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Biodegradation of styrofoam waste by ligninolytic fungi and bacteria

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Abstract. Styrofoam wastes are composed of many polymerized styrene monomers that are generally considered to be recalcitrant and are resistant to biodegradation. In this study, the ability of ligninolytic fungi and bacteria were investigated on degradation of styrofoam wastes. All the fungi and bacteria used were able to grow on agar media containing styrofoam. Fungi *Cymatoderma dendriticum* WM01, *Ceriporia sp.* BIOM3, and *Pestalotiopsis sp.* NG007 degraded 15.7%, 19.4%, and 74.4% styrofoam within 30 d, respectively. *Cerratia marcescens* BLSP4, *Bacillus subtilis* BLSP4, and *Pseudomonas aeruginosa* BLSP4 degraded 38.3%, 52.6%, and 63.4% styrofoam, respectively. SEM analysis demonstrated the appearance of micro pore in styrofoams treated with *Pestalotiopsis sp.* NG007 and *P. aeruginosa* indicating biodegradation. In addition, analysis using UATR FTIR corroborated removal of some functional groups from the degraded styrofoam were eliminated. This study showed strains of ligninolytic fungi and selected bacteria have the potential to be used in bioremediation of styrofoam wastes.

1. Introduction

Styrofoam is a type of plastic product made from polystyrene foam and is often used for food packaging. Unfortunately, it is not environmentally friendly due to its recalcitrant nature. Based on the data released, Indonesia is placed second among the world's top plastic polluters, where approximately 187.2 million tons of plastic debris was thrown into the ocean, after China that polluted the ocean with 262.9 million tons of plastic debris [1]. In Bandung city, styrofoam waste has exceeded 27.02 tons per month [2].

Styrofoam consists of a number of polymerized styrene monomers. Styrofoam has strong bonds of single carbon and double C-C bonds which resonate with each other as presented in Figure 1. The structure of polystyrene is similar to that of lignin which, along with other polycyclic aromatic hydrocarbons like phenanthrene, pyrene, benzo[a]pyrene and chrysene, is degradable by ligninolytic enzymes. Ligninolytic enzymes are non-specific oxidative enzymes activated by non-protein mediators which play important role in degrading the lignin [3]. Ligninolytic enzymes activity enhance when Albizia powder is added to the media, as Albizia powder contains 49% cellulose, 26.8% lignin, 15.6% pentose, 0.6% ash and 0.2% silica [4].



Some bacteria has been shown to use styrene as massive source of energy, however, the metabolism of styrene has been extensively studied in the genus *Pseudomonas*. Generally, *Pseudomonas* degrades styrene through two main pathways, viz., an upper and a lower pathway. In the upper pathway, styrene is oxidized by styrene monooxygenase to form styrene oxide which requires reducing electrons. Styrene oxide is then transformed into phenylacetaldehyde by the styrene oxide isomers to be later oxidized by phenylacetaldehyde dehydrogenase to form phenylacetates. In the lower pathway, phenylacetates are ligated into co-enzyme A to create coil phenylethyl, which is then broken down by some enzymes to form CoA acetyl within TCA cycle [5].

Regarding to environmental issues caused by styrofoam, various methods have been made to solve them. There has been a ban toward the use of styrofoam, where people are instead encourage to use biodegradable plastic material. A survey done by USEPA (2016) reported that styrene is rarely found in ground water, in which it was only found 295 times out of 174.000 analysis (< 0.2% of the samples) [6]. The styrene concentration was found between < 10 to 40 $\mu\text{g/L}$. Another serious problem is the high production cost of biodegradable plastics, causing higher price in the market. In addition, burning the styrofoam creates dioxin compounds and furan.

