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Isolation, Identification and Characterization of Indigenous Fungi for Bioremediation of Hexavalent Chromium, Nickel and Cobalt

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Abstract. Waste from nickel mining of Sorowako in South Sulawesi contains hexavalent chromium, nickel and cobalt metals in high concentration and may have a negative impact to the environment. Common waste treatment systems such as chemical treatment using a reducing reagent may still have a negative impact. Bioremediation using fungi or bacteria becomes more popular because it is an environmentally friendly alternative. The purposes of this study are to isolate and identify indigenous fungi that are resistant to heavy metals (hexavalent chromium, nickel, and cobalt) and are capable of reducing the concentration of metals in mining wastes. Ten fungal isolates were successfully isolated from the soils and pond sediments in the area of nickel mining in Sorowako. Selection of superior isolate was carried out by growing all the isolates on PDA medium, which contained all of the three metals. One superior isolate was identified to be able to grow on medium with concentrations of 6400 ppm hexavalent chromium, 200 ppm nickel and 50 ppm cobalt. Molecular identification and phylogenetic studies of the isolate using fungal PCR primers developed to amplify the ITS (internal transcribed spacer) region showed that the isolate sequence was very close to *Trichoderma atroviride* with 99.8% similarity. Optimum incubation time for the uptake of hexavalent chromium was 3 days, nickel and cobalt was 5 days, respectively, with an optimum pH of 4.

Keywords: heavy metals, bioremediation, phylogenetics, ITS, fungi, hexavalent chromium, nickel, cobalt
PACS: 89

INTRODUCTION

The presence of heavy metals in the environment can cause toxic effects for living things. Mining activities become one of the causes of increasing heavy metal concentration in the environment. Nickel mining waste in Sulawesi, Indonesia is known to contain heavy metals in a high concentration [1]. In addition to producing nickel, process of nickel mining also produces other heavy metals that are associated with nickel such as hexavalent chromium and cobalt [2]. According to the World Health Organization (1984), the metals of most immediate concern are cadmium, chromium, cobalt, copper, lead, nickel, mercury and zinc. The presence of such metals >5 g cm^3 in aquatic environments causes severe damage to aquatic life, killing microorganisms during biological water purification process [3]. Moreover, these metals have exacting consequences on humans such as brain damage, reproductive failures, nervous system failures, tumor formation, etc [4].

Common waste treatment systems performed include reverse osmosis, electrodialysis, ultrafiltration, ion-exchange, chemical precipitation, phytoremediation, etc. However, all these methods have disadvantages like incomplete metal removal, high reagent and energy requirements [5].

Alternative methods of metal removal and recovery based on biological materials and also called as bioremediation have been considered. Microbial communities are importance in bioremediation of metal-contaminated soil and water because microbes alter metal chemistry and mobility through reduction, accumulation, biosorption and immobilization. In related studies, metal removal abilities of various species of fungi have been investigated such as *Polyporus squamosus* [4], *Trichoderma harzianum* [6], *Evernia prunastri* [7] and *Penicillium* [8]. Indigenous fungi have a potential as bioremediation agent because it is resulted from long-term adaptation to soils with extreme properties such as high concentration of heavy metal [9].

This study was carried out to isolate, identify and characterize indigenous fungi from heavy metal-contaminated soil and sediment as well as their application potential to bioremediation of hexavalent chromium, nickel and cobalt, heavy metals present in effluents from mining wastes in South Sulawesi Province, Indonesia.

MATERIALS AND METHODS

Isolation of metal-resistant indigenous fungi

Heavy metal-resistant fungi were isolated from soils and pond sediments contaminated by nickel mining wastewater in Sorowako, South Sulawesi. Soil and sediment were suspended in sterile distilled water and then plated on potato dextrose agar (PDA) supplemented with 20 ppm $K_2Cr_2O_7$, 10 ppm $NiCl_2.7H_2O$ and 10 ppm $CoCl_2$. The cultures were incubated at room temperature for 7 days. Grown colonies of fungi were then selected and purified. Fungi isolates were maintained by sub-culturing.

Screening metal-resistant fungi

All isolated fungi were grown on potato dextrose agar (PDA) containing varying concentrations of hexavalent chromium (Cr[VI]), nickel (Ni) and cobalt (Co) and were subsequently incubated at room temperature for 7 days. The growth of fungal mycelium was observed, and the isolate which showed high resistance in metal-amended agar was selected for further study.

