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Perchlorate Reducing Microorganisms Isolated from Soils in Ussangoda Area in Sri Lanka: Candidates for Bioremediation on Mars

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ABSTRACT

The elemental composition of soils in Ussangoda National Park, Sri Lanka, (6°05'55"N 80°59'12"E) reveals similarities with Martian soils. Mars is a prominent candidate for future human colonization, but high concentrations of perchlorate in Martian soil pose a significant hazard to animal and plant life. Microbial bioremediation offers a viable solution. This study aimed to isolate microorganisms from Ussangoda soils and explore their perchlorate reduction abilities for potential applications on Mars. Microbial samples were obtained from 11 locations using random soil sampling under aerobic and anaerobic conditions. Perchlorate-reducing bacteria and fungi were isolated using anoxic media under anaerobic and aerobic conditions. One bacterial and three fungal species were isolated, with the bacteria forming biofilms at high perchlorate concentrations. Perchlorate tolerance assays showed that the bacteria tolerated up to 0.5 M perchlorate, while fungi tolerated 0.2 M to 0.5 M. The isolates' perchlorate reduction abilities were quantified using FTIR, revealing significant reductions ranging from 1.6 % to 60.9 % across different species combinations and biofilm formation in mixed cultures. These findings highlight the highest rate of perchlorate reduction at high concentrations by any microbial setting recorded so far. Further research is needed to understand how perchlorate-tolerant fungi enhance the efficiency of perchlorate-reducing bacteria. The isolated microorganisms thrived in high salt concentrations and extreme environmental conditions while maintaining good perchlorate reduction efficiency, indicating their potential for future bioremediation applications on Mars.

KEYWORDS: Bioremediation, Mars, Perchlorate, Perchlorate reducing bacteria, Ussangoda

INTRODUCTION

Perchlorate (ClO_4^-) is a monovalent inorganic chlorine oxyanion with high water solubility and stability. It is found in the environment from natural or anthropogenic sources.

Perchlorate is identified as an emerging contaminant (EC) as it pollutes water sources and causes environmental and human health impacts. On Earth, in areas such as northern Chile and the southwestern region of the United States (US) where perchlorate is identified as a major water contaminant (Steinmaus, 2016).

Mars emerges as a prominent candidate in the context of future expansions of human civilization, owing to its terrestrial nature) and recent evidence indicating the presence of water. However, the presence of perchlorate in Martian soil at concentrations ranging between 0.5 % and 1 % (Catling *et al*., 2010) indicates a barrier for human colonization due to its toxicity and negative effects on animal and plant life. Thus, in the context of colonizing Mars, removing perchlorate from the soil and water sources is essential. Physico-chemical methods such as ion exchange, adsorption, electrochemical reduction, membrane filtration and use of catalytic converters as well as phytoremediation have limitations in such endeavors (Srinivasan and Viraraghavan, 2009). Thus, microbial bioremediation for perchlorate reduction expresses a viable option.

Perchlorate-reducing bacteria (PRB) exhibit the ability to convert perchlorate (ClO_4) into harmless chloride ions (Cl^-) through enzymatic processes. This transformation is facilitated by the presence of perchlorate reductase (PcrAB), a specialized enzyme belonging to the dimethylsulfoxide reductase superfamily (Youngblut *et al*., 2016). Soil microorganism species within genera such as *Dechloromonas*, and *Azospira* (Coates and Achenbach, 2004) have demonstrated the capability to reduce perchlorate. These microbes are typically classified as either facultative anaerobic or microaerobic, indicating their ability to thrive in environments with varying levels of oxygen availability.

The applicability of PRB to reduce perchlorate in Martian soil is not widely discussed. Recent discoveries state ferric and ferrous ions have the capability to inhibit the perchlorate reduction by PRB (Levakov *et al*., 2021). As the Martian soil is rich in iron and lacks electron donors, the investigation into the effectiveness of PRB in simulated Martian soils is crucial for identifying optimal strategies to remediate the Martian soil.

The elemental composition of soils in Ussangoda National Park, Sri Lanka, reported by Vithanage *et al*., (2014) reveals similarities with the elemental composition of Martian soils reported by Oravec *et al*., (2021). Ussangoda, known for its limited biodiversity due to the high iron content in the soil, is a prime candidate for acquiring microbes that can adapt to Martian soil conditions. It is hypothesized that microorganisms inhabiting in soils of the Ussangoda area may have adaptations to reduce perchlorate while overcoming iron interference. This study aimed to isolate microorganisms from soils in the Ussangoda area and to explore their perchlorate reduction ability for future applications including bioremediation in the context of colonizing Mars.

METHODOLOGY

Study Area and Sampling Points

The study was conducted in Ussangoda National Park, located in the Hambanthota District of the Southern Province of Sri Lanka (6°05'55"N 80°59'12"E). The mean annual temperature of the Ussangoda area is 27.9 °C and records less than 1250 mm of rainfall annually including a long dry period of five months (Weerasinghe and Iqbal, 2011). The soil is characterized as serpentine with high iron content.

