Potential Use of \textit{Aspergillus flavus} strain KRP1 in Utilization of Mercury Contaminant

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Abstract

\textit{Aspergillus flavus} strain KRP1 isolated from tropical forest soil was examined for its potential use in degradation of mercury contaminant in soil. This fungal strain has shown its capability to grow best in 25ppm mercury which contaminated Potato Dextrose Agar (PDA) plate media compared with other strains. The preference pH for fungal growth was ranged from 5-7 and reached optimum on pH 6. The optimum temperature was found to be 27.5-35°C. The growth expression of fungi to the presence of mercury contaminant in media was by showing the inhibitory on growth diameter compared to non-contaminated media. The minimum inhibitory concentration (MIC) of this fungal strain to mercury was at 100ppm when there was no growth on the plate media while the growth on non-contaminated media reached maximum (5 day old). The fungal strain was also evaluated in vitro for the potential use in bioremediation of soil contaminated by mercury through observation of the growth profile and the mercury concentration in culture medium. The growth profiles of \textit{Aspergillus flavus} strain KRP1 showed considerable growth in culture medium containing mercury. This result was supported by the decrease of mercury concentration which indicates a utilization process for mercury and might have mechanism for degradation. This indicates that this fungal strain has a potential use for areas where mercury contaminant is exist.

Keywords: \textit{Aspergillus flavus} strain KRP1; mercury; biodegradation

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1. Introduction

Fungi in natural environment are ubiquitous and play the most important role as decomposers of organic material as well as nutrient cycling. Some metals such as K, Na, Mg, Ca, Mn, Fe, Cu, Zn, Co, and Ni are essential for the fungal growth, but many others such as Rb, Cs, Al, Cd, Ag, Au, Hg and Pb are not. However, these metals are able to interact with fungal cells to be accumulated through physico-chemical mechanism [1]. Fungi are known to tolerate and detoxify metals by several mechanisms such as valence transformation, intra and extracellular precipitation and active uptake [2]. The high surface, volume ratio and ability to detoxify metals are amongst reason to be considered as potential alternative for bioremediation of dilute solution of metals and metal contaminated solid wastes [3, 4]. Fungal metal transformations are divided into mobile and immobile phase. Fungal mobilization of metal occurs through heterotrophic (chemoorganotrophic) leaching. Metal immobilization is processed through biosorption or metal binding in cell [5].

Mercury in soils has a long retention times and a negative impact towards living organism [3]. Mercury polluted soil remediation is particularly important because mercury does not degrade, and thus persists almost indefinitely in the environment. Mercury in soil is firmly bound to organic matter or precipitated as sulphide, and is found in trace concentrations in soil solutions. Methods such as excavation and disposal, stabilization/solidification, electro-remediation, soil washing/leaching, and as well as thermal desorption are known to be less economic favor.

Aspergillus species is determined in utilization of metals in environment such like Aspergillus niger which is solubilize stable lead material, pyromorphite (Pb₅(PO₄)₃Cl) and methylation of metalloids to yield volatile derivatives (selenium) that could provide one means of removal. Metal immobilization process includes biosorption or metal binding in cell [5]. Others utilization of A. niger is by using its pretreated biomass in removal of inorganic (Hg²⁺) and methyl mercury (CH₃Hg⁺) from aqueous solution resulting the potential use for removal of inorganic mercury and methyl mercury ions from polluted aqueous effluent [6]. The other Aspergillus species such as A. fumigatus and A. flavus are also proven to have high tolerance to heavy metal such as Zn contaminant on textile wastewater [7] as well as Pb, Zn, Cu, and Ni from paper mill effluent [8]. Metal has been proven to be accumulated in the fungal biomass [9].

2. Material and Method

2.1. Chemical and materials

Mercury stock solution was made in concentrate solution of Hg²⁺ in order to simplify when dissolved to media. Stock solution was made by diluting 0.677 g HgCl₂ powder into 100mL sterile distilled water in aseptic technique. Potato Dextrose Agar (PDA) containing 24 g Potato Dextrose Broth (PDB) (Difco; Becton Dickonson and Company, USA) powder and 20 g standard agar per liter adjusted to pH 5.7 and autoclaved on 121°C for 15 minutes was used as media. When media has been reached about 45°C, the Hg²⁺ solution in certain concentration was mixed well in the media before it was poured into plates. All the process was done in aseptic technique. Liquid media for biosorption was provided by diluting 24g PDB powder in one liter distillate water and heat up until thoroughly dissolved. This solution was then cooled down to room temperature and adjusted to pH 5.7 before autoclaved in 121°C for 15 minutes.

The fungal strains namely FRP1, KRP3, KRP2, GRP1, FRP2 and KRP1 were obtained from fungal strains collection of Hygiene and Sanitation Laboratory, Yamaguchi University, Japan. These collections were resulted from screening and isolation of tropical forest soil at Malang, East Java, Indonesia [10]. Isolated fungal strains resulted from screening and isolation process were then stored in PDA slant as pure culture.

