Bioleaching Ability of Fungi Isolated from an Indonesian Sulfurous River Sediment

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Received: April 7, 2019
Accepted: February 22, 2020

DOI: 10.22146/ijc.44810

Abstract: *The unique characteristics of sulfurous river sediment located in Ungaran, Indonesia, are a reservoir of novel fungi with manganese bioleaching properties. Fungi are known to produce metabolic organic acids that have a potential for the industrial application of leaching metal from the ores. This application has high advantages, including low cost, low energy, and creates minimal environmental damage. Therefore, this research was performed to analyze the manganese bioleaching activities of two fungal isolates (KA2B2 and KB4B) from Indonesian sulfurous river sediment on pyrolusite and determine their phenotypic characters. These activities were investigated in terms of changes in fungal biomass, soluble manganese concentration, pH reduction, and organic acid production during 16 days of leaching. Soluble manganese concentrations were measured by atomic absorption spectrometry (AAS), whereas organic acid concentrations were analyzed by high-performance liquid chromatography (HPLC). According to bioleaching investigations, KA2B2 strain was more efficient than KB4B1 strain in extracting manganese from 0.02 g/cm^3 pyrolusite. It also produced higher levels of organic acids, such as oxalic acid and citric acid, than KB4B1 strain, proving that strain of KA2B2 could be used to extract manganese from pyrolusite. Based on the phenotypic characters, both strains were identified as genus* Penicillium.

Keywords: *pyrolusite; soluble manganese; oxalic acid; citric acid*

■ INTRODUCTION

Manganese is an important heavy metal that serves several industrial purposes. Due to an increase in its demand for use in industrial products, the development of an effective and environmentally safe method of metal extraction is necessary. Recently, bioleaching has been reported as the most effective, low-cost, and promising green technology for metal extraction. This method involves the activities of microorganism groups, such as chemoautotrophic bacteria (sulfur-oxidizing bacteria), heterotrophic bacteria, and fungi [1-4]. Fungi are a ubiquitous group of microbial communities that play a growing role as agents of geochemical change. Most previous research was conducted to isolate fungi from peculiar habitats such as acids, mine drainage, mining areas, the igneous oceanic crust, and rocks [5-8]. Fungal strains have been reported to extract several heavy metals such as gold, copper, nickel, and lithium by processes of bioleaching [9-11].

Fungal bioleaching processes, which are necessary for mining industry development, involve several mechanisms, including matrix solubilization, complex metal forming results from organic acids or amino acids excretion reaction, reduction of ferric iron mediated by oxalic acid, and metals bioaccumulation by fungal

mycelia. In addition, fungal bioleaching has advantages over bacterial bioleaching because fungi have a shorter lag phase during growth, the ability to tolerate toxic materials, a faster leaching rate, and capability to grow over a wide range of pH (from acidic to alkaline conditions) and in sulfurous environments [12-18].

A sulfurous river located in Ungaran, Indonesia, is one of the places that has the unique characteristics of a high sulfur concentration and a wide temperature range. In such sediment ecosystems, fungi can exhibit a variety of tolerance and survival mechanisms. These unique habitats can also serve as a reservoir of novel fungi with extraordinary properties. In consequent, the fungi with the potential for metal extraction could be isolated from these habitats. However, there is no recent study reported indigenous fungi from the Indonesian sulfurous river, which have potency for metals extraction. Therefore, in this study, fungi that have bioleaching ability were isolated from this habitat, and their phenotypic characters determined. Their manganese bioleaching ability was also investigated based on fungal growth, changes in pH values, manganese solubility, and organic acid production.

■ EXPERIMENTAL SECTION

Materials

The pyrolusite used in this research was collected and originated from Kliripan, Kulonprogo, Yogyakarta, Indonesia (7°51′48″S–110°07′00″E). The ore was dry ground to a mean particle diameter of 0.1–0.2 mm [19-20]. The mineralogical components of the ore samples were analyzed as 25% Mn, 30.4% Fe, and 34.0% S. These samples were subsequently used for bioleaching experiments. The second set of sediment samples were collected from the sulfurous river in Ungaran, Middle Java, Indonesia. These samples were kept at 4 °C and used only for isolating fungi.