Georeferenced sampling points (n=11) were randomly selected to represent the different areas of the study area (Figure 1).

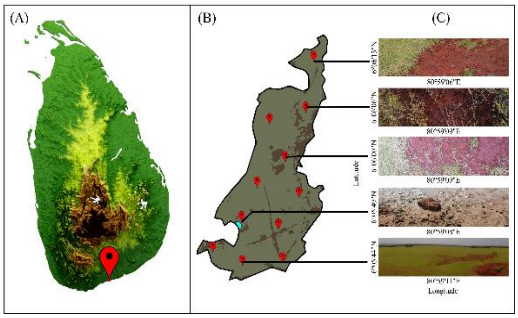

Figure 1. (A) Location of Ussangoda National Park in the map of Sri Lanka (B) Sampling points map of the Ussangoda National Park (C) Photographs of selected sampling points

Collection of Soil Samples

At each sampling point, soil samples (20 g) were aseptically collected at 5 cm depth for isolating aerobic microbes and transferred into sterilized polythene bags. Samples were collected at 15 cm depth for isolating anaerobic microorganisms employing the technique described by Kanda, (2000). Immediately after collection, each soil sample was transferred into a sterilized glass tube (15 mL) and secured with a rubber cap and the headspace gas was removed using a sterile syringe and replaced with nitrogen gas (Purity 99.9 %) to induce anaerobic conditions.

Samples were transported to the Microbial Biotechnology unit of the National Institute of Fundamental Studies in Kandy, Sri Lanka within 24 h of sample collection, and the samples were stored at 4 °C.

Isolation of Anaerobic Microorganisms

Anaerobic microorganisms in the soil samples were isolated under anaerobic conditions using a previously described anoxic growth medium (Bruce *et al.*, 1999). The growth medium was prepared in 1 L, the pH was adjusted to 7.2, and sterilized by autoclaving (20 min at 121 °C, 15 psi). A Baker Ruskinn Anaerobic Workstation was utilized under controlled atmospheric setting (5 % H_2, 10 % CO_2 and 85 % N_2) for anaerobic culturing. The growth medium was transferred to airtight culture tubes (n=11) within the workstation. Each soil sample tube under anaerobic conditions was opened and approximately 1 g of soil was introduced to the anoxic growth medium. The culture tubes were incubated in the anaerobic workstation and monitored for cell growth.

After cell growth in liquid culture tubes, solid culture plates were prepared by spread plate method by transferring 1 mL of the cell suspension on anoxic growth medium (2 % agar). Pure cultures were obtained by serial streak plate technique.

Isolation of Aerobic Microorganisms

Aerobic microorganisms in the soil samples were isolated and pure cultures were prepared using the same growth media under aerobic conditions in a laminar flow cabinet (Bruce *et al*., 1999).

Screening of the Isolates for Perchlorate Tolerance

The isolated anaerobic bacteria (n=1) were screened for perchlorate tolerance *via* an assay. The isolated strains were inoculated into the anoxic media in the presence of sodium perchlorate (0.01 M, 0.05 M, 0.1 M, 0.2 M, 0.5 M and 1 M) and 28 g/ L agar, in duplicates.

In parallel, the fungal isolates (n=3) were screened by transferring into potato dextrose agar (PDA) in the presence of sodium perchlorate (0.01 M, 0.05 M, 0.1 M, 0.2 M, 0.5 M and 1 M) in duplicates. The cultures were incubated under anaerobic conditions and colony formation was monitored in 24 h intervals to obtain the maximum perchlorate tolerance.

Determination of Perchlorate Reduction Efficiency of the Isolates by FT-IR

The distinct bacterial (A, B) and fungal isolates (W, Y & P) which indicated the ability for perchlorate tolerance were further studied by FTIR analysis. An experiment was designed with nine treatments using the bacterial isolates as pure cultures and as mixed cultures with fungal isolates (Table 1). Fungi-bacteria biofilm formation was observed in all fungi-bacteria mixed culture treatments. Further studies are suggested to confirm the formations.

All treatments were triplicated, and a control (E) was included. The prepared isolates (1.5 mL) were inoculated in anoxic growth media containing 0.2 M sodium perchlorate and incubated for 18 days.

Table 1. Isolate combinations used in different treatments

Treatment (ID)	Isolate				
	A	B	W	Y	P
E	-	-	-	-	-
A	+	-	-	-	-
B	-	+	-	-	-
C	+	+	-	-	-
AW	+	-	+	-	-
AY	+	-	-	+	-
AP	+	+	-	-	+
CW	+	+	+	-	-
CY	+	+	-	+	-
CP	+	+	-	-	+

+ - signs indicate the presence or absence of the bacterial (A, & B) and fungal (W, Y &P) isolates. E – The control treatment containing $NaOCl_4$ in 0.2 mol dm^{-3} concentration.

