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Cao Jian-bing [a c e], Li Xiao-ming [a b c], Ouyang Yu-zhu [d], Zheng Wei [a c], Wang Dong-bo [a c], Shen Ting-ting [a c], Yue Xiu [a c] & Yang Qi [a c]

[a] College of Environmental Science and Engineering, Hunan University, Changsha, 410082, P.R. China

[b] School of Environment Studies, Guangxi University, Nanning, 530004, P.R. China

[c] Key Laboratory of Environmental Biology and Pollution Control (Hunan University), Ministry of Education, Changsha, 410082, P.R. China

[d] College of Chemistry and Chemical Engineering, Jishou University, Jishou, 416000, P.R. China

[e] Huayuan Municipal Environmental Protection Bureau, Huayuan, 416000, P.R. China

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Manganese-electrolysed slag treatment: bioleaching of manganese by *Fusarium sp.*

Cao Jian-bing[a,c,e], Li Xiao-ming[a,b,c*], Ouyang Yu-zhu[d], Zheng Wei[a,c], Wang Dong-bo[a,c], Shen Ting-ting[a,c], Yue Xiu[a,c] and Yang Qi[a,c]

[a]*College of Environmental Science and Engineering, Hunan University, Changsha 410082, P.R. China;* [b]*School of Environment Studies, Guangxi University, Nanning 530004, P.R. China;* [c]*Key Laboratory of Environmental Biology and Pollution Control (Hunan University), Ministry of Education, Changsha 410082, P.R. China;* [d]*College of Chemistry and Chemical Engineering, Jishou University, Jishou 416000, P.R. China;* [e]*Huayuan Municipal Environmental Protection Bureau, Huayuan 416000, P.R. China*

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A fungi strain named *Fusarium sp.* was isolated from manganese-electrolysed slag by using a gradient dilution spread plate method, identified by 26S RNA sequence analysis and phylogenetic tree analysis, and explored for the bioleaching capacity to manganese (II) from manganese-electrolysed slag in liquid mineral medium under different environmental conditions, including system temperature, incubator rotation speed and initial pH value. DNA sequence and phylogenetic analysis indicated the name of this fungi strain, that is, *Fusarium sp.*, and higher bioleaching efficiencies (71.6%) of manganese by this fungi were observed when the bioleaching was carried out under the optimized conditions as follows: contact time: 72 h; system temperature: 28°C; inoculums concentration: 2% (v/v); incubator rotation speed: 150 rpm; pH 4.0. Because of its low cost, environment friendliness and better efficiency, the bioleaching technique will have a significant impact on manganese-electrolysed slag pollution mitigation.

Keywords: manganese-electrolysed slag; bioleaching; fungi, manganese, treatment

1. Introduction

There are large resources of manganese in the west of Hunan province, China. Manganese-based industries produced plentiful electrolytic manganese, MnO_2, $MnSO_4$, and therefore every year millions of tons of manganese-electrolysed slag are discarded as waste after manganese has been extracted from these ores [1,2]. An enormous amount of electrolytic manganese slag is produced from the electrolytic manganese metal (EMM) industry as a main solid waste; about 6–9 tons of the solid waste is discharged into the environment for every ton of EMM [3]. Their improper management in the past resulted in the mobilization of heavy metals to the surrounding environment, contributing to contamination of soil substrates, destruction of soil texture, shortage of nutrients, destruction of ecological landscape, pollution of groundwater, decrease in biological diversity, etc. In order to resolve the above problems, it is important to develop a suitable and economical technology for the removal of heavy metals from those tailings. It can be re-utilized as a secondary source and recycling of the slag can offer economic benefits through the recovery of the valuable materials, as well as the preservation of the raw materials in the interest of sustainable development [4]. Presently, recovering manganese from this kind of slag to produce manganese ingot or compounds, such as MnO_2, $MnSO_4$ or $MnCl_2$, is one of the main methods of recycling this waste. The approach is usually used is the extraction of manganese using heated sulfuric acid or hydrochloric acid solution as a solvent [5,6]. However, the extraction efficiency of manganese obtained by these methods is generally low, especially for slag with low manganese content [7]. However, many different methods have been used to leach manganese from low manganese ores and nodules [8–11]. Bioleaching processes are based on the ability of microorganisms to transform solid compounds and result in soluble and extractable elements that can be recovered.