Instrumentation

The pH of the leachate was measured by using a digital pH meter (Metrohm). Dissolved manganese was analyzed by using a flame atomic absorption spectrophotometer (Hitachi, Z-2000) [21]. The organic acid components were investigated using high-performance liquid chromatography (HPLC, Knauer) equipped with a Zorbax C18 column (250 mm × 4.6 mm) and a detector with diode array at 210 nm. The mobile phase was composed of 0.01 mol/L KH_2PO_4-H_3PO_4 (pH 2.6) and 3% methanol (v/v) at 0.5 mL/min flow rate. Finally, to quantify organic acids, an external standard method has been used [22].

Procedure

Fungal isolation and screening

One gram of the second sample was diluted in 9 mL sterilized H_2O and was inoculated into sucrose liquid medium with a pulp density of pyrolusite of 0.02 g/cm^3 (final pH: 6.0). The mixture was incubated at 30 °C with rotary shaking at 120 rpm. The sucrose liquid medium consisted of the following composition (gL^{-1}): sucrose (100), $NaNO_3$ (1.5), KH_2PO_4 (0.5), $MgSO_4{\cdot}7H_2O$ (0.025), KCl (0.025), and yeast extract (1.6) [9]. After 7 days of incubation, it was plated on sucrose solid medium with a pulp density of pyrolusite of 0.02 g/cm^3. This culture was incubated for 7 days until single colonies as pure cultures of the fungal strain were obtained. Sixteen pure strains were isolated from the second sample and then transferred into a sterilized liquid sucrose medium with a pulp density of pyrolusite of 0.02 g/cm^3. Through analyzing shifts in media pH values, fungal strains were selected within 7 days based on their ability to significantly lower the pH of the medium from more than 7.3 to 3.0. These strains were selected for further experiments.

Bioleaching investigations

Two fungal strains selected (KA2B2 and KB4B1) were incubated on potato dextrose agar slants for 7 days. After incubation, their spores were washed from the cultures using a physiological saline sterile solution (9 gL^{-1} NaCl). Spores were enumerated under a microscope at 400× magnification using a hemocytometer of 1 mm depth, and their number was determined at about 10^7 spores/mL with a physiological saline sterile solution [23].

One milliliter part of the spore suspension was inoculated into 100 mL of sterilized sucrose medium with a pulp density of pyrolusite of 0.02 g/cm^3 in a 250 mL

Erlenmeyer flask. Each strain was incubated at 30 °C and 120 rpm for 16 days. On experimental days 0, 2, 4, 6, 8, 10, 12, 14, and 16, samples were collected from each flask, centrifuged for 10 min at 5000 rpm and filtered through a 0.42 μm Whatman membrane filter. The filtered samples were then measured for their value of pH, soluble manganese concentration, and organic acids. Whereas, the filter paper containing mycelia was then dried at 80 °C for 24 h for fungal biomass measurements.

Phenotypic characterization

Both strains were further observed for their phenotypic characters based on a macroscopic and microscopic investigation in two types of identification media: Czapek yeast auto lysate (CYA) and malt extract auto lysate (MEA). Fungal cultures were incubated following the recommended of previously mycology researches [24-25]. The macroscopic observations studied included the diameter of the colony, the color of obverse and reverse colonies, and the presence or absence of exudates. Whereas, microscopic characters of the fungal isolates were observed using an optical microscope.

Statistical analysis

The data were analyzed using a Statistical Analysis Systems (SAS 9.4, SAS Institute, Inc., Cary, NC, USA) software package. Statistical analysis differences between average values were analyzed using analysis of variance (ANOVA) and Duncan Multiple Range Test (DMRT) method, respectively. The results were reported as means of three replicates. Their mean values were then compared using a significance level of $p < 0.05$.

■ RESULTS AND DISCUSSION

In this investigation, two fungal cultures (strain KA2B2 and KB4B) were isolated and selected from the five strains for subsequent observations. These selections were evaluated by analyzing shifts in media pH values produced during acidolysis by fungal strains. As reported by previous researchers, the main mechanism of bioleaching by fungal strains is the acidolysis of organic acids [9,26]. Both filamentous fungal strains were observed to significantly lower the pH of the medium from more than 7.3 to 3.0 in 7 days.