Biodegradation by biological agents such as microorganisms including fungi and bacteria is one of beneficial. Biodegradation could be conducted by biological agents such as microorganisms including fungi and bacteria. In this research, three kinds of fungi were used, including *Pestalotiopsis sp.* (NG007), *Ceriporia sp.* (BIOM3), and *Cymatoderma dendriticum* (WM01). The biodegradation process done by white rot fungi involved ligninolytic enzymes which consists of lignin peroxidase (LiP), laccase, and manganese peroxidase (MnP).

In this research, three different bacteria including *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Serratia marcescens* were also used. Those bacteria have the ability to degrade the monomeric hydrocarbon components of styrofoam [7,8]. Therefore, biodegradation process becomes more environmentally friendly as it triggers no secondary impacts.

Currently, biodegradation agents of styrofoam is mostly limited to only bacteria belonging to the genus *Pseudomonas*. There has not yet been any study reporting the potential use of ligninolytic enzymes present in the fungus, *Pestalotiopsis sp.*, for styrofoam degradation process. Hence, it is necessary to conduct deeper research on the use of ligninolytic enzymes from white rot fungi in styrofoam degradation process. This research was conducted to determine the most resistant of white rot fungi and bacteria which have better potential in degrading styrofoam materials. The hypothesis of this study relates to the belief in the potentials of ligninolytic enzymes in degrading styrofoam compounds. It is expected to give significant contribution to the knowledge upon the potentials of ligninolytic enzymes produced by fungi and bacteria in styrofoam degradation process.

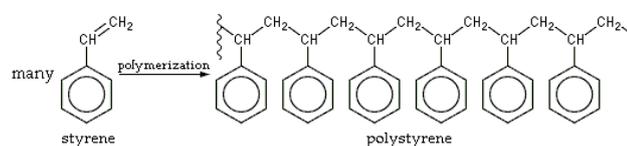


Figure 1. Structure of polystyrene [9]

2. Materials and Methods

2.1 Materials

Three fungal isolates were used in this study, *Pestalotiopsis sp.* (NG007), *Ceriporia sp.* (BIOM3), and *Cymatoderma dendriticum* (WM01). Three bacterial isolates were *Pseudomonas aeruginosa* (BLSP4), *Bacillus subtilis* (BLSP4), and *Serratia marcescens* (BLSP4). Malt Extract Agar (MEA), Nutrient Agar (NA), Malt Extract Broth (MEB), and Nutrient Broth (NB) were purchased from Difco.

Styrofoam solution consisted of 70% alcohol, dichloromethane, dimethylformamide, and Tween 80 surfactant.

2.2. Media and Isolate Preparation

2.2.1. Agar media preparation. MEA was used for regenerating fungi, and NA for bacterial regeneration. Both media were also used for styrofoam degradation tests on solid surface. MEA consisted of glucose 20 g/L, malt extract 20 g/L, hypolypton 1 g/L and the addition of yeast extract 15 g/L, meanwhile NA media composition weighed as 20 g/L. Both types of media, separately were dissolved and heated respectively in 300 ml of distilled water, then sterilized by autoclave. Each medium was divided into 15 petri dishes sterile.

2.2.2. Liquid media preparation. MEB and NB were prepared separately in 1 liter of distilled water. MEB media consisted of glucose 20 g/L, wheat extract 20 g/L, and hypolypton 1 g/L, meanwhile NB media composition weighed as 28 g/L. Both media, separately were dissolved in 1 liter of distilled water, stirred, heated. After boiling, 100 mL of each was poured into 300 mL Erlenmeyer and sterilized by autoclave.

2.2.3. Isolates culture preparation. All of the fungal isolates were inoculated to MEA, while the bacterial isolates were inoculated to NA with sterile inoculation needles. The cultures were then incubated for 7 d for fungi and 2 d for bacteria.

2.3. Styrofoam degradation test

2.3.1. Styrofoam solution for agar media. As much as 0.1 g of styrofoam was soaked into 70% alcohol for surface sterilizing. After that, styrofoam was dissolved in 100 mL of dichloromethane until the solution was homogenous. The properties of dichloromethane are volatile so they can easily remove from the layer of the agar medium. The concentration of styrofoam solution was 1000 ppm.

