50-6

[5] Adukwu E C, Allen S C H and Phillips C A 2012 *J. Appl. Microbiol.* **113** 1217.

[6] Striebich R C, Smart C E, Gunasekera T S, Mueller S S, Strobel E M, McNichols B W and Ruiz O N 2014 *Biodegradation* **93** 33.

[7] Wang X, Jin D, Zhou L and Zhang Z 2016 *Genome Announc.* **4** 1675.

[8] Lawrence M J and Gareth D R 2000 *Adv. Drug Deliv. Rev.* **46** 89.

[9] Wu Y, Luo Y and Wang Q 2012 *Food Sci. Technol.* **48** 283.

[10] Madigan M T, Martinko J, Stahl D and Clark D 2012 *Biology of Microorganism* (San Fransisco: Pearson) p 102.

[11] Borsodi A K, Micsinai A, Kovács G, Tóth E, Schumann P, Kovács A L, Bödői B and Márialigeti K 2003 *Int. J. Syst. Evol. Microbiol.* **53** 555.