Changes in Fungal Biomass, pH, and Manganese Concentration during the Bioleaching Process

Bioleaching experiments were conducted at pH 6 and 30 °C with a particle size of 0.1–0.2 mm and agitation at 120 rpm. The initial inoculum size was 10^7 spores/mL, the pulp densities of pyrolusite were 0.02 g/cm^3 and the incubation time was 16 days. In this research, the two fungal strain *Penicillium* KA2B2 and *Penicillium* KB4B1 were selected and compared as bioleaching agents. Fig. 1 compares the changes in fungal biomass, pH, and yield of extracted manganese between the two strains during 16 days of bioleaching investigations.

Penicillium KA2B2 yielded larger biomass and had a shorter lag phase compared with *Penicillium* KB4B1. During 4 days of *Penicillium* KA2B2 growth, the biomass had a maximum value of 30 g/L, and the pH of the medium steadily decreased, reaching 1.5 on the 16th day. This increase in the biomass value and decrease in pH at the fourth day indicated that *Penicillium* KA2B2 was in its logarithmic growth phase. A similar trend of increasing biomass and declining pH values during the logarithmic growth phase of *Penicillium* KA2B2 was also observed in the culture of *Penicillium* KB4B1. In comparison, *Penicillium* KB4B1 took longer to reach the logarithmic growth phase, which was observed at 6 days' incubation. The maximum value of its fungal biomass was 18 g/L, and the minimum pH was 2.6.

The acidification phenomenon in both cultures led to manganese solubilization, as shown by the increase in their yield of leached manganese. *Penicillium* KA2B2 extracted as much as 25% of the manganese from pyrolusite under optimal conditions at 8 days' incubation. On the other hand, *Penicillium* KB4B1 extracted less (20%) manganese from pyrolusite under optimal conditions at 10 days' incubation. As reported by previous researchers, the phenomenon of culture acidification is caused by organic acids provided by strains, which are important in the metal bioleaching process. These acids could supply both protons and metal-complexing anions, leading to free metal cations released. The reactions between organic acids (oxalic acid,