2.3.2. Styrofoam solution for liquid media. As much as 990 mg of styrofoam was soaked into 70% alcohol for surface sterilizing. Then, the styrofoam was dissolved in 49.5 mL of dimethylformamide (DMF) and 0.5 mL of tween 80 was added into the solution. The concentration of styrofoam solution was 20.000 ppm.

2.4. Resistance test of isolates to styrofoam

Isolates resistance test were conducted on the media in order to determine the ability of isolates to survive against styrofoam solution. These test used 2 types of agar media which is NA and MEA. MEA medium was used to test the three fungal isolates, while NA medium was used to test the three bacterial isolates. To each medium, 2 mL of styrofoam solution (in dichloromethane) was added and the solution was shaken evenly on the entire surface of the agar medium. The solvent dichloromethane was used because they have a high volatility levels and evaporated rapidly, so that only styrofoam layer will be left in the agar media. The cultures then were incubated at 25 °C for 7 d for fungal isolates and 2 d for bacterial isolates.

2.5. Styrofoam degradation test on liquid media

Medium used in this styrofoam degradation test were MEB and NB, with the volume of each medium was 20 mL. One millilitre of styrofoam solution with a concentration of 20.000 ppm of dimethylformamide solvent and mycelial of fungal or bacterial culture were added to each medium. After that, the liquid medium containing styrofoam solution and isolates was incubated at 25 °C for 30 d [10]. After the incubation period was over, the fungal mycelial or bacteria cultures were separated from filtrate and then dichloromethane was added until the volume reached 50 mL. Styrofoam solution was extracted using a separating funnel and evaporated in a rotary evaporator.

Extracted compounds in the vials were allowed to dry at room temperature, and weighed when completely dry. The weight loss of styrofoam was then calculated (W1). The value of WO can be measured through the concentration of 1.000 ppm of styrofoam solution, the degradation efficiency value (DE) are obtained from the equation below [11]:

$$DE\% = \frac{WO-W1}{W1} \times 100\% \quad (1)$$

Description:

DE% = Degradation Efficiency Value

WO = Weight of control

W1 = Weight of sample

2.6. Fourier Transform Infra Red (FTIR) test

Assessment of morphological damage due to polymer degradation test results was observed using spectrum two FTIR with UATR (Perkin Elmer, USA) from 4000 – 450 cm⁻¹ with total scan minimum 16 times.

2.7. Styrofoam degradation test on soil media

A total of 30 jar bottles were prepared for storing styrofoam in the soil. This degradation test consist of treatments with styrofoam either with the bacteria isolate *P. aeruginosa* or styrofoam with fungal isolate NG007, keeping control treatment without any organism. These experiments were performed in three replications. A total of 15 jar bottles were filled first with black soil (top soil) 3 g, 3 g of glucose, 3 g of sengon wood powder, 12 mL of sterilized water.

To each bottle, a styrofoam plate (2 cm x 2 cm) with a thickness of 0.5 cm (soaked in 70% alcohol for 5 min for surface sterilization) was inserted. Then the bottle with styrofoam plate was incubated with fungal isolate NG007 or homogenized with *Pseudomonas aeruginosa* bacteria isolate. Next, the bottle treatment was covered with soil and incubated for 30 days at 25°C [10]. After 30 days, styrofoam plates were rinsed with aquades, and weighed using the same calculation [11].

2.8. Morphological analysis with Scanning Electron Microscopy (SEM)

The morphological damage due to polymer degradation test results was observed by using SEM The sample was coated with gold splashes for 30 minutes. Then, SEM was activated with a voltage of 20 kV.

