JBAT 6(2) (2017) 168 – 174

Jurnal Bahan Alam Terbarukan

Terakreditasi: SK No.: 36b/E/KPT/2016
http://journal .unnes.ac.id/nju/index.php/jbat

p-ISSN 2303 0623
e-ISSN 2407 2370

Biodegradation of Chrysene by Consortium of *Bacillus cereus* and *Pseudomonas putida* in Petroleum Contaminated-Soil on Slurry-Phase Bioreactor

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DOI 10.15294/jbat.v6i2.11404

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Article Info

Article history:
Received
September 2017
Accepted
December 2017
Published
December 2017

Keywords :
Biodegradation;
Chrysene;
Bacillus cereus;
Pseudomonas putida;
slurry bioreactor

Abstract

Pollution by chrysene compounds in the polluted soil of petroleum, due to exploration activities, production and disposal of petroleum waste into the environment causing serious damage to the ecosystem environment, became the target of processing with bacteria as a model of remediation of pollution sites. Thus, the study focused on the use of a bacterial consortium to degrade chrysene in petroleum-contaminated soil. The study was conducted by mixing 20:80 (% wt) of contaminated soil with water. The consortium of *Bacillus cereus* and *Pseudomonas putida* 10%(v/v) and 15%(v/v) bacteria with ratios; 2:3; 1:1; 3:2 is inserted into the slurry bioreactor. Biodegradation process is run with agitation of 100 rpm and temperature 26°C – 30°C and in aeration. Identification of chrysene using gas chromatography–mass spectrometry (GCMS) and bacterial populations with haemycitometer. The initial concentration of chrysene is 24.48 ng/µL. After 49 days remediation period for a 10% (v/v) reduced chrysene bacteria consortium and bacterial populations were 8.68 ng/µL; 7.56 ng/µL; and 8.07 ng/µL; with biodegradation rate is 67.01%; 69.10%; And 64.54%. As for the 15% (v/v) bacteria consortium with the same ratio, chrysene was degraded to 2.60 ng/µL; 1.57 ng/µL; and 2.02 ng/µL and the measured chrysene biodegradation rate was 89.39%; 93.58%; And 91.73%. These findings suggest that the percentage of low crude oil is degraded because of the increasing concentration of crude oil.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs), a large group of xenobiotic pollutants, are common, persistent and recalcitrant contaminants (Hidayat & Tachibana, 2015) found in soils, waterslides, sediments and their fate in nature is a major environmental concern because of the dangerous or potential toxicity, mutagenicity, and carcinogenicity of higher organisms and resistant to microbial degradation (Cerniglia, 1992; Kanaly & Harayama, 2000; Hadibarata et al., 2009). PAHs of low molecular weight, composed of two or three aromatic rings, may decompose under favorable conditions; PAHs with four or more rings are recalcitrant to biodegradation and can last for prolonged periods in the environment. Chrysene is a high molecular weight PAH consisting of four unified benzene rings. Among PAHs, chrysene is classified as a priority pollutant by US EPA (Smith et al., 1989; Harvey, 1991). Humans may be exposed to these compounds from various sources, such as through work, environment, food, and etc. (Prak & Pritchard, 2002; Liu et al., 2001).

Polycyclic aromatic hydrocarbons can be reduced from polluted soil by incineration, thermal desorption, and soil washing, but these methods are expensive and have negative effect to the environment. Another way is bioremediation as a competitive alternative to convert pollution into less toxic form (Hidayat & Tachibana, 2015). Microbial degradation is believed to be one of the main ways to clean up the chrysene-contaminated environment. Microbial communities have

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ISSN 2303-0623
e-ISSN 2407-2370

Table 1. Workplace Exposure limit of Chrysene

	Workplace Exposure Limit of Chrysene
OSHA[a])	The legal airborne permissible limit (PEL) is 0.2 mg/m^3 (as coal tar pitch volatile, benzene-soluble fraction) average over an 8 hour work shift
NIOSH[b])	The recommended airborne exposure limit (REL) is 0.1 mg/m^3 (as coal tar pitch volatiles, cyclohexane-extract-able fraction) average over a 10 hour work shift
ACGIH[c])	Recommends that exposure by all routes to be controlled to levels as low as low possible
Kep.Men LH No. 128 tahun 2003	The final value requirement of petroleum sludge processing in biological weight in dry weight for PAH and in it containing chrysene is 10 µg/g.