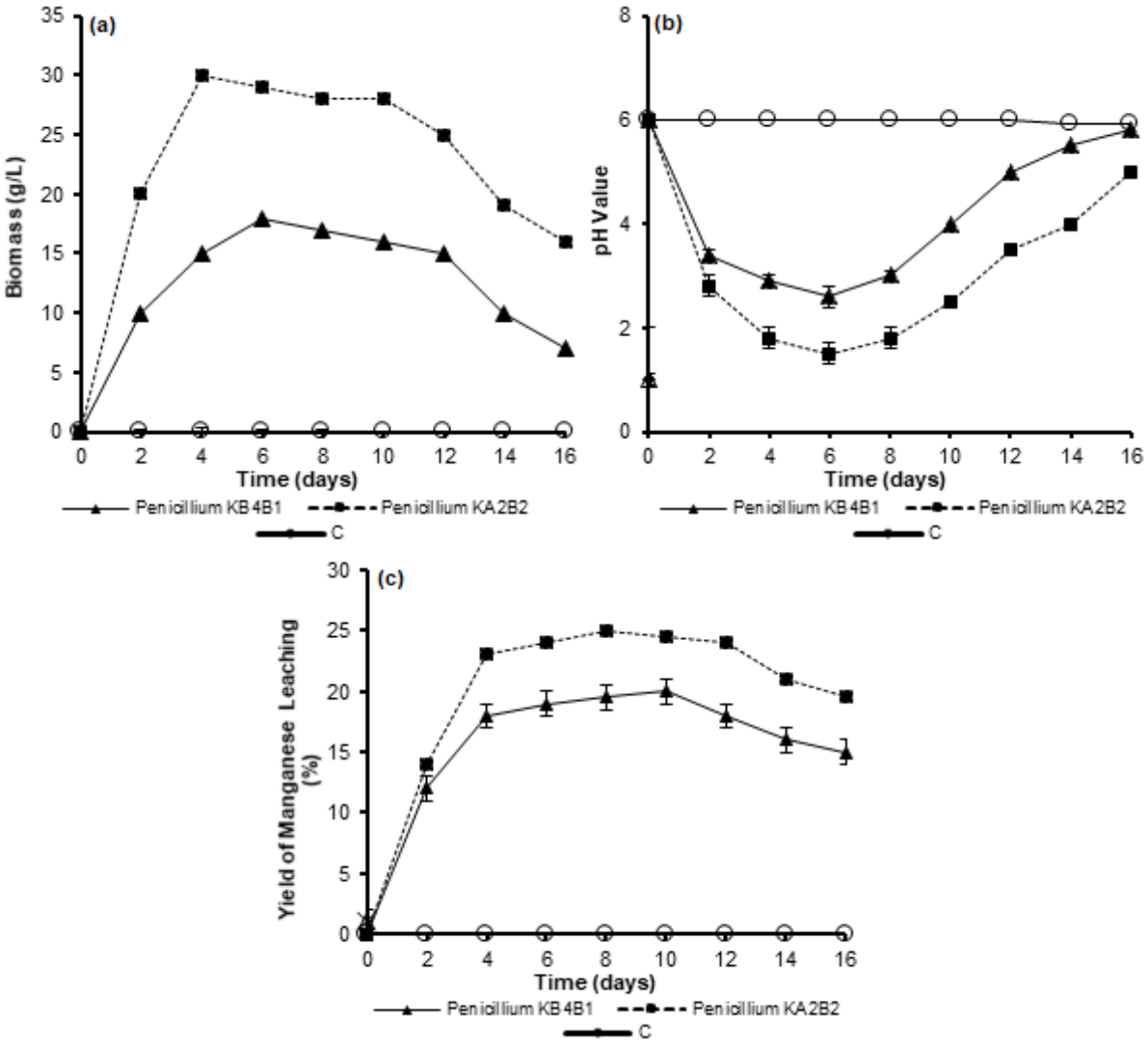

Fig 1. Changes in biomass (a); pH value (b); and manganese yield (c); between *Penicillium* KB4B1 and *Penicillium* KA2B2 at a pulp density of pyrolusite of 0.02 g/cm^3 during 16 days of leaching

citric acid) and metallic ion (M^{n+}) are described below [27-28].

The dissociation (1, 2) and complexation reactions (2, 3) of oxalic acid are:

$$C_2H_2O_4 \rightarrow C_2HO_4^- + H^+ (pK_{a1} = 1.25) \quad (1)$$

$$C_2HO_4^- \rightarrow C_2O_4^{2-} + H^+ (pK_{a2} = 4.14) \quad (2)$$

$$n[C_2HO_4^-] + M^{n+} \rightarrow M[C_2HO_4]_n \text{ (Oxalic metallic complex)} \quad (3)$$

$$n[C_2O_4^{2-}] + 2M^{n+} \rightarrow M_2[C_2O_4]_n \text{ (Oxalic metallic complex)} \quad (4)$$

Whereas the dissociation (5, 6, 7) and complexation reactions (8, 9, 10) of citric acid are:

$$C_6H_8O_7 \rightarrow C_6H_7O_7^- + H^+ (pK_{a1} = 3.09) \quad (5)$$

$$C_6H_7O_7^- \rightarrow C_6H_6O_7^{2-} + H^+ (pK_{a2} = 4.75) \quad (6)$$

$$C_6H_6O_7^{2-} \rightarrow C_6H_5O_7^{3-} + H^+ (pK_{a2} = 6.40) \quad (7)$$

$$n[C_6H_7O_7^-] + M^{n+} \rightarrow M[C_6H_7O_7]_n \text{ (Citric metallic complex)} \quad (8)$$

$$n[C_6H_6O_7^{2-}] + 2M^{n+} \rightarrow M_2[C_6H_6O_7]_n \text{ (Citric metallic complex)} \quad (9)$$

$$n[C_6H_5O_7^{3-}] + 3M^{n+} \rightarrow M_3[C_6H_5O_7]_n \text{ (Citric metallic complex)} \quad (10)$$

Organic acid analysis by HPLC revealed that the *Penicillium* KA2B2 culture reached optimum production of citric acid and oxalic acid at 13.5 and 17 mmol/L, respectively. In contrast, the maximum production of citric acid and oxalic acid of *Penicillium* KB4B1 were 5 and 8 mmol/L, respectively (Fig. 2).