2.2. Experimental Methods

The fungal strains namely FRP1, KRP3, KRP2, GRP1, FRP2 and KRP1 were chosen based on their consistency to grow on third MEA, PDA and Czapek media. The fungal strain was first refreshed on non-mercury PDA plates media by placing a small piece chunk containing fungal mycelia in the centre of the plates and being incubated on 30°C for 5 days. This process was repeated twice before using it for treatment. For the fungal strain selection, a 7
mm and 5 day old mycelia plug of each strain was placed on the centre of both mercury and non-mercury amended PDA plates in triplicate and incubated on 30°C. The growth diameter of the fungal strains was measured every 24 h for 5 days. Concentration of the media was occupied to contain 25 ppm Hg\(^{2+}\). The mycelia growth measurement was conducted by cultured a 7 mm diameter mycelia plugged from the edge of 5 day old fungal strain culture on PDA, then placed on the centre of each plate agar, and incubated for 7 day at 30°C in three replicates [11]. Fungal strain with highest ratio of growth diameter on 7 day observation was the selected fungal strain for further study.

The ratio of growth diameter observation was aimed at finding out the strength of fungal strain to grow on mercury contaminated PDA media comparing to non-contaminated media. The formula used to calculate the ratio of growth diameter is as follow:

\[
\text{Ratio of growth diameter} = \frac{M}{S} \quad (1)
\]

Where: 
- \(M\) = Growth diameter on treatment (Hg\(^{2+}\) contaminated media)
- \(S\) = Growth diameter on control (non-contaminated media)

The optimum pH for the mycelia growth was conducted by growing the fungal strain on PDA media adjusted at pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, and 7.0 with 1 mol/L NaOH or HCl. The mercury contaminated media was done by amended 25ppm Hg\(^{2+}\) on sterile media in aseptic technique. A 7 mm mycelia plug was removed from 5 days old culture of the fungal strain grown on PDA media. Each plug was placed on the centre of the plate, and then incubated for 5 days at 30°C. To investigate the optimum temperature, a 7 mm cork borer mycelia plug was removed from 5 day-old cultures of the fungal strain grown on finding optimum pH PDA media, placed each plug in the centre of plate, and was incubated for 5 days at 25°C, 27.5°C, 30°C, 32.5°C, and 35°C. The mycelia growth measurement was the average values obtained from three replicates [11].

Minimum inhibitory concentration (MIC) means the concentration which inhibits the growth of fungal strain. A 7 mm plug mycelia was placed in PDA plate media (pH 5.7) amended with Hg on various concentrations i.e. 5, 15, 25, 50, 100, 150 and 200ppm. Media was previously autoclaved in 121°C for 15 min, cooled to 45°C, then added with desired concentration of Hg in aseptic technique. The inoculated plates were incubated at 30°C for 5 days. Growth diameter was observed daily to have ratio of growth diameter value.

Growth profiles observation was examined in 250mL Erlenmeyer flask containing 100mL Potato dextrose Broth (PDB) medium which was inoculated with 2x10^7 spore/mL. The pH of the medium was maintained at 5.7 – 6 during thr study using 1 mol/L NaOH or HCl. All glassware was washed with 5% HNO\(_3\) and rinsed with deionized water to remove contaminated metals on glassware. The culture was incubated at 30°C with shaking at 100rpm (EYELA, Japan) for 28 days. Every 4 days, the culture was filtered and the dry weight of the mycelium was determined. The average values were obtained from three replicates.

The degradation of mercury by Aspergillus flavus strain KRP1 was examined in 250mL Erlenmeyer flask containing 100mL PDB medium. The medium was contaminated with Hg\(^{2+}\) solution and maintained to have 10ppm of Hg\(^{2+}\). The observation was done as in growth profile. The mercury degradation was determined on the basis of total mercury concentration on the liquid medium. Every 4 days a 6mL sample of each broth culture was pipette into centrifugation tube and centrifuged for 15 min at 6000rpm. One milliliter of the supernatant was pipette into 200mL Erlenmeyer flask and diluted with deionized water into 100mL to measure the mercury concentration using CVAAS Hiranuma 200. The average values were obtained from three replicates.

Results and Discussion

2.3. The Selection of Fungi

Fungal strain KRP1 shows the highest capability to consistently grow on mercury containing PDA. See Fig. 1. This strain also has the highest capability on eliminating the toxicity effect of mercury shown by the highest ratio of growth compared to the growth on non-contaminated media. Thus, this strain was chosen for further study in order to know more about its capability in mercury contaminated media as well as its potential on remediation of mercury contaminant.
Fungal survival in the presence of toxic metals mainly depends on intrinsic biochemical and structural properties, physiological and/or genetics adaptation, including morphological changes and environmental modification of metal speciation, availability and toxicity [1]. This fungus showed highest growth diameter in mercury contaminated media as an indication of fungus hardiness. The ratio of growth diameter of fungal strain KRP1 also showed the highest ratio compared to other strains. This indicates that the presence of mercury contaminant inhibits the growth of fungal strain KRP1 less than others. The other strain such as KRP2 showed high tolerance as well, however this strain had lower growth diameter showing lower capability to grow in mercury contaminated media. According to these two reasons, it can be said that fungal strain KRP1 showed the resistance or tolerance to mercury contaminant.