3. Results and Discussion

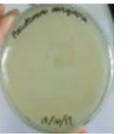
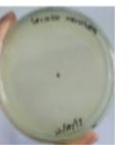
3.1. Resistance test of isolates to styrofoam

This test was conducted to determine the ability of isolates to survive on the presence of styrofoam in the media. The result of the test is underlying further tests to see styrofoam degradation ability in the soil [12]. As shown in Table 1, strain NG007 has the strongest ability to survive among other fungal isolates, while *Pseudomonas aeruginosa* appears as the bacteria with the strongest survival rate indicated from the growth of white hyphae in fungi and the number of colonies formed by the bacteria. The highest survival rate of fungi was showed by NG007, BIOM3 and WM01, respectively. The fungal isolate NG007 showed the highest survival rate because it has a higher lignin peroxidase enzymes activity compared to BIOM3 and WM01. Thus, the fungal isolate NG007 could degrade more substrate (styrofoam) and use carbons from styrofoam as one of the carbon source alternatives for hypha growth.

On the other hand, the highest survival rate of bacteria was showed by *P. aeruginosa*, *B. subtilis*, and *S. marcescens*, respectively. *P. aeruginosa* has the highest survival rate due to carbon supplies from the soluble styrofoam. It has main enzymes which change styrene (styrofoam) into phenylacetic CoA that consists of monooxygenase, styrene oxide isomerase, and phenylacetaldehyde

dehydrogenase. Phenylacetic CoA will change into acetyl-CoA by enzymes supports so that acetyl-CoA could enter the Krebs cycle [5].

Table 1. Survival rate test of fungi and bacteria isolates on styrofoam solution for 7 day of incubation.

Isolate	Resistance level	Growth 0 d	Growth 7 d	Isolate	Resistance level	Growth 0 d	Growth 7 d
<i>Pestalotiopsis</i> sp. NG007	+++++			<i>P. aeruginosa</i>	+++++		
<i>Ceriporia</i> sp. BIOM3	++++			<i>B. subtilis</i>	+++		
<i>C. dendriticum</i> WM01	++			<i>S. marcescens</i>	+		

Note: (+) very weak, (++) weak, (+++) moderate, (+++++) strong, (+++++) very strong

3.2. Styrofoam degradation in liquid medium

The degradation of styrofoam solution in liquid medium by fungi is shown in Figure 2. It is presented that (a) out of the three types of fungi, isolate NG007 has the highest degradation level at 74.43% from the other fungi (BIOM3 and WM01). The result of styrofoam degradation by bacteria is shown in Figure 2, resulting to the finding that (b) the highest degradation level was obtained by *P. aeruginosa* at 63.43%.

The degradation test of styrofoam solution in liquid medium was conducted to measure the degradation level of styrofoam by fungi and bacteria in malt extract liquid media and nutrition broth that are rich of carbon and nitrogen. Carbon and nitrogen act as sources of microbial growth which could support enzymes production [13].

The results showed that NG007 and *P. aeruginosa* have the highest degradation level compared to other isolates, possibly due to higher ligninolytic enzyme activities. The ligninolytic enzymes had important roles in degrading the styrofoam by oxidizing methoxy group in aromatic nonphenolic ring. *P. aeruginosa* is one of *Pseudomonas* genus which has styABCD operon as upper enzyme pathway code (catabolism enzyme). Thus, it makes *P. aeruginosa* capable of degrading styrofoam more efficiently compared to other bacteria [14].

The result from FTIR is presented in Figure 3, (a) there were some functional groups that identify the styrofoam including the C-H aromatic stretching vibrations within 3025.96 cm⁻¹ spectrum. The second peak of stretching mode of C-H was found in 2921.30 cm⁻¹ spectrum. The next peak was found at 1601.47 presenting the stretch of C=C. The peak found at 755.27, 696.06 cm⁻¹ presents the monosubstituted buckling of C-H. Within the comparison to the structure of control functional groups of styrofoam in liquid medium in the form of liquid nutritional stock which had been incubated for 30 days as seen in Figure 3, it can be seen that (b) there is no significant changes in the solution, in which only higher water content was found in 3369.09 cm⁻¹ spectrum at absorbance level of ± 90 %T.

