Optimization of Benzene and Toluene Biodegradation by *Aspergillus niger* and *Phanerochaete chrysosporium*

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Abstract - **Benzene and toluene can cause contamination in environments. Utilization of microorganism to degrade these compounds could be favorable approach to discover a convenient biodegradation agent. Fungi have an important role in the degradation of petroleum hydrocarbon (PHC). The aim of this study was to evaluate the potential of benzene and toluene degrading molds *Aspergillus niger* and *Phanerochaete chrysosporium*. These molds were selected to check the Lignin Peroxidase (LiPs) activity and formulate the medium conditions for improving degradation process. Optimization of medium conditions (pH and nutrient concentrations) for benzene and toluene degradation was applied under *in vitro* conditions. Molds were grown in sawdust media with pH settings (4, 5, 6 and 7) and nutrients (25%, 50%, 75% and 100%) followed by the addition of benzene and toluene. The percentage of degradation was analyzed using Gas Chromatography (GC). The optimal degradation conditions of benzene and toluene by *A. niger* were at pH 6 with nutrient concentration of 75%, while *P. chrysosporium* at pH 7 with nutrient concentration of 25%. All samples that had been polluted by benzene and toluene did not show LiPs activity, however non polluted samples showed LiPs activity 0,528 U/ml in *A. niger* and 0,275 U/mL in *P. chrysosporium*.**

Keywords- *Aspergillus niger, Benzene, Biodegradation, Phanerochaete chrysosporium, Toluene*.

INTRODUCTION

Benzene, toluene, ethylbenzene and xylene (BTEX) are petroleum monoaromatics hydrocarbons which are known as hazardous pollutants. BTEX is the most widely produced chemical from industrial sources, such as paint, gasoline, avtur, plastics and so on [1]. Annual production of benzene was up to 8-10 million tons and 5-10 million tons for toluene [2]. Hydrocarbon pollutants will lead devegetation, contamination of drinking water, reduction of plants and animals reproduction, moreover mortality of organisms [3].

Microorganisms have been shown the capability to degrade BTEX compounds, especially bacteria and fungi. However, the capabilities of BTEX biodegradation have focused on bacteria rather than fungi. Biodegradation of BTEX mediated by fungal has been assayed with white-root fungi. BTEX pollutants degradation by the white-rot fungi *Phanero*chaete *chrysosporium* showed that the organism degraded BTEX efficiently. Benzene and toluene degradation by *P. chrysosporium* were observed around 9,6% for benzene and 35,8% for toluene after 5 days incubation at 25°C [4]. *Aspergillus* sp. utilized hydrocarbon pollutants as a carbon source in metabolism process [3]. Hydrocarbon pollutant such as toluene can be degraded up to 92% by *A. niger* after two weeks in batch reactor [5].

The ability to degrade recalcitrant pollutants by wood-degrading white-root fungi was due to mineralization of lignin. Ligninolytic conditions which are associated with two enzymes: lignin peroxidase (LiPs) and manganese-dependent peroxidase (MnPs) enable biodegradation, primarily was observed during secondary metabolism triggered by nutrient starvation. Several environmental factors also determined biodegradation including temperature, pH, oxygen, climatic conditions and nutrient availability. The presence of alternative carbon sources, the physical

conditions of oil and the appropriate metabolic abilities of microorganisms established the results of biodegradation process as well [4]. However, biodegradation of benzene and toluene by *A. niger* and *P. chrysosporium* had not been reported. This paper reports the optimal conditions for the degradation of benzene and toluene by *A. niger* and *P.* chrysosporium.

METHODS

Microorgenisms

The pure culture of *Aspergillus niger* and *Phanerochaete chrysosporium* were taken from Center for Application of Technology of Isotope and Radiation (PAIR), National Nuclear Energy Agency of Indonesia laboratory in potato dextrose agar (PDA) (Oxoid) media slants at 4°C.