The recovery of manganese from manganiferous ores by bioleaching with different kinds of microorganisms has been extensively investigated by many workers, and microbiological processes also have been proposed to be less hazardous [12]. The present study was initiated with the isolation of a fungus, *Penicillium citrinum*, which is capable of leaching manganese ore. Acharya et al. [13] then studied the mechanism of bioleaching manganese ores. *Fusarium sp.*, which was firstly discovered in Hibiscus mutabilis, can growth in many plants, and can produce toxin. Recently it was extensively used for researching on effecting the growth of plants [14]. It was not used in researching on bioleaching; in this paper, we isolated a *Fusarium sp.* from manganese-electrolysed slag, and

*Corresponding author. Email: rachel_725@163.com

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there were no studies with the bioleaching of manganese from manganese-electrolysed slag based on the microorganism that exists in manganese-electrolysed slag itself. The purpose of this study was to study the optimal bioleaching condition of bioleaching manganese from manganese-electrolysed slag using a fungus that was isolated from manganese-electrolysed slag.

2. Materials and methods

2.1. Materials

The manganese-electrolysed slag study comes from the manganese-electrolysed slag dam located 40 km south of Jishou city in central China's Hunan province. The manganese-electrolysed slag samples and mould-containing sludge were transported to the laboratory and stored at 4°C for 24 h prior to their use. The characteristics of manganese-electrolysed slag (per dry weight) were measured as follows: the pH value of the manganese-electrolysed slag was 6.72; Mn of manganese-electrolysed slag was 35,450 mg/kg per dry weigh.

Czapek's Culture Medium was used for fungi isolation. This was prepared by dissolving 30 g sucrose, 2 g $NaNO_3$, 1 g K_2HPO_4, 0.5 g KCl, 0.5 g $MgSO_4$ and 0.01 g $FeSO_4$ (all the reagents were analytically pure) in 1 l deionized water before autoclaving at 121°C for 20 min. The solid medium was prepared by adding 15–20 g agar to the above medium before autoclaving [15].

2.2. Isolation strains

Manganese-electrolysed slag was spread on Petri plates containing Czapek's Medium and was incubated at 27 ± 2°C. Cells from micro colonies on these plates were isolated by micromanipulation. The fungi cells were purified to aseptic conditions by streaking the cells repeatedly on the Czapek's Medium agar plate with 20% NaCl five times. The purified fungi cells were transferred to sterilized liquid media. These liquid cultures were tested for bacterial contamination by plating on bacteriological media. Isolated and purified fungi cultures were identified according to morphological properties.

2.3. DNA extraction, polymerase chain reaction amplification and sequencing

The method of DNA extraction was as follows. Three pellets were resuspended in 1.5 ml of lysozyme solution (0.15 mol l^{-1} NaCl, 0.1 mol l^{-1} ethylene diamine tetraacetic acid disodium (Na2EDTA), 10 mg ml^{-1} lysozyme, pH 8), 0.5 ml lysis buffer [10% sodium dodecyl sulfate (SDS), 0.1 mol l^{-1} NaCl, 0.5 mol l^{-1} Tris–HCl, pH 8] and 0.5 ml phosphate buffer (0.1 mol l^{-1}, pH 8) and shaken at 37°C on an orbital shaker at 225 rpm for about 30 min, and 0.5 ml chloroform/isoamyl alcohol (24:1) was added into the tubes. Then, the tubes were homogenized at 2800 rpm for 10 min and centrifuged (6000 × g, 3 min). The upper layers were transferred to fresh tubes and 0.5 ml sterile deionized water was added into the former tubes to wash the pellets with centrifugation (6000 × g, 1 min). Both the upper layers were mixed and centrifuged at 12,000 × g for 5 min, and then were treated with 0.6 vol of isopropanol for 1 h at room temperature. The mixtures were centrifuged at 16, 000 × g for 10 min, and the precipitated crude DNA was washed twice with 0.7 ml ice-cold 70% ethanol. The crude DNA was dissolved in 600 μl Tris-EDTA (TE) buffer (10 mmol l^{-1}Tris–Cl, 1 mmol l^{-1} EDTA, pH 8) after being dried under vacuum.