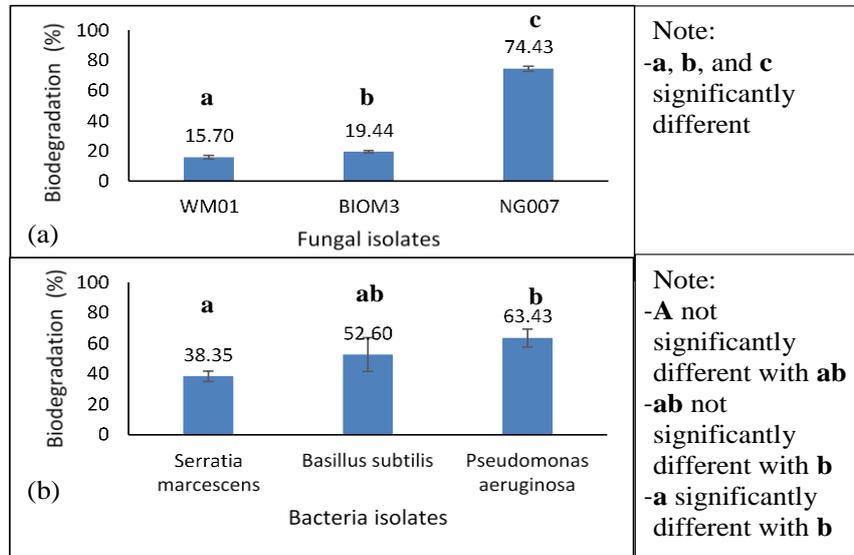


Figure 2. Styrofoam (solution) degradation by fungal (a) and bacteria (b) in liquid medium

The following FTIR spectrum presented in Figure 3 (a) and (b) is the comparison between styrofoam degradability of fungi isolate NG007 and bacteria isolate *P. aeruginosa*. In the degradation spectrum of isolate NG007, some functional groups of aromatic C-H and monosubstituted C-H stretching and other stretching functional groups of C-C, C-H were released. While, C-H has its quantity decreased as seen in its level of absorbance. Identical peak corresponding to completely integrated styrofoam was observed in the FTIR spectrum of *P. aeruginosa* has been found, yet seen from its level of absorbance, there has been a decrease in the intensity of each functional group.

Table 2. Functional group of styrofoam degradation in liquid medium.

Peaks					
Control Styrofoam (before) (cm ⁻¹)	Control Styrofoam liquid medium (cm ⁻¹)	Treatment NG007 (cm ⁻¹)	Treatment <i>P. aeruginosa</i> (cm ⁻¹)	Functional Group	References (cm ⁻¹)
3025.96	3025.94	-	3025.95	C-H aromatic	2970-3070
2921.3	2919.65	2928.58	2921.51	C-H stretch	2840-3000
1601.47	1602.12	1651.74	1646.25	C=C stretch	1620-1680
1028.60	1028.63	1023.64	1198.74	Buckling C-H	1000-1250
696.06	696.36	-	695.21	Buckling C-H monosubstitution	690-770