[a]) OSHA – Occupational Safety and Health Administration

[b]) NIOSH – National Institute for Occupational Safety and Health

[c]) ACGIH – American Conference of Governmental Industrial Hygienists

considerable potential for improving oil contaminated deposits and removing chrysene from aqueous solutions (Ramsay et al., 2000; Tam et al., 2002). High-molecular weight PAHs such as Chrysene and benzo[a]pyrene are difficult to degrade while low molecular weight PAHs such as phenanthrene and naphthalene are degraded efficiently (Yamada et al., 2003). Chrysene is on the right to know Hazardous Substances List because it is cited by OSHA, ACGIH, NIOSH. Some of these agencies have also prescribed the workplace exposure limits of chrysene as well (Table 1) (Patel & Ali, 2014).

Bioremediation technique using bioreactor is an ex-situ bioremediation development. Slurry bioreactor is not only used to degrade waste in liquid phase and slurry but also solid/soil waste (Robles-Gonzales et al., 2008). The employment of microorganisms in the biodegradation of hydrocarbons over chemical or conventional treatment is preferred for many reasons. First of all, the potential and selected microbes can alter raw and crude oils in beneficial ways and the resulting end products are comparatively safer to the environment and all living beings. Microorganisms have been employed for bioremediation of hydrocarbon-rich waste material products, along with their various recalcitrant noxious compounds, which are finally converted into environmentally friendly products. These microbes utilize waste material as carbon substrate, increase their population, and ultimately biodegrade hydrocarbon products to nontoxic products, such as H_2O and CO_2 (Prakash et al., 2014). Very little information is available on the metabolic pathway of high molecular weight PAHs but the overall route of pathway is known (Figure 1). It is now understood that the initial step in the aerobic catabolism of a PAH molecule by bacteria occurs via oxidation of the PAH to a dihydrodiol by a multi-component enzyme system (Patel & Ali, 2014).

Microorganisms play an important role in the degradation of aromatic hydrocarbons in terrestrial and aquatic systems. The use of microorganisms for bioremediation requires knowledge of the metabolic pathways of aromatic compounds in organisms. However, the success of bioremediation has been limited by the failure to eliminate high molecular weight PAHs (Wilson & Jones, 1993) such as chrysene. To eliminate chrysene, degraders should be able to get enough biomass and mineralization ideally and grow on chrysene as a source of carbon and energy (Alexander, 1999). This Study have shown that there is a great diversity of microorganisms which are capable of degrading low molecular weight PAHs such as naphthalene and phenanthrene. Relatively, few organism have been observed to degrade high molecular weight PAHs such as pyrene and chrysene. Studies have shown that low molecular weight hydrocarbons are metabolized by pure strain and biodegradation of high molecular weight hydrocarbons required the combined efforts of different populations (Kanaly et al., 2000). PAH compounds are transformed throughout a series of different metabolic reaction by microorganisms in the real environment. Therefore, the study of PAH (chrysene) metabolism by bacterial consortia is important. It will provide new insights for improving future studies on bioremediation of environmental pollutants.