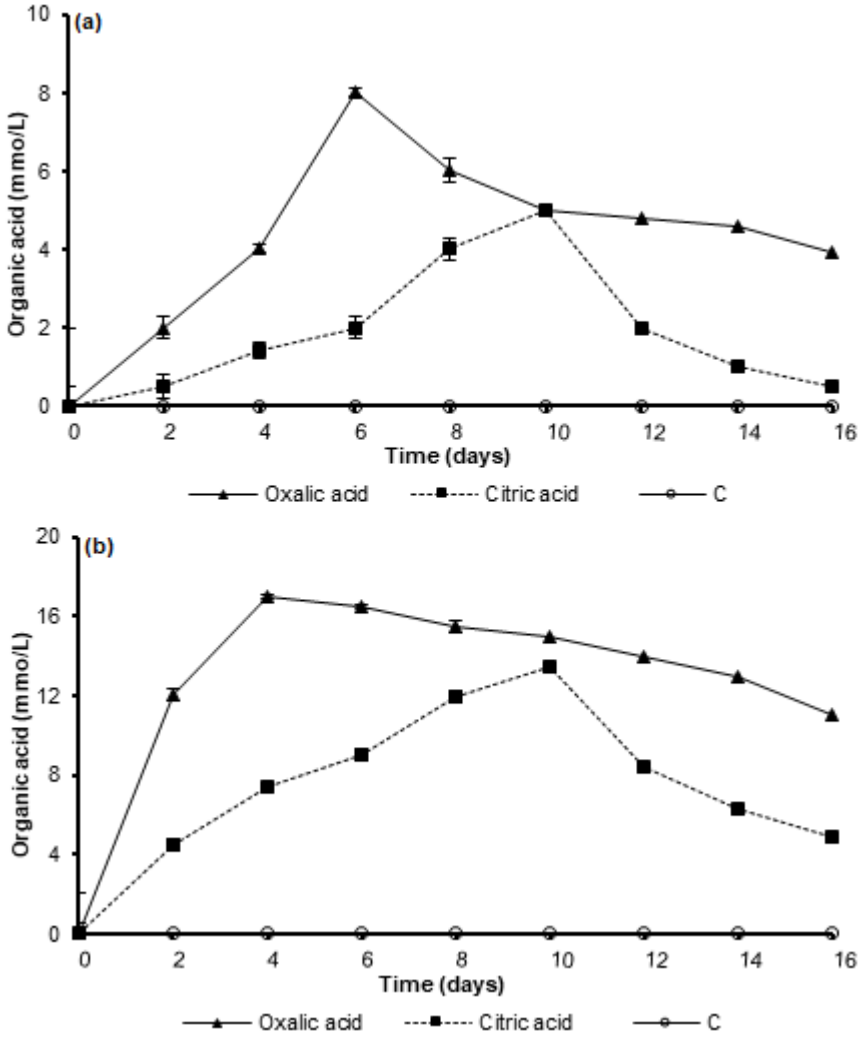

Fig 2. Organic acid production in *Penicillium* KB4B1 (a) and *Penicillium* KA2B2 (b) at pulp densities of pyrolusite of 0.02 g/cm^3 in the 16-day leaching process

There were similar trends of oxalic acid and citric acid secretion in *Penicillium* KA2B2 and *Penicillium* KB4B1. Within the logarithmic growth phase of these fungi, the production of oxalic acid was highly increased, and then slowly decreased; however, a significant amount of oxalic acid remained in the medium at the end of the incubation. In contrast, the production of citric acid by both strains occurred within the stationary phase, which was characterized by a decreased rate of growth in these fungi. In comparison with pH measurements, it is clear that the increase in citric acid and oxalic acid secretion by the two strains occurred at pH values less than 3. When the pH of the medium measured was above pH 3, acid production was reduced.