An organism may directly and/or indirectly rely on several survival strategies for example, methallothienein synthesis mechanism in *Saccharomyces cerevisiae* to Cu²⁺ by binding or precipitating it around the cell wall and intracellular transport [12]. In terms of bioremediation, fungi are able to process the target compound through enzymatic breakdown (cometabolism), uptake and concentrate within its body (accumulation), and even use the target compound as carbon source. However, fungi are often more proficient at cometabolism and accumulation process. Oxidative enzyme, that plays a major role and the excreted organic acids and chelators by fungus, is involved in cometabolism process and makes many toxic chemicals mineralized by fungi which are already highly oxidized [13].

### 2.4. Fungal identification and characteristic

Based on the morphology of colonies (see Fig. 2) and cells based taxonomic investigation, strain KRP1 belonged to *Aspergillus* sp. [14]. This fungal strain was identified as *Aspergillus flavus* strain KRP1 based on partial 320 bp sequences of the 18S rRNA amplicon and the comparison in the GenBank databases showed 98% nucleotide base homology to *Aspergillus flavus*.

The effect of pH and temperature on the mycelial growth of *A. flavus* strain KRP1 in vitro (see Fig. 3.) showed its ability to grow pH ranged 5.5-7. As *Aspergillus* genera has high capability to grow in highly aerobic environment and oxygen-rich environment (loose soil), it demonstrates its oligotrophic characteristic which is capable to grow in less-nutrient environment so was this strain. *A. flavus* strain KRP1 growing in wide range of temperature that is 30-35 °C (Fig. 1.B) showed its preference in hot environment like the general type of *A. flavus* which favors to hot dry condition and has optimum growth on temperature 37°C and readily grow between the temperatures of 25-42°C.
2.5. Minimum inhibitory concentration (MIC)

The response of *A. flavus* strain KRP1 on the presence of various mercury concentrations in media tends to follow second order reaction leading to the death of organism if it is exposed to high concentration of mercury (see Fig. 4). The minimum inhibitory concentration was reached at 100 ppm concentration where the growth of this fungal strain was completely inhibited.

The presence of mercury on media seems to be toxic to these fungal strains, however in certain level they are still able to grow. Fungi are known to be good in accumulating heavy metals onto their mycelium and spores [13]. This mechanism will decrease the risk of heavy metals absorption by cultivating crop in contaminated agricultural soil in order to prevent it to be consumed by human. Thus, this *A. flavus* strain KRP1 has potential to be applied in contaminated soil with mercury.

*Aspergillus* sp. was present and dominant in heavy metal contaminated sites as well as *Fusarium* sp., *Penicillium* sp. and *Mucor* sp. (14, 15). One of *Aspergillus* species namely *Aspergillus niger* was proven to be able to resist various high concentration of heavy metals such as Cd, Co, Cr, Cu, Ni, Pb and Zn (15). As a biological leaching agent of heavy metals from contaminated soil, *A. niger* exhibits a good potential in generating a variety of organic acids effective for metal solubilization. These acids effectively removed the exchangeable, carbonate and oxide fraction of Cu, Cd, Pb and Zn (23). Research development regarding bioremediation by using *A. flavus* has begun to consider. Heat inactivated (killed) *A. flavus* biomass was suitable to be used as biosorbent for the removal of Pb(II) and Cu(II) ions from aqueous solution (29) also for As (III) (20). Applicative use of *A. niger* and *A. flavus* in removing heavy metal was also done on paper mill effluent and showed capability to accumulate Pb, Zn, Cu, and Ni (31).
2.6. Growth profile and utilization of mercury by means of total mercury

The growth profile of *Aspergillus flavus* strain KRP1 was further monitored on 28 days based on mycelia dry mass using potato dextrose broth medium (see Fig. 5.). Growth phase included “lag” phase, the acceleration phase that occur at the “exponential” phase and then “decelerate” phase, followed by stationary phase and lyses (cell death) (British Mycological Society). The “exponential” phase was during 8 days where there was progressive increase of dry mass weight called as growth on up to the first 8 days of cultivation. A “decelerate” phase followed by “stationary” phase occurred between 12th and 20th day of cultivation; and then a slight increase was on next days.

The growth profile of *A. flavus* strain KRP1 in potato dextrose broth medium contained 10ppm of mercury. The progressive increase of mycelial dry mass during the first days of incubation was accompanied by decreasing of total mercury from the medium. Degradation of mercury from Potato Dextrose Broth culture medium occurred during the progressive increase of mycelia dry mass in the first 8 days of incubation. The rate of increasing of mycelia dry mass was coupled with the decreasing of the mercury concentration in the culture medium (see Fig. 5.). This indicates a utilization process occurred and could be said that a mechanism of degradation was possessed.

The total mercury concentration dropped most notably on 8th days of cultivation and continued until 28 days of cultivation. This result indicates that the utilization process for mercury took place and perhaps showed that the study on fungal strain might possess a mechanism for degradation of mercury contaminant.

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