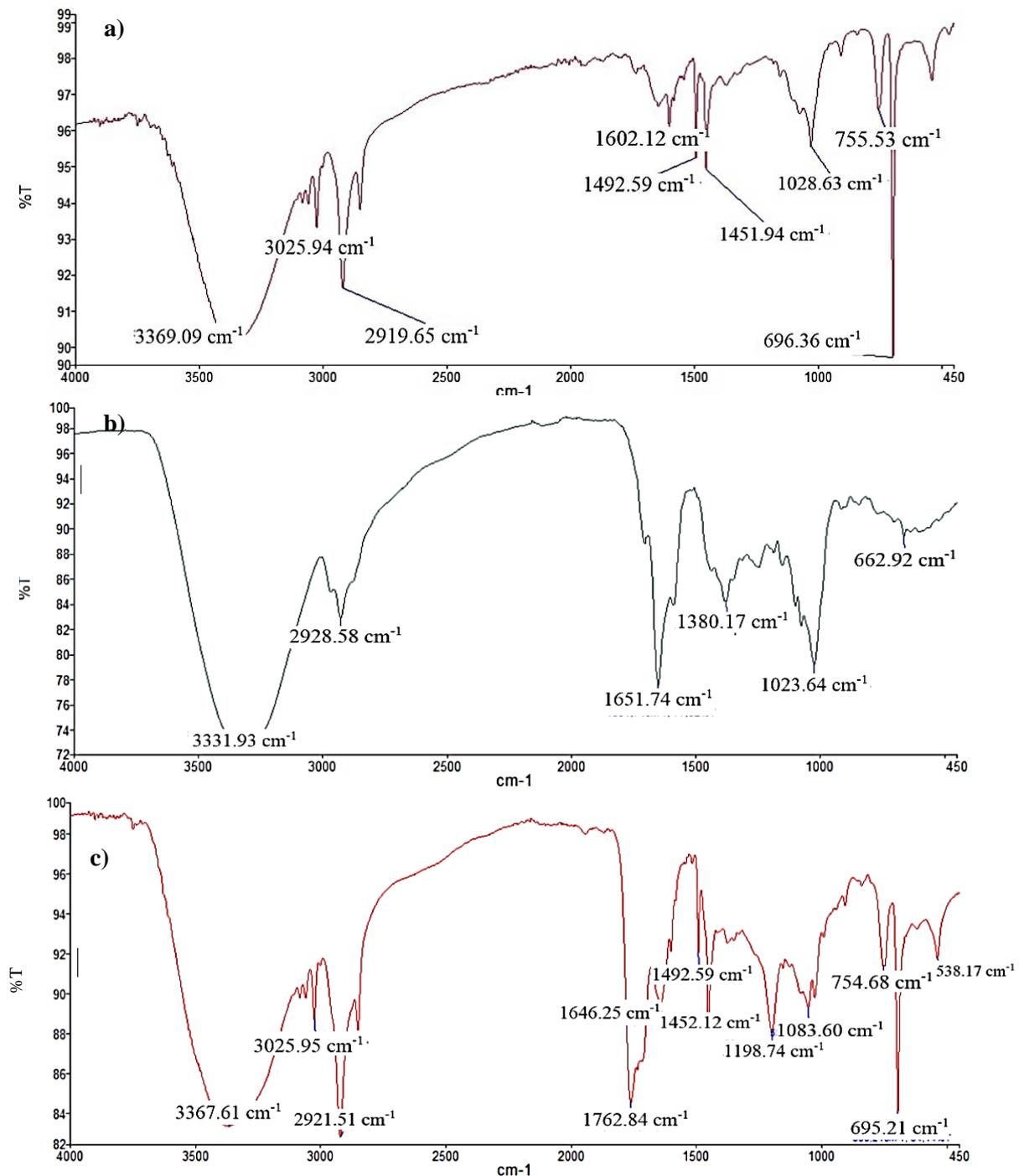


Figure 3. Styrofoam FTIR peaks control in liquid media (a) treated with fungi isolate NG007 (b) and isolate *P. aeruginosa* (c).

The results of FTIR test from both first styrofoam control and styrofoam control in liquid media had complete identity of functional group of styrofoam which consists of C-H aromatic, C-H stretch, C=C stretch, buckling C-H and buckling C-H monosubstitution. There was a degradation of styrofoam which was indicated by removal of identity of styrofoam functional group, that were C-H aromatic and buckling C-H monosubstitution. Moreover, there was a decrease transmittance within functional group

of C-H stretch, C=C stretch and buckling C-H which indicate their decreasing due to the degradation by ligninolytic enzymes of fungi isolate NG007.