Identification of a selected fungal isolate by direct sequencing of ITS region

A fungal culture was grown in potato dextrose broth at room temperature for 3 days. The mycelium was used to extract genomic DNA by using the UltraClean™ Microbial DNA Isolation Kit (MO BIO Laboratories, Inc.) according to the manufacturer's instructions with several modifications. The universal ITS primer pairs ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'TCCTCCGCTTATTGATATGC-3') were used to amplify ribosomal total ITS region [10]. PCR amplification was conducted using these sets of primers with the following program: 35 cycles of denaturation at 95°C for 30 sec, annealing at 59°C for 30 sec and a final extension step at 72°C for 2 min. The PCR product was purified before DNA sequence analysis using *DNA Purification Kit* from Geneaid, and was sequenced by direct sequencing method at the 1st Base Bioengineering Technology Service Co., Ltd. (Singapore). The sequence of the ribosomal total ITS region from the isolate was used as query to determine the genus and species of its closest eukaryotic relative using BLASTN. The phylogenetic tree was constructed using the neighbour-joining method in the MEGA5 program. Bootstrap analysis with 1000 replicates was also conducted in order to obtain confidence levels for the branches.

Effect of incubation period and pH on fungal growth as biomass

Effect of incubation period was determined by incubating the cultures in different periods (1-7 days) of incubation. The cultures were grown in sterile 200 mL Erlenmeyer flask containing 50 mL of potato dextrose broth (PDB), which were supplemented with 20 ppm Cr[VI], 20 ppm Ni and 20 ppm Co, and were inoculated with spore suspension of a selected isolate ($1x10^6$ spore/mL). Flasks were placed on orbital shaker (150 rpm) at room temperature. Effect of different pH (3.0-8.0) was studied in the above prescribed condition. Samples of all experiments were analyzed identically in duplicate. Samples of each day were filtered and biomass was dried at 80°C in oven up to constant weight. After drying, biomass was weighed to determine the effect of different cultural conditions on the growth of fungal strains. Metal content was analyzed after digestion. Cr[VI] concentration was determined using 1,5-diphenylcarbazide method, and KSCN method was used to determine Co. Ni concentration was determined by using Atomic Absorption Spectroscopy (AAS).

RESULTS

Ten indigenous fungi were able to be isolated from contaminated soils and sediments with their metal resistance levels as given in Table 1 & 2. From the first step of screening at concentration 200/100/100 ppm of Cr[VI], Ni and Co, there was no fungi able to grow (data not shown). Thereafter, the second step of screening was undertaken by varying Cr[VI] concentrations (200-12800 ppm) in the presence of Ni and Co in 50 ppm concentrations (Table 1). From this step, two isolates were able to grow at high concentration of Cr[VI], and the isolate anoa 3 was selected as a superior isolate due to its growth ability at the highest concentration of Cr[VI]. For further study, the isolate anoa 3 was used to be identified and characterized. Using alignment sequence in BLASTn the isolate anoa 3 was identified as *Hypocrea lixii* or known as *Trichoderma harzianum* with query coverage 92% and maximum identity 98%. The homology of the isolate anoa 3 was also studied by a phylogeny tree, inferring that it had a closest genetic relationship with *Trichoderma atroviride* (99.8% similarity) (Figure 1).

Table 1. Screening the fungal isolates grown in potato dextrose agar (PDA) supplemented with different concentrations of three metals of Cr[VI], Ni and Co

Isolate code	Concentration of Cr[VI]/Ni/Co (ppm)			
	0/0/0	50/25/25	100/50/50	200/100/100
Anoa 3	++++	++++	++++	-
Anoa 5	++++	++++	++++	-
Pt 1-1	+	+	+	-
Pt 1-2	+++	+	+	-
Pt 1-3	+	+	+	-
Pt 1-4	++++	++	+	-
Pt 1-5	++	+	+	-
Pt 2-1	+++	++	+	-
Pt 2-2	+++	+++	++	-
Matano	+++	+++	+	-

Note: ++++ : extremely high growth; +++: high growth; ++: moderate growth; +: low growth; - : no growth

Table 2. Screening the fungal isolates grown in potato dextrose agar (PDA) supplemented with different concentrations of Cr[VI] at Ni and Co concentration of 50 ppm