The DNA was amplified using the TaKaRa Fungi Identification polymerase chain reaction (PCR) Kit (Code No. D317). The PCR mixture (50 μl) contained 1 μl template, 25 μl PCR Premix, 0.5 μl D1/D2 forward primer (20 pmol/μl), 0.5 μl D1/D2 reverse primer (20 pmol/μl) and 23 μl dH2O. The PCR was performed in a DNA Engine Thermal Cycler (PTC-200, BIO-RAD, USA) with a hot starting performed at 94°C for 5 min, followed by 30 cycles of 94°C for 0.5 min, 55°C for 0.5 min and 72°C for 1 min, followed by a final extension performed at 72°C for 5 min. Sequencing was then performed at Nanjing Boya Biotechnology Company, Limited (Nanjing, China). The sequence was compared against the GenBank database using the NCBI Blast program. The 26S rDNA sequence of strain J62 has been deposited in GenBank under accession No. FJ532369.

2.4. Bioleaching with fungi

2.4.1. Bioleaching conditions

Investigation of the bioleaching behaviour of fungi was performed in an Erlenmeyer flask by inoculating a fungi biomass in 100 ml sterilized (to ensure that the bioleaching process was conducted by the fungi only) Czapek's Culture Medium. For each strain, 0.044 g dry wet of fungi biomass, that is, 2% (v/v) inoculum concentration, was used. The initial pH of the liquid medium was adjusted to 4.0. The whole solution system was placed in an HZQ-F160 incubator at 28°C for 144 h with shaking at 150 rpm. Then, the biomass was separated by centrifugation at 5000 rpm for 5 min and the supernatants were analysed for the residual Mn^{2+} concentration. All experiments were carried out in triplicate. The reproducibility, expressed as the standard deviation of the results, was less than 7%.

The bioleaching efficiencies of Mn^{2+} on fungi strains can be estimated by bioleaching efficiencies $= C_1/C_0 \times 100\%$, where C_0 is the initial mass of Mn^{2+} in the manganese-electrolysed slag and C_1 is the final mass of Mn^{2+} in the medium.

2.4.2. Determination of Mn^{2+} concentration

The concentration of Mn^{2+} (C) in solution was determined by Atomic Absorption Spectrophotography (AAS). The

detection wavelength was at 279.5 nm. The standard curve method was used for quantification.

3. Results and discussion

3.1. Identification of the strain

The microorganisms were isolated according to Section 2.2. The strain was identified according to Section 2.3. The phylogenetic tree analysis was carried out by the Dalian Baobiology Company. The phylogenetic tree constructed by the gene sequence of the strain is portrayed in Figure 1, which shows a close relationship between the fungi and *Fusarium sp.* Phylogenetic identity was determined by comparing the partial 16S rRNA gene sequences of the clones with sequences in GenBank using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and a phylogenetic tree was constructed with MegAlign in Lasergenge 7.0 (DNASTAR Inc., Madison, WI, USA) using the Clustal V method. Sequences with a similarity of more than 97% were considered to belong to the same species [16].

3.2. Bioleaching of manganese (II) from manganese-electrolysed slag by **Fusarium sp.**

The effectiveness of bioleaching is highly dependent on the physical, chemical and biological factors in the system [17]. Among these affecting factors, such as the nature of the contaminated material, substrate concentration, solids concentration, temperature, oxygen, pH, bacterial strain and cell concentration [18] and temperature, pH value and oxygen play an important role in optimization of the bioleaching process.

In this paper, we investigated the effects of three parameters, that is, system temperature, incubator rotation speed and initial pH value, on the bioleaching of Manganese (II). The results were as follows.