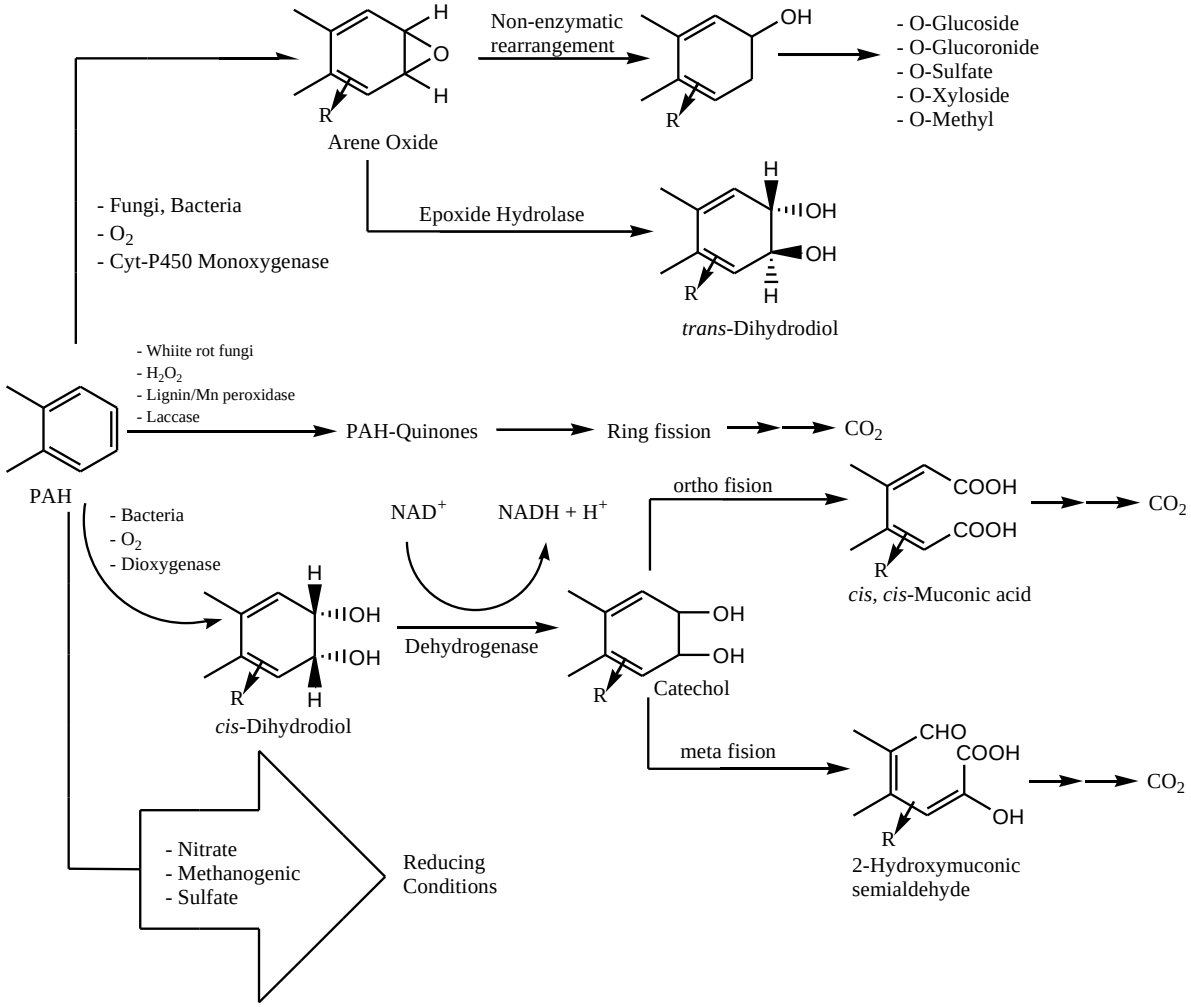

Figure 1. Proposed pathway for microbial catabolism of polycyclic aromatic hydrocarbons (Adapted from Cerniglia (1992))

Most of the bacteria used in the degradation of petroleum-contaminated areas are aerobic bacteria such as *Bacillus cereus* and *Pseudomana putida* bacteria. *Bacillus cereus* is one of the minority sporeforming, aerobic bacteria are recognized as pathogenic bacteria. These bacteria are prokaryotic, unicellular, and do not contain structures that are restricted in the cytoplasm. *Bacillus cereus* belongs to the Bacillus species, single-celled organisms, short rods usually in the form of long chains. In general, *Bacillus* sp. has been identified as petroleum hydrocarbon degraders (Ghazali, et al*.,* 2004; Das & Mukherjee, 2007) and is known as naphthalene and pyrene degraders (Zhuang, et al*.,* 2002). Pseudomonas putida bacteria are included in the Pseudomonadaceae family and are easily found in soil, water and on surfaces in contact with soil or water. These bacteria are known to utilize aromatic hydrocarbon compounds such as toluene, xylene and methyl benzoate as the only carbon source. The objective of this research is to know the method of using *Bacillus cereus* and *Pseudomonas putida* bacterial consortium to degrade chrysene on the slurry phase in ex situ contaminated petroleum.

MATERIALS AND METODS

Sample and Polluted Soil Treatment

The petroleum contaminated soil sample was obtained from Pertamina-Petrochina Tuban, wellpad B. The contaminated soil was removed after being separated from the rocks and foliage, then sterilized to obtain the polluted soil sample free indigenous bacteria.