Isolate Code	Concentration of Cr[VI] ppm						
	200	400	800	1600	3200	6400	12800
Anoa 3	+++	+++	+++	+++	+++	+	-
Anoa 5	+++	+	+	+	+	-	-
Pt 1-1	-	-	-	-	-	-	-
Pt 1-2	-	-	-	-	-	-	-
Pt 1-3	-	-	-	-	-	-	-
Pt 1-4	-	-	-	-	-	-	-
Pt 1-5	-	-	-	-	-	-	-
Pt 2-1	-	-	-	-	-	-	-
Pt 2-2	-	-	-	-	-	-	-
Matano	-	-	-	-	-	-	-

Note: +++: high growth; +: low growth; - : no growth

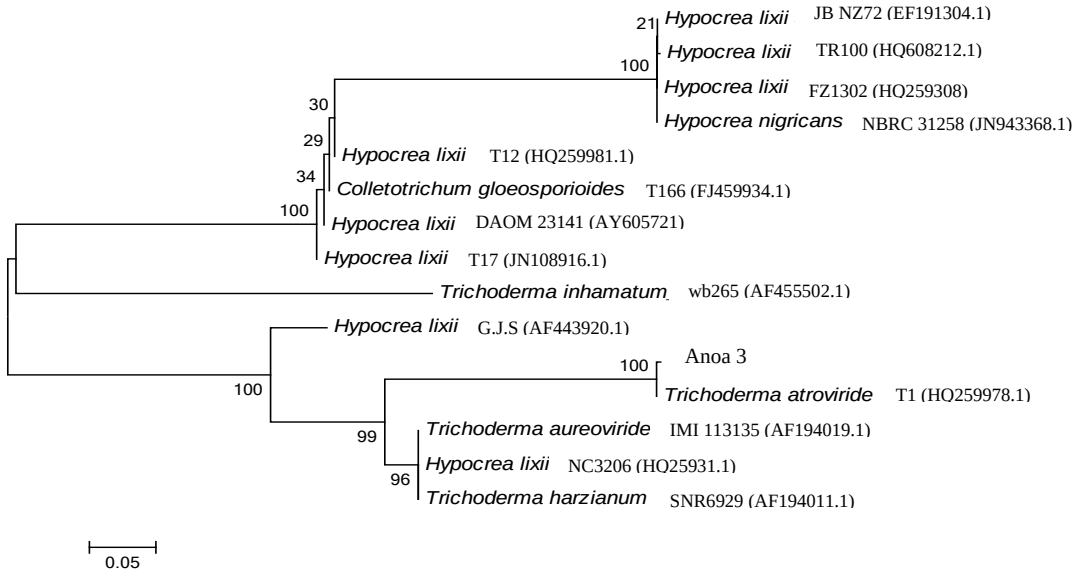

Figure 1. Phylogenetic tree of the isolate anoa 3 constructed using the neighbour-joining method with the Kimura two-parameter distance. Numbers at nodes indicate percentages of bootstrap support derived from 1000 replications. Bar, 0.05 substitutions per nucleotide position

Metal removal at various incubation periods of culture was tested using Cr[VI], Ni and Co concentration of 20 ppm. Every metal had different optimum days to remove. Hexavalent chromium gave the best uptake by the isolate anoa 3 at 3 days of incubation with 94.85% removal, whereas the best uptake for nickel and cobalt was at 6 days of incubation with 82.53% and 90.38% removal, respectively (Figure 2).

Different pH levels within the range of 3 to 8 were studied for their effect on metal removal by the isolate anoa 3 using Cr[VI] (20 ppm), Ni (20 ppm) and Co (20 ppm) for 3 days of incubation. Effect of pH on growth of the isolate anoa 3 during removal of those three metals showed maximum uptake for three metals at pH 4,

where concentrations of hexavalent chromium, nickel and cobalt in medium were reduced by up to 94 %, 68% and 60%, respectively (Figure 3).