3.2.1. Influence of temperature on the bioleaching efficiencies

The effect of temperature ranging from 22 to 33°C on leaching efficiency after 144 h is shown in Figure 2(a) and the variation of pH value during bioleaching process is shown in Figure 2(b). As shown in the figure, temperature had an obvious effect on the bioleaching efficiencies. The bioleaching efficiencies were raised from 48% at 22°C to 71% at 28°C. The best temperature for fungi growth was 28–30°C, so when the temperature was lower than that, the fungi's growth will be deferred; when the temperature was higher than that, it will inhibit the growth. It was supposed that this was the reason for temperature's effect on bioleaching efficiencies. This result showed the same trend on bioleaching metals by microbe [19]. Five temperatures showed the same trend: the bioleaching efficiencies increased constantly with time extending. *Fusarium sp.* had prodigious bioleaching efficiencies at the front 72 h after

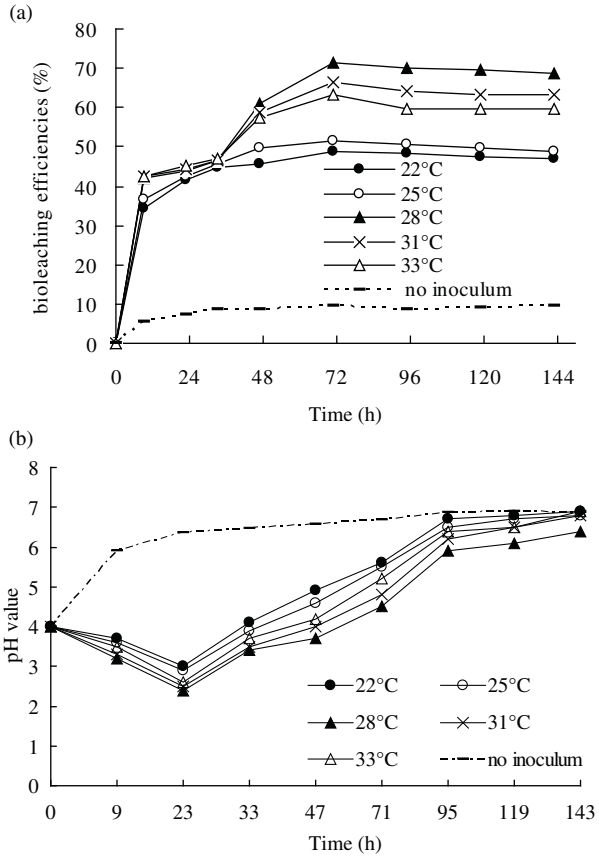

Figure 2. (a) Effect of temperature on Mn^{2+} bioleaching rate, where data measured at min 0 represents the value that was obtained at the beginning of the experiment, and the initial concentration of Mn^{2+} was 0. (b) The variation of pH value during the bioleaching process.

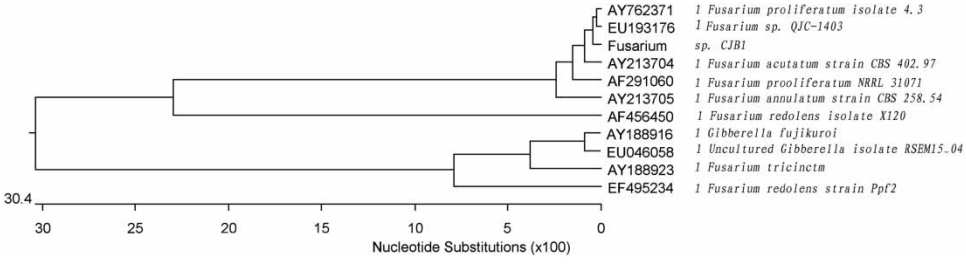

Figure 1. The phylogenetic tree of the fungi.

