Screening and molecular identification of Cr(VI)-resistant *Trichoderma* isolated from ex-tin mining soil in Bangka Belitung Province, Indonesia

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Abstract. Several major industries in Indonesia such as the textile industry, electroplating industry, and the pharmaceutical industry, can generate a considerable amount of chromium (Cr) wastes, hexavalent chromium [Cr(VI)]. Unfortunately, the wastewater treatment plant systems of these industries may be partially unqualified, causing heavy pollution in several water bodies such as rivers and lakes. The aim of the present study was to discover and identify Cr(VI)-resistant fungi as bioremediation agents of removing the Cr(VI) from the environment. Seven fungal isolates from tin mining soil of Bangka Belitung province were found tolerant to high Cr(VI) concentration. All fungal isolates grew well at 0.25 mM of Cr(VI), and five fungal isolates still tolerant at a concentration of 2 mM. Molecular identification based on the ITS (internal transcribed spacer) rDNA region showed that these isolates belong to *Trichoderma crassum* (2 isolates), *T. virescentiflavum* (3 isolates), and *T. aff. tomentosum* (2 isolates).

1. Introduction
The release of heavy metals into the environment (terrestrial and water ecosystems) as a result of the activities of various types of industries and mineral mining always increases over time to time, thus posing a significant threat to the environment and public health due to their persistence in nature and toxicity [1]. These heavy metals include Arsenic (As), Cadmium (Cd), Lead (Pb), Mercury (Hg), and Chromium (Cr). The Indonesian Ministry of Environment and Forestry No. 55/2015 has identified chrome and 15 other non-organic chemicals as mandatory parameters for wastewater effluent [2].

Chromium (Cr), especially hexavalent chromium [Cr(VI)], is one of major toxic compounds produced from the wastewater of several major industries such as textile, electroplating, and pharmaceutical industries. Cr, in fact, is an essential micronutrient for the sugar, lipid, and protein metabolism in mammals [3]. However, at high level concentration, Cr in a hexavalent state [Cr(VI)] if enters the cells causes cancer, activation of apoptosis, and cell death in human and animal [4].

The conventional methods for removing heavy metals (including Cr) such as physical methods (reverse osmosis, electro-dialysis, ion-exchange, photocatalysis, and membrane filtration) and chemical methods (chemical precipitations using various chemicals) have been reported effective in reducing Cr(VI) [3, 5]. However, these methods still have various limitations such as high operational cost, high energy consumption, secondary pollutants production, and most of these methods are only able to perform well under high metals condition [5]. Therefore, cost-effective technology is necessary in the industrial wastewater management worldwide.
Mycoremediation, a form of bioremediation of various compounds by fungal-based application, has begun to attract the attention of mycology and bioremediation researchers in recent years, because an effectiveness of the fungal activities in mobilizing and immobilizing heavy metals [6, 7]. This application is also potentially cost-effective and environmentally safe for removing a wide array of toxic compounds from industrial wastewater and polluted environments [6, 7]. Several fungal genera such as Acremonium, Aspergillus, Fusarium, Paeclomyces, Penicillium, Phanerochete, Pichia, Pleurotus, Saccharomyces, and Trichoderma have been reported successfully in remediating Cr through biosorption, biotransformation, and bioaccumulation mechanisms [3]. Among these fungi, a member of Trichoderma appears as one of promising fungal groups in mycoremediation technology due to their tolerance to various heavy metals, ability in producing various metabolites and enzymes, and highly adaptable to various environmental conditions [8, 9, 10, 11].

The objective of the present study was to isolate Cr(VI)-resistant Trichoderma isolates from the extraneous mining soil and to examine the optimum condition of the Cr(VI) reduction activity by the Trichoderma isolates. The identity of the Cr(VI)-resistant Trichoderma isolates was determined by molecular phylogenetic analysis based on the sequence of the Internal Transcribed Spacer (ITS) of ribosomal DNA.

2. Materials and Methods

2.1. Soil sampling and isolation of Cr(VI)-resistant fungi

Cr(VI)-contaminated soil was collected from the BatuBelubang village, Bangka Belitung province, Indonesia (S: 02º11’409”, E: 106º11’265”). A total 25 soil samples (@ 1 Kg) was collected from 25 imaginary plots with a depth of 20 cm at each sampling point. Cr(VI)-resistant fungi were isolated from the contaminated soil using 0.1 × YTPG (Yeast Tryptone Peptone Glucose) medium [12] with the following composition (L-1): peptone 0.25 g, tryptone 0.25 g, yeast extract0.5 g, glucose 0.5 g, MgSO4.7H2O 30 mg, CaCl2.2H2O 3.5 mg. A total of 5 g of soil was added into liquid 0.1 × YTPG medium with the addition of potassium dichromate (K2Cr2O7) 2 mM, and then incubated for 60 minutes on a rotary shaker with a speed of 130 rpm at room temperature. After incubation, 0.2% of the solution was taken and put into liquid 0.1 × YTPG medium by adding K2Cr2O7 0.25 mM, and then incubated at room temperature for 5-7 days on a rotary shaker with a speed of 130 rpm. 0.1 mL incubated sample was taken and then spread onto 0.1 × YTPG agar medium amended with K2Cr2O7 0.25 mM and incubated for 5-7 days at room temperature. The fungal colonies were purified and then stored at 4°C for further use.