Preparation of inoculums

A. niger and *P. chrysosporium* were grown in potato dextrose broth (PDB) (Difco) with mechanical shakers in room temperature (28-32°C) for 4 days. Afterwards, the cultures were spread on the surface of PDA in petri dishes at 32°C for 4 days. The grown molds were taken 1 x 1 cm^2 for cultivation in 50 mL PDB with mechanical shakers in room temperature (28-32°C) for 4 days as starter.

Preparation of sawdust medias

PDB media was made by mixing 4,8 g PDB and added with nutrients: KH_2PO_4 (Merck) 0,04 g, K_2HPO_4 (Merck) 0,04 g, and $MgSO_4.7H_2O$ (Merck) 0,2 g in 200 mL distilled water. Variations of nutrient percentage were made by adding distilled water in 25% (v/v), 50% (v/v), 75% (v/v) and 100% (v/v). The pH of each medium with different percentage of nutrients was set in 4, 5, 6 and 7 by the addition of NaOH or H_2SO_4. Sawdust media was prepared by putting 1 g of sawdust in a small bottle then 2 mL PDB was added and autoclaved for 15 min at 121°C. After sterilization, 1 mL of *A. niger* and *P. chrysosporium* starters were added to the sawdust media and incubated for 7 days (inoculated sawdust media).

Preparation of pollutant

Pollutant was prepared by mixing 5 mL benzene and 15 mL toluene (1:3). Each of inoculated sawdust media was dripped with 1 mL pollutant mixture then incubated for 24 hours at room temperature (polluted sawdust media).

Construction of standard curve

Benzene and toluene were diluted using ethanol with concentrations of 40,000 ppm, 60,000 ppm, 80,000 ppm, 100,000 ppm. The solutions were used as standard solutions and tested using Gas Chromatography (GC) Shimadzu GC14-B model.

Benzene and toluene biodegradation assay

The polluted sawdust media was added by 5 mL ethanol. A total of 1,5 mL mixture was put in a microtube and centrifuged at 8,000 rpm for 10 min. The supernatant was taken for biodegradation assay using GC. The GC was set at 50°C in injector, 190°C in column, 280°C in detector for 20 minutes. The supernatant was taken as much as 1 μL and injected to the GC. The results were shown in form of peaks with certain area and retention time.

Lignin peroxidase (LiPs) assay

The polluted sawdust media was added by 5 mL acetate buffer. A total of 1,5 mL mixture was put in a microtube and centrifuged at 12.000 rpm 4°C for 10 min. The supernatant was taken as the sample for LiPs assay using spectrophotometer. A total of 0,4 mL veratyl alcohol, 0,8 mL acetate buffer and 1,8 mL distilled water was mixed in the test tube and analyzed using spectrophotometer with 310 nm wavelength at room temperature. Before being analyzed, 800 μL of the samples and 100 μL of H_2O_2 were added to the test tube, homogenized and tested with a spectrophotometer (t-0). All samples were put back to the test tube and incubated for 20 min before retesting (t=1). One unit of LiPs activity is defined as the amount of enzyme that causes conversion of 1 μmol (10^{-6}) veratyl alcohol per min. The calculation of LiPs activity was carried out using the following formula [6].

$$\text{LiPs Activity (U/mL)} = \frac{\Delta OD \times Vtotal\,(mL) \times 10^6}{\epsilon Max \times d \times Venzim(mL) \times t} \quad (1)$$

Note:
ΔOD = Absorbance difference
V*total* = 4 mL
∈*max* = Molar absorption of veratyl alcohol 9300/M.cm
d = Inner thickness of cuvet (cm)
V*enzim* = 0.8 mL
t = time

RESULT AND DISCUSSION

Effect of pH in benzene and toluene degradation

One of the most important parameters affecting microbial growth is pH. Certain pH series favor nourishment and replication of microbes [7]. The effect of different pH (4-7) on the benzene and toluene biodegradation by *Aspergillus niger* and *Phanerochaete chrysosporium* was examined. The maximum degradation percentage of benzene and toluene in *A. niger* was observed on pH 6 (Picture 1), where as *P. chrysosporium* on pH 7 (Picture 2). Generally, fungi prefer acidic to neutral condition, while bacteria in neutral to alkaline environment [8].

