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Isolation and characterisation of a rock solubilising fungus for application in mine-spoil reclamation

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ABSTRACT

The soil and water loss in rock mining areas is an extremely serious problem, while microbes play significant roles in soil remediation of those areas. In this study, a fungal strain *Gongronella* sp. NF-15 was isolated from the abandoned dolostone mines, and its mechanism of promoting dolostone dissolution was explored by analyzing the changes of pH value, Ca^{2+} concentration, Mg^{2+} concentration, organic acid concentration, micromorphology of rock surface, and rock particle diameter under controlled experimental conditions. After incubation of strain NF-15 with dolostone, the pH value continuously decreased significantly within 15 d, while the concentrations of Ca^{2+} and Mg^{2+} rose rapidly (1.8–2.7 times higher than that of control group). High performance liquid chromatography (HPLC) analysis showed that organic acids (succinic acid, citric acid, oxalic acid, malic acid, and lactic acid) were secreted by the strain NF-15 in the process of dolostone dissolution, and succinic acid was found more effective than other organic acids in lowering the pH and accelerating dissolution of rocks by Pearson correlation and Structural Equation Model (SEM) path analysis. In addition, the ultrastructure observation of the rock exhibited the significant erosion by fungal hyphae. Thus, strain NF-15 was suggested to combine with environmental remediation technologies to solve the problems of thin soil layer and mineral deficiency in destroyed dolostone mines. These results not only enrich the available microbial resources but also provide a new strategy for rehabilitating abandoned land in rock mining areas.

1. Introduction

The over-exploitation of rock mineral resources has seriously caused environmental damage, resulting in the abandonment of a large number of mining areas [1]. In China, the area of destroyed land caused by mining has reached up to 2.0×10^7 hm^2, and is increasing at a rate of more than 3.3×10^5 hm^2 per year [2]. Carbonate rock is widely distributed around the world, and accounts for 55% of the sedimentary mantle in China [3]. As is well-known, dolostone is one of the two types of carbonate rock regarded as an extremely versatile non-metallic mineral resource [4,5]. The long-term exploitation of this rock mineral broke the original ecological balance in mining areas, leading to an inhospitable environment with huge loss of soil and water, vegetation recession, rock bareness, and productivity loss [6].

The ecological restoration of abandoned mining areas has been proven incomplete relying on natural recovery [7,8]. Therefore, a variety of ecological restoration techniques have been developed and applied to these regions [9–11]. The external-soil spray seeding is one of the important techniques widely used in recent years. In this technique, soil, fertilizer, matrix material, and plant seeds are mixed together as spray seeding material which is then sprayed on bare palisades by high-pressure spray gun. In this way, the nutrients for plant growth can be provided, thus fast vegetation restoration can be realized [12]. However, due to the lack of available soils on the surface of rock, the poor rock interface fusion and extremely low survival rate of vegetation lead to the failure of long-term maintenance through external-soil spray seeding technique in destroyed rock mining areas [13]. Therefore, the key to solve these problems is via thickening the thin soil layer on the surface of rocks, and thus improving the efficiency of spray seeding technique.

Recently, microbial communities have been proposed as an economical and eco-friendly method to restore the destroyed environment by promoting the formation of mineral soils and improving the nutrient cycling in destroyed areas [14]. Although the profound geochemical activities of bacteria with respect to anaerobic environments attracted considerable attention [15],

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fungi are of great importance in aerobic environment, particularly in the existing rock surface, soil, and the plant root-soil interface [16]. Fungi have an accelerating effect on weathering due to their abundant presence and their capacity to bridge distances by virtue of mycelial growth [17,18]. Usually, fungi decompose rocks using both physical and chemical methods. Studies have shown that fungi are likely to accelerate physical weathering by extending hyphae into cracks, expanding and contracting hyphae according to the environmental variation, and forming secondary minerals to further disrupt rocks [19,20]. Fungal hyphae could extend up to 12 mm deep into carbonate rock as long as the nutrients and water levels are agreeable. The familiar boring fungi, which have the capacity to produce micropores on rocks and accelerate the decompose of rock, especially the fungi like *Lithopythium* sp. and *Ostpacoblabe* sp. [21]. In addition, the chemical weathering agents produced by fungi including proton-based and ligand-based agents can also help to increase the decomposing of rocks. The proton-based agents consist of respiratory carbonic acid and other acids produced in the environment directly surrounding the fungal hyphae [22]. Ligand-based weathering agents contain organic anions, lichen acids, siderophores, polyphenolic acids, and acid polysaccharides [23]. The low molecular weight organic acids belonging to both above groups, such as oxalic acid and citric acid, are of the most importance in weathering of rocks [24]. The main chemical weathering functions of fungi are acidolysis, complexation, and oxidation/reduction of Fe and Mn [25,26]. So far, plenty of species in fungal phyla Ascomycota [27], Basidiomycota [28], and Zygomycota [29] have been reported to have selective weathering capabilities for different types of carbonate rock such as limestone and calcite.