All fungal isolates were further screened on mineral agar medium(glucose 1 g, K2HPO40.5 g, NaCl0.5 g, MgCl21 g, NH4NO30.5 g, yeast extract0.5 g, agarose 1.5 g in 100 mL of distilled water). K2Cr2O7 1 mM was added into the medium after sterilization. Fungal isolates were inoculated on the mineral agar medium and incubated for 5 days at room temperature. During the incubation period, the fungal colony growth was observed. Resistance of selected fungus at different concentrations of chromium was carried out on mineral medium amended with K2Cr2O7 up to 2.0. Incubation was carried out for 6 days at room temperature.

2.2. DNA extraction, PCR amplification and sequencing

Genomic DNA extraction of the Cr(VI)-resistant fungus was conducted by growing the fungus mycelium in Potato Dextrose Broth (PDB) (Difco, USA) for 5 days at room temperature on a rotary shaker (120 rpm). The DNA extraction process of the mycelium was carried out using the Genaid® Genomic DNA Mini Kit (Plant) GP100 kit (Geneaid, Taiwan) according to the manufacture instructions.

A PCR amplification of the ITS (Internal Transcribed Spacer) region was carried out using a primer pair of ITS5 (forward) (5'-GGAAAGTAAAGTCTGTAACAAGG-3') and ITS4 (reverse) (5'-TCCTCCGCTATTGATAT-3') [13]. A total 30 µL PCR mixture composed of 1.2 µL DNA template (100 ng/µL), 3 µL Dream Taq Buffer (containing MgCl2) (Thermo scientific, USA), 3 µL 2 mM dNTP (Thermo scientific, USA), 0.6 µL, 0.75µL Dream Taq polymerase (Thermo scientific, USA), and 20.85 µL ddH2O. PCR reaction was carried out at the following condition: pre-denaturation at
94°C for 5 mins, denaturation at 94°C for 30 s, annealing at 52°C for 30 s, extension 72°C for 30 s, and final extension at 72°C for 7 mins. The PCR product was further electrophoresed using 1% agarose gel at 100 V for 15 minutes. A 1 kb Ladder (Thermo Scientific, USA) was used as a marker. The agarose gel was further soaked in an Ethidium Bromide (EtBr) for 30 mins and further visualized using Gel Doc™ XR+ Gel Documentation System (Bio-Rad, USA). The PCR products were sent to 1stBASE (Malaysia) for nucleotide sequencing.

2.3. Phylogenetic analysis

Newly nucleotide sequences from the ITS rDNA region were edited and assembled using the ChromasPro version 1.7.7 software (Technelysium, Australia). Homologous reference sequences from the GenBank (http://www.ncbi.nlm.nih.gov) was searched using BLAST (the Basic Local Alignment Search Tool). Multiple sequence alignment of Trichoderma sequences with homologous sequences from the GenBank was conducted using MUSCLE (multiple sequence comparison by log-expectation) [14] in MEGA (Molecular Evolutionary Genetics Analysis) version 7.0 software [15]. Phylogenetic analysis was carried out using the Neighbor Joining (NJ) method implemented in the MEGA version 7.0 [15]. The strength of internal branches in the phylogenetic tree was tested by bootstrap (BS) analysis in 1000 replications. The Maximum Composite Likelihood was selected as model of the NJ analysis for the dataset. Pairwise deletion was used to treat gaps in the alignment. Sequences of *Trichoderma stromaticum* CBS 101875 (NR_077128) and *T. effusum* strain strain DAOM 230007 (DQ083008) were used as outgroup. Bootstrap values (> 50%) are displayed on the phylogenetic tree.

3. Results and Discussion

3.1. Results

3.1.1. Isolation of Cr(VI)-resistant fungi

Nine isolates of Cr(VI)-resistant fungi from ex-tin mining soil samples were obtained using selective medium (0.1× YPTG medium amended with 0.25 mM K₂Cr₂O₇) (Figure 1). At a concentration of 1 mM K₂Cr₂O₇, seven isolates (A, C1, D1, D2, D3, E1, E2) were found being resistant to chrome (Figure 2). These seven isolates were also resistant to chrome at 2 mM K₂Cr₂O₇.

![Figure 1. 6-days old colonies of nine Cr(VI)-resistant fungi isolated from ex-tin mining soil in Bangka Belitung province, Indonesia.](image-url)
Figure 2. 12-days old 1.0 mM Cr(VI)-resistant fungal isolates isolated from ex-tin mining soil in Bangka Belitung province, Indonesia.

In the growth assay at three different concentrations of chrome (0.25 mM, 1 mM, and 2 mM), highest growth rate was found at 0.25 mM concentration (Figure 3), followed by 1 mM and 2 mM concentrations, respectively. This showed that chrome affects the growth of all Cr(VI)-resistant fungal isolates. At 2.0 mM of chromium concentration, isolate A showed highest colony growth followed by isolate C1 and D3, respectively (Figure 3).