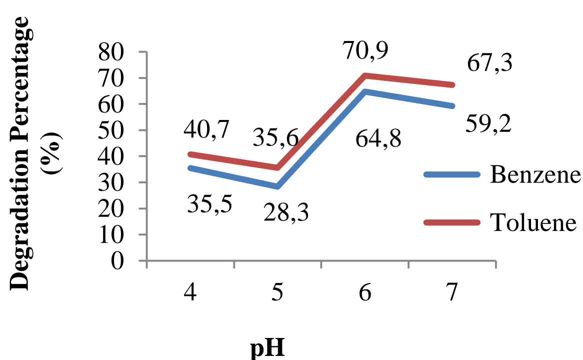

Figure 1. Effect of pH on degradation percentage of benzene and toluene by *Aspergillus niger*

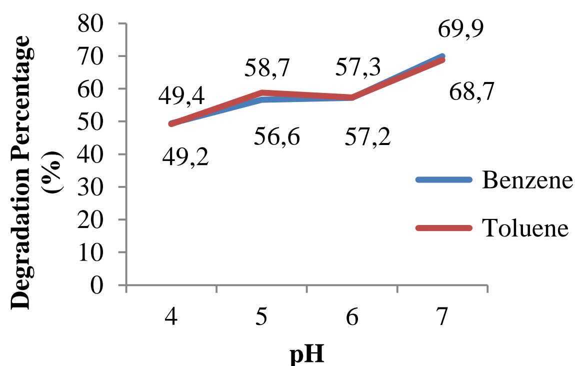

Figure 2. Effect of pH on degradation percentage of benzene and toluene by *Phanerochaete chrysosporium*

The growth of mold biomass and extracellular enzyme production can be effected by pH. The optimal pH of *A. niger* was reported between 6-7 at 30°C [9]. This result is in accordance with the degradation percentage results of benzene and toluene by *A. niger* with the highest at pH 6 and continued by pH 7. Higher degradation result was observed in toluene than benzene. This result is consistent with Jung and Park [10] report that in a mixture benzene, toluene and m-xylene (BTX), benzene was more difficult to degrade than m-xylene and toluene respectively. Benzene ring group has a high resonance (electron delocalization) and tend to be stable, so that the energy needed to break the benzene ring is greater.

Larasati *et al.* [11] reported that *P. chrysosporium* was growing at pH 4-7. *P. chrysosporium* was able to degrade polycyclic aromatics hydrocarbon (PAH) optimally at pH 7 and temperature 30℃ [12] in accordance with the degradation percentage results of benzene and toluene by *P. chrysosporium* with the highest at pH 7. Degradation of pollutants was occurred due to the breakdown of hydrocarbon compounds that produce CO_2, H_2O and biomass. Toluene will be used by molds as the initial carbon source because of its methyl groups activate electrophilic substitution of the benzene ring so that it is more reactive and easier to degrade, then benzene is degraded when used as the only substrate [13].

Effect of nutrients in benzene and toluene degradation

Variation of nutrients was aimed to determine the optimal nutrient concentration, so that the molds can growth and do various metabolic activities especially degrading pollutants. The effect of different nutrients (25%-100%) on the benzene and toluene biodegradation by *A. niger* and *P. chrysosporium* was examined. The maximum degradation percentage of benzene and toluene in *A. niger* was observed on 75% (Picture 3), whereas *P. chrysosporium* was varies 25% on benzene and 75% on toluene (Picture 4).