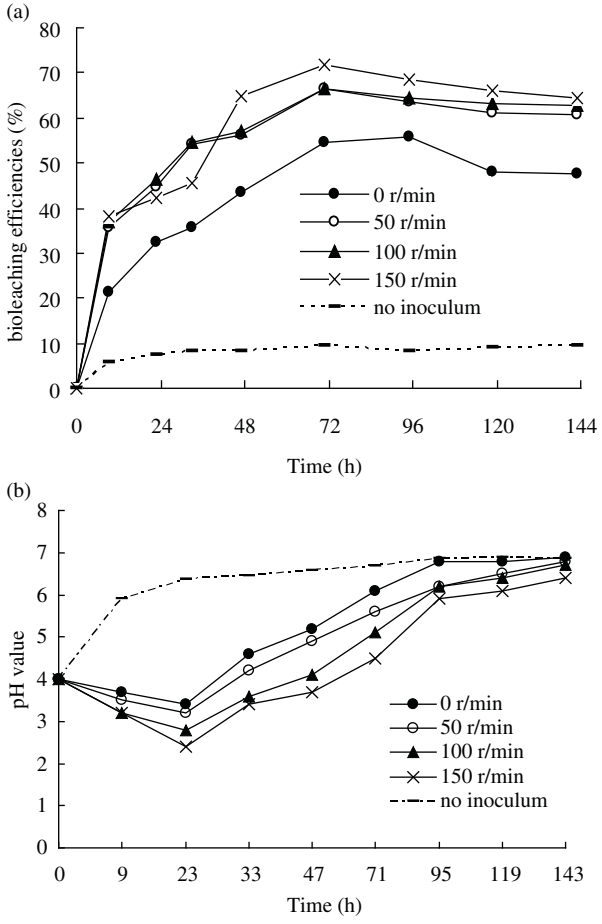

Figure 3. (a) Influence of the incubator rotation speeds on bioleaching ratio, where data measured at min 0 represent the value that was obtained at the beginning of the experiment, and the initial concentration of Mn^{2+} was 0. (b) The variation of pH value during the bioleaching process.

being incubated at 28°C, and reached 71.6%. We choose 28°C as the optimum temperature by considering all the results and economic benefit.

3.2.2. *Influence of incubator rotation speed on the bioleaching efficiencies*

The effect of rotation speed ranging from 0 to 150 r/min on leaching efficiency after 144 h is shown in Figure 3(a) and the variation of pH value during bioleaching process is shown in Figure 3(b). The bioleaching efficiencies increased rapidly, and then decreased slowly. For static cultures and 150 r/min cultures the bioleaching efficiencies were 54.6% and 71.6%, respectively, for 72 h. This showed that *Fusarium sp.* had a low requirement regarding rotation speed, but sufficient oxygen supply in the environment was beneficial to Mn^{2+} bioleaching, because this strain is aerobe. High rotation could lead to plasmatorrhexis. We choose 150 rpm as the optimum rotation speed.

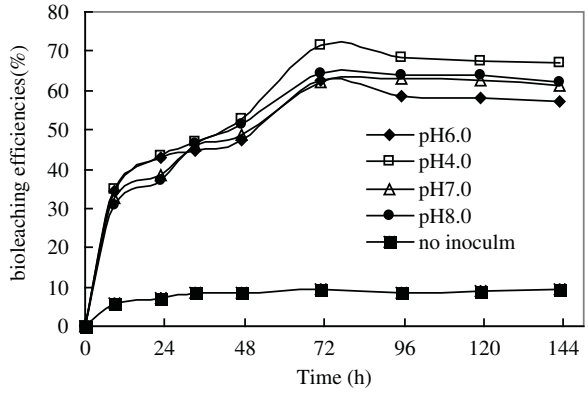

Figure 4. Effect of the initial pH value on manganese bioleaching rate, where data measured at min 0 represent the value that was obtained at the beginning of the experiment, and the initial concentration of Mn^{2+} was 0.

3.2.3. *Influence of initial pH on the bioleaching efficiencies*

The effect of pH value ranging from 4 to 9.6 on leaching efficiency after 144 h is shown in Figure 4. We can see that the strain had high bioleaching efficiencies at those four pH values. In addition, for each of the pH values, the trend of the bioleaching efficiencies was the same, that is, the bioleaching efficiencies increased rapidly in the 72 h, and then decreased a little. The reason for the similar trend was the growth of the fungi. At pH 4 after 72 h, the bioleaching efficiencies reached the highest level, namely 71.6%. The result showed the same trend to Mehta et al.'s research [15] on manganese leaching by *Aspergillus niger*. We chose pH 4 as the optimum bioleaching pH value.

3.2.4. *Regulation of the acidity of culture medium by* Fusarium sp.

Different microorganism species have their own optimum growth pH value. The pH value of the environment will have both a direct and indirect effect on microorganism cells, for example, effect on uptaking of nutrients, enzyme adaptability in metabolism reaction, toxicity of environmental harmful material and so on. However, at the same time the life course of the micro will change the external environment pH value. Regulation of the acidity of culture medium by *Fusarium sp.* at different pH values is shown in Figure 5. In this figure the pH value clearly decreased before 48 h cultured. After 48 h the pH value tended to be stable. However, the pH increased obviously after 48 h and it tended to stay around pH 7. Although they had different initial pH values, after culturing the pH value finally settled between 6.2 and 6.8 at the end of the bioleaching period. This can explain why *Fusarium sp.* can change the harsh pH environment, and it was significant for environmental remediation.