Figure 3. Growth assay of seven Cr(VI)-resistant isolates at different concentrations.
3.1.2. Molecular phylogenetic analysis

The phylogenetic tree generated from the ITS rDNA sequence showed that the seven Cr(VI)-resistant isolates from ex-tin mining in Bangka Belitung province belong to the genus Trichoderma (Figure 4). Sequences of D1 and D2 nested in the same clade with *T. tomentosum* strain DAOM178713A (DQ085432) and *Hypocrea albocomea* strain GJS 92-78 (DQ018116), but with a low bootstrap support (BS) (< 50%). Because D1 and D2 sequences are closely related to *T. tomentosum* strain DAOM178713A, thus both sequences are tentatively named as *Trichoderma aff. tomentosum* strain D1 and D2. Further analysis is necessary to identify the two isolates (D1 and D2) by adding sequences from elongation factor 1-α (TEF 1-α) and RNA polymerase II gene (RPB2). In addition, nucleotide sequences of A, C1 and D3 isolates were identified as *T. crassum* because they form a monophyletic clade with *T. crassum* strains of DAOM 164916 (EU280067) sequence with high BS value (95%). In this clade, the sequences of A, C1 and D3 form an independent lineage with 72% BS. The remaining isolates, E1 and E2, were determined as *T. virescentiflavum* because they nested in the same clade with sequence of *T. virescentiflavum* strain PC 278 with high bootstrap support (84% BS). The E1 and E2 sequences are identical (100% BS).

3.2. Discussion

Several members of the genus *Trichoderma* such as *T. atroviride*, *T. viride*, and *T. harzianum* have been reported resistance to heavy metals such as copper, zinc and cadmium, Cu(II), and chromium [16, 17, 18, 19]. Among these heavy metals, chromium, in the form of hexavalent chromium [Cr(VI)], is very toxic and carcinogenic to living things [16]. A contamination of Cr(VI) to water resources and soil may occurs if handling of waste containing Cr(VI) is not good. As available conventional methods such as chemical precipitation, ion exchange using resin, and adsorption using various materials (e.g. activated carbon) showing unsatisfactory results and expensive [20], it is necessary to discover an alternative method that cheap and effective in removing Cr(VI) from the industrial wastewater.
effluent. Among available biological treatment methods, mycoremediation emerges as one of promising method to remove heavy metals such as Cr(VI), in wastewater management.

In the wastewater treatment by mycoremediation method, fungal mycelia were usually immobilized [20, 21, 22, 23] or was grown on natural lignocellulose materials as carriers such as wood chips and similar lignocellulosic materials [24]. The immobilized mycelium or colonized wood chips were usually put in the bioreactor or wastewater treatment ponds. These methods were successfully reduced heavy metals contents, dyes, and other pollutants from the wastewater [20, 21, 22, 23, 24]. In the large-scale application of contaminated soil, lignocellulose material colonized by mycelium mixed with soil and then the surface was tightly closed like a composting process [25]. This process is not only low cost, but also provides rapid reaction rate and self-heating. Nitrogen supplementation is necessary to optimize the mycoremediation process in this method [25].

This study showed that three species of Cr(VI)-tolerant fungi from ex-tin mining soil belong to the genus Trichoderma (Figure 4). These include T. crassum (2 isolates), T. virescentiflavum (3 isolates), and T. aff. tomentosum (2 isolates). In the previous study, T. viride was reported as Cr(VI) removal agent in an experiment of using airlift bioreactor as wastewater treatment technology [8]. Therefore, this is the first report of T. crassum, T. virescentiflavum, and T. aff. tomentosum as Cr(VI)-tolerant fungi. Among them, T. crassum isolate A, C1, and D3 appear as the most resistant fungi to chrome (Figure 3). Two isolates of T. aff. tomentosum, isolate D1 and D2 also showed resistance to high concentration of chrome (Figure 3).

Little information has been known regarding the mechanism of fungal resistance to chrome. It is possibly related to the ability of fungi to absorb and accumulate various heavy metals in the fungal cell wall. This is because fungi have a cell wall structure that can bind several types of metals [26]. In addition, biotransformation of Cr(VI) to Cr (III) form in fungal cell wall is possible (8,27). The mechanism involves biosorption through periplasmic wall, followed by intracellular accumulation, and several direct enzymatic reactions [28] or indirectly with metabolites [29]. In Cr(VI)-resistant Trichoderma [8], reduction of carbon sources in the cellular metabolism processes is the key in the Cr(VI) biotransformation to Cr(III).

4. Conclusions
Seven fungal isolates from ex-tin mining in Bangka Belitung province, Indonesia, exhibited Cr(VI)-resistant activity. Molecular identification based on the ITS (internal transcribed spacer) of rDNA determined these isolates as Trichoderma crissum (2 isolates), T. virescentiflavum (3 isolates), and T. aff. Tomentosum (2 isolates).

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