Medium and bacterial preparations of *Bacillus cereus* and *Pseudomonas putida*

The liquid medium is prepared by mixing 24 grams of NBA (nutrient browth agar) with 1%

glucose and 1% yeast extract in 1 L distillated water. After sterilization with autoclave at 121°C, the medium is cooled to a temperature of 28°C. After cold, bacterial (*Bacillus cereus* and *Pseudomonas putida*) removal is carried out from the media agar tilt to into the new medium by using ose aseptically in the laminar flow. Then the new media (starter) is incubated at a temperature of 30oC and 70 rpm for 1 day. The bacteria were then calculated by Counting Chamber and after 48 hours, the total bacteria were 1.13 x 10^7 Cell/mL.

Biodegradation Process

The Chyrsene biodegradation process is carried out ex situ in the Wastewater Treatment Laboratory, Department of Chemical Engineering – Institut Teknologi Sepuluh Nopember Surabaya. The polluted soil is formed into slurry by mixing soil and water with a ratio 20:80. This process takes place in a slurry phase bioreactor (Figure 2) with stirring and aeration. The biodegradation temperature between 26–30°C and pH 6–8. This process takes place aerobically, so the air is passed continuously to maintain the condition of the process. The medium containing *Bacillus cereus* and *Pseudomonas putida* was added to a bioreactor with a concentration of 10% and 15%(v/v) (bacterial ratio of 3:2; 1:1 and 2:3) and once biorekator control (without addition of bacteria). Bioremediation process occurs for 49 days. Every 7 days, the soil is taken from bioreactor and extracted with n-hexane for 16 hours. The oil is separated from the solvent by vacuum evaporator for 7–10 minutes.

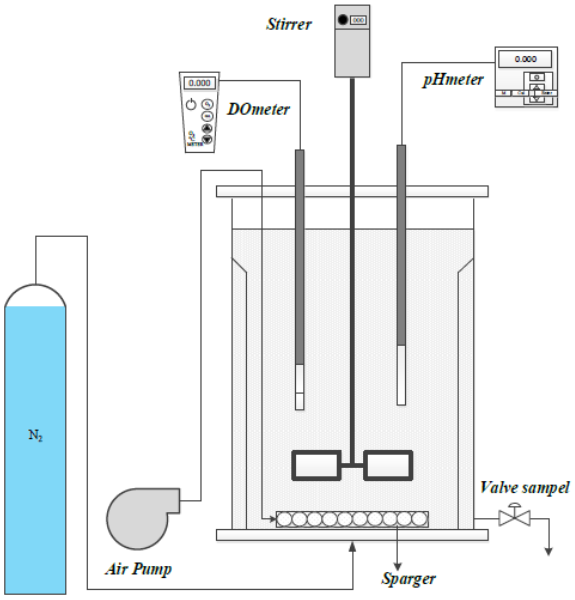

Figure. 2 Slurry Bioreactor

Chrysene Analysis

Chrysene degradation analysis on petroleum contaminated soil with GCMS (gas chromatography–mass spectrometry). 10 g of soil samples (slurry) in the extract within a Soxhlet apparatus with 100 mL of n-hexane and 10 g Na_2SO_4 hidrate to remove water (EPA Method 3540C). The extracted oil was then analyzed by Chrysene at the Oseonography Research Center LIPI Jakarta, with Thermo Trace ISQ 1310 LT (Single quadrupole Mass Spectrometer) method Supelco Standard QTM PAH Mix 47 930-U quantitatively. The percentage of chrysene biodegradation was calculated by using equation 1.

$$Degradation(\%) = \frac{[Chrysene]_0 - [Chrysene]_n}{[Chrysene]_0} x100 \quad (1)$$

RESULTS AND DISCUSSION

Polluted Land Characteristics

Based on Indonesia Minister of Environment Decree No. 128, 2003, PAHs (Chyrsene) in soil contaminated with petroleum is too much above the quality standard. Therefore the soil needs to be processed before it is released into the environment.