On the other side, the styrofoam degradation by bacteria *Pseudomonas aeruginosa* showed that the identity of styrofoam functional group was intact but the transmittance was decreasing which indicated that the degradation happened due to metabolism activities of *Pseudomonas aeruginosa* by changing styrene (styrofoam) into acetyl-CoA that could enter the TCA cycle [5].

3.3. Styrofoam degradation in soil

This research used samples of soil with 30.17% water content and pH level at 7.93 as the medium. Top soil samples were obtained from sites around Research Center for Biomaterials-LIPI area. In the control treatment of styrofoam flakes, there were no signs of damages or pore enlargement found after observation under SEM as presented in Figure 4 (a) and 6 (b).

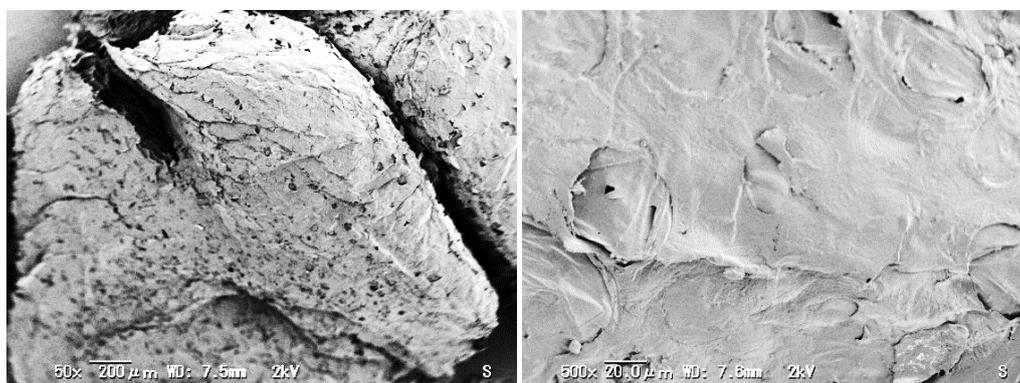


Figure 4. Styrofoam surface observed under SEM, styrofoam control treatments with 50X magnitude (a) and 500X magnitude (b).

Styrofoam can be degraded by *P. aeruginosa* in the soil with or without using *Albizia* powder. Enlarged pores were found in the degraded styrofoam by *P. aeruginosa* added with *Albizia* powder as seen in Figure 5 (a) 50X (left) and 500X magnification (right), respectively. A slightly different SEM result of the degraded styrofoam by *P. aeruginosa* without *Albizia* powder is presented in Figure 5 (b) 50X (left), and 500X magnification, respectively.

Isolate NG007 showed the highest ability to degrade the styrofoam in the soil with or without *Albizia* powder. Micro pore was found in the surface of styrofoam after degraded by NG007 with the addition of *Albizia* powder as presented in Figure 5 (c) 50X (left) and 500X magnification (right), respectively. The result from SEM observation of treatment without *Albizia* powder showed not significantly different with that of addition of *Albizia* powder Figure 5 (d) 50X (left) and 500X magnification, respectively which is not significantly different from the SEM result of the degradation done by isolate NG007 added with *Albizia* powder treatment.