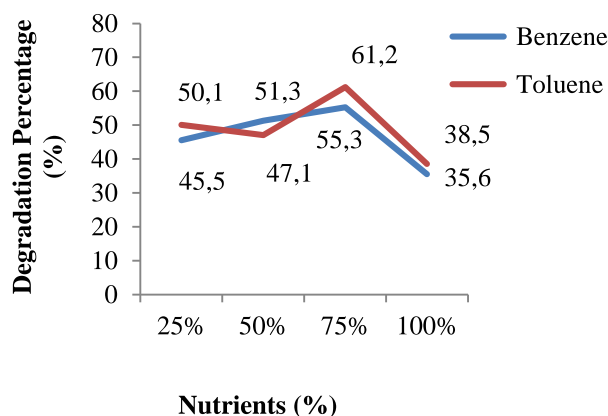

Figure 3. Effect of nutrients on degradation percentage of benzene and toluene by *Aspergillus niger*

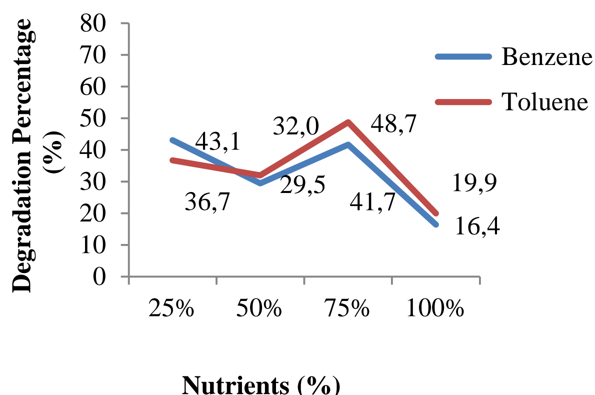

Figure 4. Effect of nutrients on degradation percentage of benzene and toluene by *Phanerochaete chrysosporium*

Nutrient variations were carried out by adding nutrient solutions and mineral salts in the form of PDB, KH_2PO_4, K_2HPO_4 and $MgSO_4.7H_2O$ at 25%, 50%, 75% and 100% for growth and release metabolic products [11]. Availability of Mg^{2+} in media that was obtained from $MgSO_4.7H_2O$ is needed to stabilize ribosome, membrane and protein synthesis [14]. Magnesium also functions as activator for various types of enzymes and helps the growth of cell [15]. Potassium and phosphate content were obtained from KH_2PO_4 is needed as cell metabolism and various transport processes control through cell membrane. A high potassium element that was obtained from K_2HPO_4 is needed to stabilize pH of the medium [16].

Lignin Peroxidase (LiPs) Activity

LiPs activity was also measured during the degradation process of benzene and toluene. All samples that had been polluted by benzene and toluene did not show LiPs activity. However, LiPs activity in non polluted samples showed positive results, 0,528 U/ml in *A. niger* and 0,275 U/mL in *P. chrysosporium.* LiPs activity in *P. chrysosporium* was lower than *A. niger*. Zacchi *et al*. [17] showed that LiPs activity by *P. chrysosporium* after 4 days incubation in submerged culture with continuous agitation and cellulose as carbon source was 0,2-0,4 U/mL. Moreover, LiPs activity by *A. niger* after 7 days incubation at 30°C in Czapek-Dox broth media was 3,6 U/mL [18]. It can be concluded that the incubation period and the substrate affect the LiPs activity, besides the ability of the mold itself.

Previous study has suggested that white rot fungi can secrete LiPs enzyme but not specifically only mineralize lignin compounds as sawdust media that we had used [18]. Biodegradation of pollutants has been reported to be related to availability of LiPs in ligninolytic conditions. However, LiPs activity under ligninolytic conditions using sawdust was not available when degraded benzene and toluene after 24 hours incubation. These results are supported by Yadav and Reddy [4] that benzene and toluene degradation occurred in the first 2 days when molds in primary metabolism. Extracellular enzymes such as LiPs are secreted when molds in secondary metabolism in 3-4 days.

CONCLUSION

This study investigated the optimization of pH and nutrient concentrations for benzene and toluene degradation by *A. niger* and *P. chrysosporium.* The optimal conditions for benzene and toluene degradaton by *A. niger* were found at pH 6 with nutrient concentration 75% then *P. chrysosporium* at pH 7 with nutrient concentration of 25%. LiPs were not produced under polluted condition but under non polluted condition obtained higher by *A. niger* 0,528 U/mL then *P.chrysosporium* 0,275 U/mL.

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