Oxalic acid biosynthesis from glucose occurs when oxaloacetate is hydrolyzed to oxalate and acetate, catalyzed by cytosolic oxaloacetase, while citric acid is formed as an intermediate in a cycle of tricarboxylic acids involving a polysaccharide such as sucrose [29]. Therefore, sucrose present in the medium during the bioleaching experiment was used as a substrate by the strain for oxalic acid and citric acid biosynthesis. Glucose was first released from sucrose, then absorbed and catabolized to two molecules of pyruvate. In the following step, it was converted into oxaloacetate and acetyl-CoA, and finally, citric acid formed by condensation of these two precursors, which was then secreted from mitochondria and mycelia [30-32]. From

these results, it was clear that the increase in citric acid production by the cultures was followed by a reduction of the concentration of oxalic acid present in the cultures.

Compared to previous studies, both fungi were about 54% lower in extracting manganese than *Aspergillus niger* PTCC 5210 isolated from mining deposits in India [29]. Those fungi were also 22% lower in extracting metals than *Penicillium chrysogenum* Y5 isolated from heavy metal contaminated areas [33]. In this study, the low ability of metal extraction from both fungi due to the optimization processes of bioleaching has not been done. Optimization of the parameters that enhanced fungal bioleaching was related to their production of metabolic organic acids, including organic carbon, nitrogen, phosphorous, micronutrient ($MgSO_4$ and $MnSO_4$), and aeration.

Phenotypic Characters of Fungi

For phenotypic identification, fungal isolates were characterized according to previously researchers [24-25]. Macroscopic observations showed heterogeneity between the fungal isolates that being examined (Table 1). The data obtained exhibit that colony diameters of fungal strain KA2B2 varied over a wide range on different agar media: from 3.6 mm (CYA) to 14.3 mm (MEA). In addition, the examined fungal cultures displayed variations in surface and reverse colony colors. Strain KA2B2 had a green to the grey surface and orange to bright yellow colony color on CYA. Its surface and reverse colony colors on MEA were green and orange to bright yellow, respectively.

Observation of fungal microscopic characters such as conidiophore branching and its elements are key to the identification of fungi. Two types of fungal conidiophores, metulae, phialides, conidia, and stipe characters, were observed (Table 2). Fungal strain KA2B2 had a mono-verticillate conidiophore branching pattern. The metulae had a compact terminal character. The type of fungal phialides was ampuliform. The conidia size was less than 10 μm, with a globose and smooth morphology. Fungal strain KB4B1 also had a mono-verticillate conidiophore branching pattern. The metulae had a tuberculate character. The fungal phialides were cylindrical and short with a wide neck. The conidia size was also less than 10 μm, with a globose and smooth morphology. Thus, based on both macroscopic and microscopic investigations, both of strain KA2B2 and KB4B1 was identified as genus Penicillium.

■ CONCLUSION

Penicillium KA2B2 isolated from Indonesian sulfurous river sediment is highly efficient for manganese extraction with a pulp density of pyrolusite of 0.02 g/cm^3. The culture extracted manganese by producing oxalic acid and citric acid from the sucrose in the medium. These investigations indicate that oxalic acid

Table 1. Macroscopic characters of two selected fungi isolated from sulfurous river sediment

Strain	On CYA			On MEA		
	Surface colony color	Reverse colony color	Diameter (mm)	Surface colony color	Reverse colony color	Diameter (mm)
KA2B2	Green-gray	Orange to bright yellow	3.6	Green	Orange to bright yellow	14.3
KB4B1	Dark green	Green	33.3	Dark green	Yellow	33.3

Table 2. Microscopic characters of two selected fungi isolated from sulfurous river sediment

Strain	Conidiophore Branching Pattern	Metulae	Phialide	Conidia	Stipes
KA2B2	Mono-verticillate	compact terminal	Ampuliform	< 10 μm, globose, smooth-walled	Rather long, smooth-walled, vesiculate
KB4B1	Mono-verticillate	tuberculate	Cylindrical, short, and wide neck	< 10 μm, globose, smooth-walled	Relatively short

and citric acid contribute significantly to manganese leaching from pyrolusite. This strain is strongly recommended for further studies in field observations.

■ ACKNOWLEDGMENTS

This research was financially supported by KEMENRISTEK DIKTI Indonesia with the Research Implementation Letter No: 173/UN1/DITLIT/DIT-LIT/LT/2018. We express our thanks to technicians from Microbiology and Falitma Laboratory, Biology Faculty, and also *Laboratorium Penelitian Pusat Terpadu*, Universitas Gadjah Mada, Yogyakarta, Indonesia.

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