3.2.5. *Variation in the binding forms of heavy metals*

The different forms of heavy metals have different energy states, and affect not only the efficiency of bioleaching but also the bioavailability of heavy metals after bioleaching. Metals in exchangeable fractions, and Fe/Mn oxide-bound fractions, are considered to be more mobile, dangerous and bioavailable. The organic matter/sulfide-bound and residual metals are considered to be more stable and non-bioavailable than metals in exchangeable and Fe/Mn oxide-bound fractions. However, it was necessary to describe the partitioning of heavy metals into different fractions before and after the bioleaching. We then used optimized Community Bureau of Reference (BCR) sequential extraction [20] to measure the manganese-electrolysed slag.

The bioleaching had a significant impact on changes in partitioning of heavy metals. Figure 6 shows variation in the binding forms of heavy metals before and after the bioleaching process. Manganese, which was mainly bound to the exchangeable carbonate fraction and residual fraction in raw manganese-electrolysed slag, mainly bound to the organic fraction and residual fraction after the bioleaching process. The organic fraction and the exchangeable carbonate fraction had a great change before and after the bioleaching process. We hypothesized that this strain can produce some organic acids, and in the bioleaching process, these acids would react on the manganese-electrolysed slag, so the manganese would be released. In addition, this would be in accordance with the pH value changes in bioleaching process. Our hypothesis will be validated in future research.

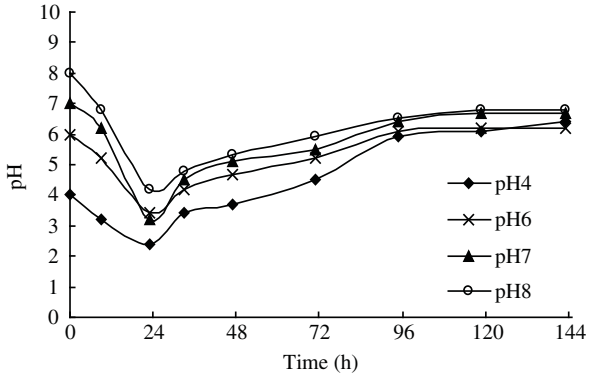

Figure 5. Culture medium acidity regulated by *Fusarium sp.*

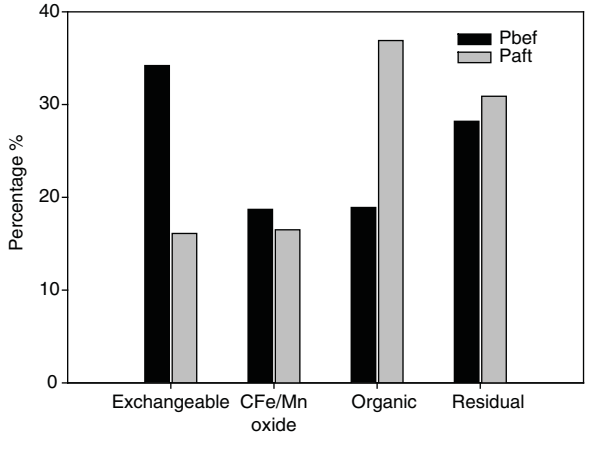

Figure 6. Form percentage of Mn^{2+} before and after bioleaching; the initial concentrations of Mn^{2+} before and after bioleaching were 35,450 mg/kg and 10,067.8 mg/kg, respectively.

4. Conclusion

The temperature and pH value had a great influence on bioleaching manganese from manganese-electrolysed slag, while rotation speed had a slight influence on it. The high manganese bioleaching efficiency of 71.6% was attend by *Fusarium sp.* at 28°C, 2% (v/v) inoculum concentration, 150 rpm rotation speed, pH 4.0 for 72 h. The pH value changes during the bioleaching process showed that some organic acids would be produced by the fungi's metabolism, and this needs to be proved by further research.

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