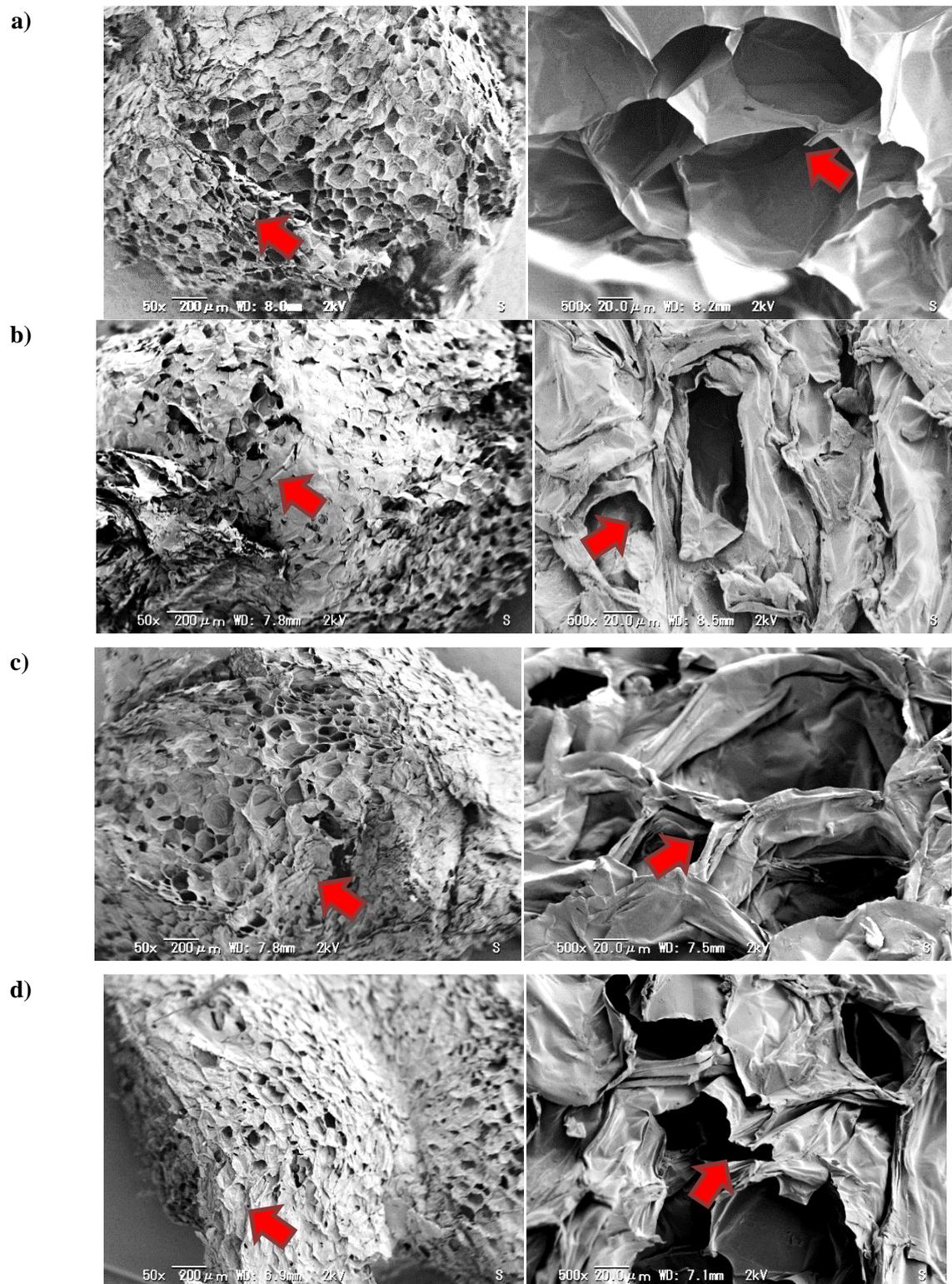


Figure 5. Styrofoam degradation by: (a) *P. aeruginosa* added with *Albizia* powder; (b) *P. aeruginosa* without *Albizia* powder; (c) NG007 added with *Albizia* powder; (d) NG007 without *Albizia* powder with 50X (left), and 500X magnification (right) during 30 d of incubation.

4. Conclusion

Pestalotiopsis sp. NG007 and *P. aeruginosa* BLSP4 showed the ability to degrade styrofoam, which previously dissolved in DMF, under liquid medium. Analysis with FTIR and SEM corroborates the degradation of styrofoam by these microorganisms.

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