Table 2. Soil Characteristic from drill sites at Pertamina – Petrochina East Java (PPEJ) Tuban

Parameter	Characteristics
Color	Brown
pH	9.1
Temperature (°C)	28
Aromatic concentration (ng/µL)	
Benzene	26.44
Toluene	121.00
Xylene	129.00
Chrysene	24.48

Effect of Bacterial Consortium on Chrysene Degradation

Polyaromatic Hydrocarbons are the most disturbing component in petroleum contaminated soil, because of the potential risks to human health and the environment. PAHs is one of the parameters of the success of bioremediation process before it is released into the environment in accordance with applicable laws and regulations. The final limit value of PAHs processed products is 10 µg/g (KepMen LH No: 128 in 2003). Chrysene is a 4-ringed hydrocarbon polyaromatic compound, which is quite difficult to reduce. The process of

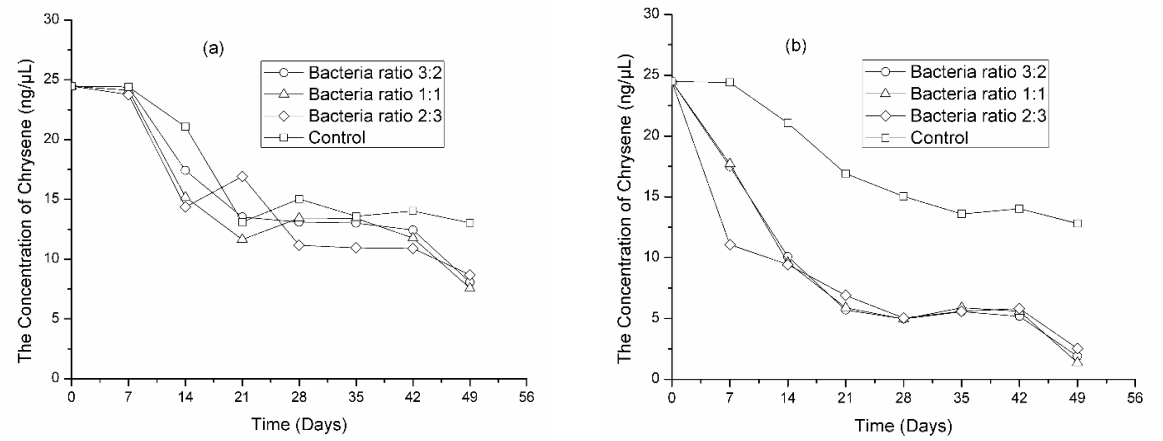

Figure 3. The Relationship of Concentration Chrysene againts Time. a) Bacteria concentration 10%(v/v); b) Bacteria concentration 15%(v/v)

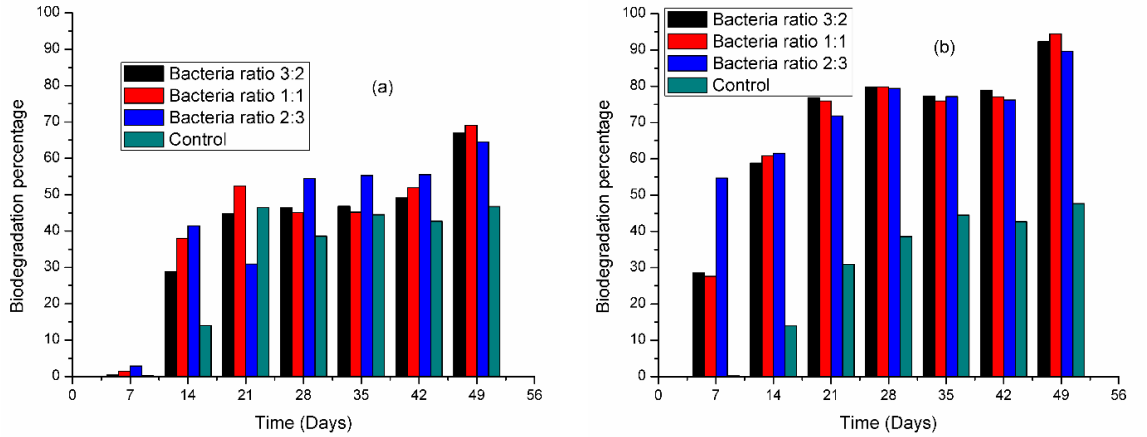

Figure 4. The Relationship of Biodegradation Percentage against Time. a) Bacteria concentration 10%(v/v); b) Bacteria concentration 15%(v/v)

biodegradation of chrysene by combining *Bacillus cereus* and *Pseudomonas putida* bacteria is expected to accelerate the process of reduction of chrysene contaminants in oil contaminated areas. Figures 3a and 3b show a significant decrease in chrysene degradation during the 49 days remediation period. Chrysene reduction, both at 10% and 15% (v/v) bacterial concentrations showed that during the remediation period, the bacterial consortium significantly breakdown chrysene compounds and use them as the only carbon source.