Based on the above research, we tried to isolate a fungal strain with the potential to be added into the matrix of external-soil spray seeding, and thus solving the problem of thin soil layers in rock mining areas. As is well known, fungi have been proven effective in the restoration of destroyed metal mining areas and oil fields [30–32]; however, few studies focus on the feasibility of the application of fungi in rock mining areas. Moreover, in reported researches regarding the microbial deterioration mechanism of fungi, only simple comparisons of the results were conducted and more reliable statistical methods were scarce. Therefore, in this study we isolated a fungal strain from the rock surface in a dolostone mining area. Subsequently, this strain was cultivated with dolostone mineral to determine its rate of rock solubilization. In addition, the weathering mechanism was also investigated by using structural equation model (SEM) analysis in the current research.

2. Materials and methods

2.1. Rock particle sample

The rock samples used in this experiment were obtained from a dolostone rock mine in Mufu Mountain, Nanjing, China. Rocks were rinsed by distilled water and dried for 12 h at 80 °C followed by pulverization. The samples were sieved with a 200 mesh sieve to make sure the particle size less than 90 μm. The rock particles were then autoclaved at 121 °C for 20 min. The major elemental compositions of rock powder samples were evaluated by X-ray powder diffraction (XRD, Ultima IV, PIGAKV, Japan). To obtain a uniform surface, according to the requirement of this technique, the samples were homogenized and compacted on the sample holder [33]. During the data collection, the samples were spun in order to minimize the preferred orientation effect and get the best peak profile. The major elemental compositions are as follows: CaO 42.35%, MgO 27.03%, Fe_2O_3 1.97%, K_2O 1.74%, SiO_2 1.36%, and Al_2O_3 0.62%.

2.2. Isolation and screening of fungal strain

Fungal isolation was conducted using the method described by Ruibal et al. [34] with minor modification. Briefly, the rocks obtained from Mufu Mountain (Nanjing) were rinsed with 96% (v/v) ethanol to reduce the interference of dust and airborne spores. The small superficial fragments from the outer layer (20 cm^2 area, 3 mm deep) of rock were removed with a sterile knife. These fragments were then pulverised sterilely and washed out with sterile water. Washed samples were treated with a 10 × serial dilutions method [35]. Each dilution (200 μL) was spread over rose bengal chloramphenicol agar (5.0 g peptone, 10 g glucose, 1.0 g KH_2PO_4, 0.5 g $MgSO_4$, 20 g agar, 100 mL 1/3000 rose bengal, 0.1 g chloramphenicol per liter deionized water) plate, and then incubated at 25 °C (2–7 d) to isolate individual colonies. Based on the morphology, various colonies were selected and purified with the routine method.

The purified isolates were cultured in 10 mL of potato dextrose broth (PDB) at 30 °C with agitation at 200 rpm for 24 h. After the incubation, 1.0 mL of each isolate was obtained for cultivating in 50 mL of selection medium (20 g glucose, 1.0 g K_2HPO_4, 2.0 g $NaNO_3$, 0.5 g $MgSO_4$, 0.5 g KCl, 0.01 g $FeSO_4 \cdot 7H_2O$, 5.0 g rock particle sample, and 1 L deionized water) at 30 °C with agitation at 200 rpm for 7 d. Meanwhile, 1.0 mL of inactivated PDB was added to the selection medium as the control. The concentrations of Ca^{2+} and Mg^{2+} in fermentation broth were measured after 7 d. Briefly, the fermentation broths were centrifugated at 8000 rpm for 15 min, and the supernatant was sterile filtered using a 0.45-μm Millipore filter. Subsequently, 5.0 mL of filtrate was acidized with 65% HNO_3 ($1.0 < pH < 2.0$), and then diluted to 100 mL with deionized water. The concentrations of Ca^{2+} and Mg^{2+} were analyzed by inductively coupled plasma emission spectrometer (ICP, Vista MPX, Varian, USA). The fungal strain NF-15 was selected for further exploration due to its capacity to release the highest amounts of Ca and Mg elements from rock sample, comparing to the other tested strains (Fig. 1).

2.3. 26S rDNA D1/D2 domain sequence analysis

Genomic DNA of strain NF-15 was successfully extracted following the protocol of Universal Cenomic DNA Extraction Kits Ver.3.0 (TaKaRa code: DV811A, TaKaRa, Japan). The PCR amplification was conducted by Fungi Identification PCR Kit (TaKaRa code: D317, TaKaRa, Japan). The forward primer sequence was 5′-CGCCACCCTTTTCCCAGTCACGAC-3′, and the reverse primer sequence was 5′-GAGCGGATAACAATTTCACACAGG-3'. Briefly, 25 μL of PCR premix, 0.5 μL of forward primer, 0.5 μL of reverse primer, and 100 ng of genomic DNA template were contained in 50 μL of PCR reaction mixture. The PCR amplification conditions were as follows: The initial denaturation at 94 °C for 5 min, followed by 30 cycles composed of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and then extension at 72 °C for 1 min. The whole reaction process was ended with a 5 min final extension at 72 °C. The PCR products were purified by PCR purification kit (FroggaBio, USA), and the purified samples were sequenced by TaKaRa Bio Inc. (Dalian, China). The sequencing result was imported in NCBI (http://blast.ncbi.nlm.nih.gov/) database for searching the similar sequences, so that the possible genus of this strain could be identified. The phylogenetic tree showing relationship between NF-15 and other reference strains was constructed with neighbor-joining (NJ) method through MEGA 7.0 software.

2.4. Microcosm experiments

After 0, 2, 5, 9, 15, 22, and 30 d of incubation in selection