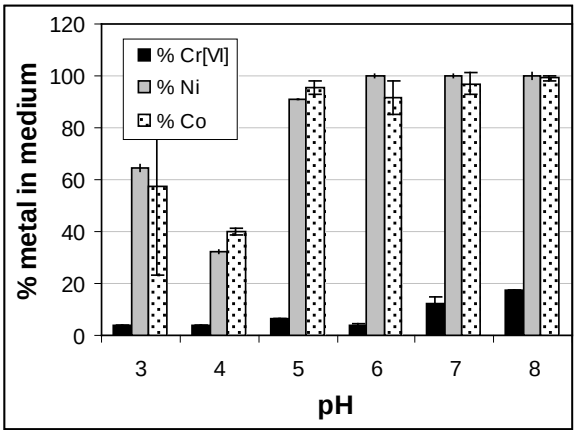

Figure 2. Percentage of residual metal in potato dextrose broth (PDB) medium supplemented with Cr[VI] (20 ppm), Ni (20 ppm), Co (20 ppm) inoculated with the isolate anoa 3 and incubated for different periods of time.

for every metal. Maximum uptake for hexavalent chromium, nickel and cobalt was noticed in 3 days, 6 days, and 5 days, respectively. This phenomenon can be occurred due presumably to the existence of competitive binding of metal ion with cell surface [1].

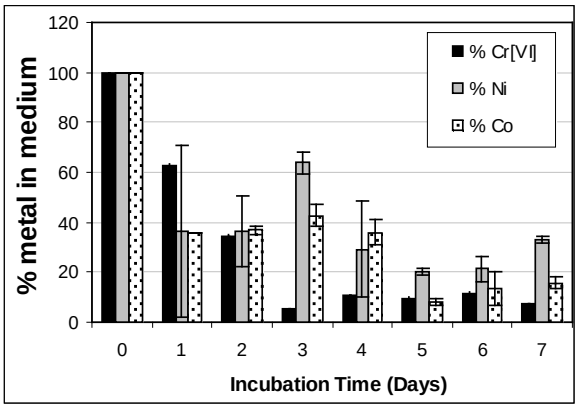

Figure 3. Percentage of residual metal in potato dextrose broth (PDB) medium supplemented with Cr[VI] (20 ppm), Ni (20 ppm), Co (20 ppm) inoculated with the isolate anoa 3 after 3 days of incubation at room temperature at different pH values.

DISCUSSION

Fungi are used widely in industrial fermentation and bioremediation [10]. Fungi are preferred over other organisms because they are easier to remove from liquid substrates. Nickel ores contain several other metals as minor, such as hexavalent chromium and cobalt [11], and at the mining waste, these associated metals will produce as pollutant. Thus, it is expected from the present study, the fungal isolates should be able to remediate all three metals at the same time.

An isolate of fungi (anoa 3) was isolated as a powerful fungal strain by having a highest resistance to the three metals Cr[VI], Ni and Co, reaching concentrations of 6400 ppm (Table 2). These metal concentrations greatly exceed the maximum permissible concentrations of Cr (400 ppm), Ni (50 ppm) and Co (20 ppm) in soils [22]. The phylogenetic analysis revealed that the isolate anoa 3 was more closely related to *Trichoderma atroviride* (99.8% similarity) (Figure 1). A previous study has reported that the genus *Trichoderma* has the potential for bioremediation of heavy metals such as Cr[VI] and Ni [6].

Metal removal capacity of the selected isolate (anoa 3) to a certain extent was time dependent system (Figure 2). A progressive increase in the uptake of all three metals was noticed at different periods of time

Metal ion binding mechanism occurs when function groups such as carbonyl, amino, thiol, hydroxyl, phosphate and hydroxicarbonyl located in the cell walls bind metal ion [13] and the mechanism is strongly influenced by the characteristics of the metal ion [14].

pH is the most important biosorption parameter because it influences both metal speciation and cell surface metal binding sites [15]. In the present study, the highest metal removal was observed at pH 4 (Figure 3). Several studies have reported that an optimum pH around 4 is as an ideal condition for metal removal and the metal uptake decreases at high pH levels [16, 17]. Likewise, the metal binding increases with pH due to the decrease of hydronium ions in the system, as they also compete for binding sites [18]. In the present study, metal removal was noticed at pH 3 to 5 with the maximum metal removal at pH 4 after 3 days of incubation (Figure 3). At pH value above 7 the uptake of metals was reduced, presumably metals exist as hydroxide colloids and precipitate at alkaline pH, resulting decrease in sorption rate [19] or due to osmotic changes and hydrolyzing effect [20-21].

CONCLUSION

In the present study, a fungal strain which was capable of removing hexavalent chromium, nickel and cobalt

from the aqueous solution was successfully isolated. By using the phylogenetic analysis, this strain was identified as *Trichoderma atroviride*. The finding of this study has indicated that this fungal strain can also be used as a biological agent for bioremediation of wastewaters and sites contaminated with metals such as Cr[VI], Ni and Co.

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