The decrease in chrysene concentration occurred fastest on day 0 to day 21. Further reduction kept chyresen reduced but not as much as in the first week. According to Leahy & Colwell (1990), bacterial growth in the first week is called the adaptation phase, which will occur three mechanisms, is the induction or secretion of specific enzymes, there is a genetic change that affects the ability of bacterial metabolism, resulting in an increase in the ability of organisms to reduce certain components. According to Nugroho (2006) the level of biodegradation has been shown to be highest in saturation, followed by light aromatics, with high molecular weight aromatics and polar compounds. This makes the bacteria in the adaptation phase prefer to reduce the alkenes and monoaromatics compounds first.

Chrysene concentrations decreased day by day for all bacterial ratios, albeit slowly. This, suggesting that chrysene compounds having 4 benzene rings, tends to be difficult to degrade. However, the difference is quite noticeable in 42 to 49 days of remediation period, where for all bacterial ratios (3:2; 1:1; 2:3, and control) at 10% and 15 (v/v) give a positive response to chrysene degradation by a combination of *Bacillus cereus* and *Pseudomonas putida bacteria*. The reduction of contaminants (chrysene) has decreased very rapidly, respectively, were, 24.48 to 8.07; 24.48 to 7.56; 24.48 to 8.68 ng/uL; for a concentration of 10% (v/v). While at a larger bacterial concentration 15% (v/v) was 24.48 to 1.89; 24.48 to 1.37; 24.48 to 2.53 ng/uL; but for control, chrysene reduction

slows down, this is due to bacteria in the control bioreactor, it is difficult to use bacteria as a carbon source, because of the limited availability of biological elements. It differs in other bacterial ratios, which can utilize carbon sources as energy in metabolizing itself in remodeling contaminants (chrysene) in petroleum-contaminated soil.

In Figure 4, it shows that in the treatment with the addition of bacteria 10% and 15% (v/v), cause the value of chyrsene can be reduced quite well every week. The greater the chyrsene concentration decreases, the greater the percentage of biodegradation increases. This can be known by increasing the biodegradation percentage of hydrocarbon compounds from the 7th day interval up to the 49 days. The best degradation percentage was achieved on the bacterial ratio 1:1 was, 69.10% at bacteria concentration 10% (v/v) and 94.41% bacteria concentration 15% (v/v). While in control, chyrsene reduction only reached 46.75% in 49 days remediation period. While in the treatment without the addition of bacteria (control), although the value of chyrsene decreased, but the biodegradation percentage is relatively lower than the treatment of bacterial addition. This is because natural bacteria in the soil have not succeeded in degrading petroleum hydrocarbons effectively. It is suspected that the group of bacteria that dominate the contaminated soil is an indigenous microbe group that cannot utilize petroleum hydrocarbon compounds for its growth, so its degradation rate is slow (Nugroho, 2006).

CONCLUSION

The slurry bioreactor method can be used for the biodegradation process in degrading chyrsene. Reduction of chrysene concentrations occurred in each bioreactor (bacterial ratio), either without bacterial addition or with bacterial addition and chrysene degradation which best occurred in bioreactors with 15% bacterial concentration (v/v) bacterial ratio of 1: 1 with chrysene concentrations at the end the remediation period was 1.37 ng/µL, with a biodegradation percentage was 94.1%.

ACKNOWLEDGEMENTS

This research was supported by funds provided by the Directorate General of Higher Education-Ministry of Research Technology and Higher Education Republic of Indonesia. The author also expressed his gratitude to, Prof. Dr. Ali Altway, MS; Dr. Ir. Sri Rachmania Juliastuti, M.Eng and Prof. Dr. Suprapto, DEA, who has provided many suggestions for the development of this research.

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