After the incubation, 10 mL of culture was treated with 100 mg of KBr. The formed pellets were analyzed using FTIR. Average absorption values were calculated using Origin Pro 2024 software. The perchlorate ion functional group of the spectra was identified by referring to the FT-IR peak values for sodium perchlorate stated by Nyquist and Kagel (1971). The presence of perchlorate ion species is commonly related to the strong absorption bands around 1050-1150 cm^{-1} range.

The perchlorate reduction efficiency of each treatment was calculated using the following equation.

$$PRE = \frac{(P_C - P_S)}{P_C} \times 100\ \% \ldots\ldots\ldots (1)$$

where, P_C = The FTIR peak value of the control sample at 1100 cm^{-1}, P_S= The FTIR peak value of the sample at 1100 cm^{-1}

Statistical Analysis

Analysis of variance and Tukey means comparison test was performed to analyze the data using IBM SPSS statistics software (Version 29.0.2.0).

RESULTS AND DISCUSSION

Bacterial and Fungal Isolates

Two distinct bacterial isolates (A & B) and three distinct fungal isolates (W, Y & P) were isolated from the Ussangoda soil samples. The colony characteristics of fungal isolates are given in Table 2 and Figure 2 and bacterial isolates are given in Table 3.

Table 2. Characteristics of fungal isolates from Ussangoda soil samples

Isolate	Colony		
	Color	Texture	Morphology/ other
Y	Yellow	Velvety	Center of the colony changes to dark green at maturity, Parallel grooves and exudate formation, Dark red soluble pigments on the reverse side.
W	White	Powdery	The center and margins of the colony remain white throughout lifecycle. No grooves, exudate, or pigment formation.
P	Pink	Velvety	Hyaline hyphae with a globose head observed in micrographs.

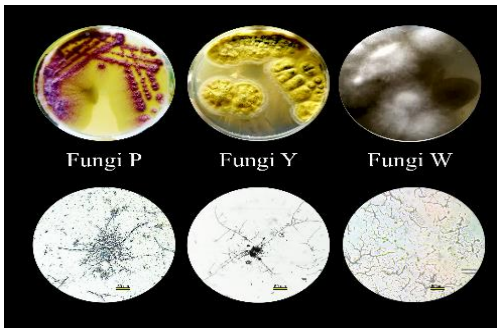

Figure 2. Fungal isolates P, Y and W, culture plate view (Upper), microscopic view (400x) (Lower)

Table 3. Characteristics of bacterial strains isolated from Ussangoda soil samples

Isolate	Colony characteristic		Cell morphology
	Color	Shape	
A	Golden yellow	Circular	Staphylococcus
B	White	Circular	Staphylococcus

Perchlorate Tolerance of the Isolates

Based on the duration for colony initiation, the perchlorate tolerance of fungal isolates Y and P was found to be up to 0.2 M $NaClO_4$, while bacterial isolate B and fungal isolate W tolerated up to 0.5 M $NaClO_4$ concentrations (Table 4). None of the isolates grew in 1 M $NaClO_4$ medium. At $NaClO_4$ concentrations higher than 1 M, a different colony morphology was observed in Bacteria A, with the colony color changing from white to golden yellow. This new bacterial setting was subsequently considered a new treatment (Bacteria setting A) in the study.

Table 4. Duration for colony initiation in Perchlorate Tolerance Assay

Isolate	Duration for colony initiation (Days)					
	0.01[1]	**0.05**[1]	**0.1**[1]	**0.2**[1]	**0.5**[1]	**1**[1]
A	2	2	5	7	25	X
W	2	2	3	5	9	X
Y	2	3	5	7	X	X
P	2	5	7	7	X	X

Bacteria (A) Fungi (W, Y & P), X- No growth was observed. [1]NaOCl4 concentration in each treatment 0.01–1 mol dm^{-3}

Within the limitations of this study, the perchlorate tolerance of fungal isolates was assessed only in solid PDA media containing sodium perchlorate. This medium may not provide optimal growth conditions for the isolated fungi. To obtain more accurate results on actual tolerance, it is recommended to use a range of growth media (both solid and liquid). For example, DMSZ growth medium (3 % malt extract, 0.3 % soya peptone) and other media optimized for facultative anaerobic fungi should be considered.

When conducting the perchlorate susceptibility assay, it was noticed that sodium perchlorate concentrations higher than 1 M interfered with agar solidification. Therefore, it is suggested to use liquid media for future studies using sodium perchlorate concentrations higher than 1 M.

Perchlorate Reduction Efficiency by Different Microbial Combinations

FTIR analysis confirmed the perchlorate reduction activity of the different bacterial and fungal isolates by indicating a peak in the absorbance spectrum at 1100 cm^{-1} (Figure 3).

Bacteria setting A alone reduced perchlorate by 32.37 %, while bacteria setting B alone achieved a 35.98 % reduction (Figure 4). As noted in the perchlorate susceptibility assay, at an initial perchlorate concentration of 0.2 M, bacteria setting B may have transformed into bacteria setting A under the experimental conditions, leading to the higher reduction rate observed in bacteria setting B.

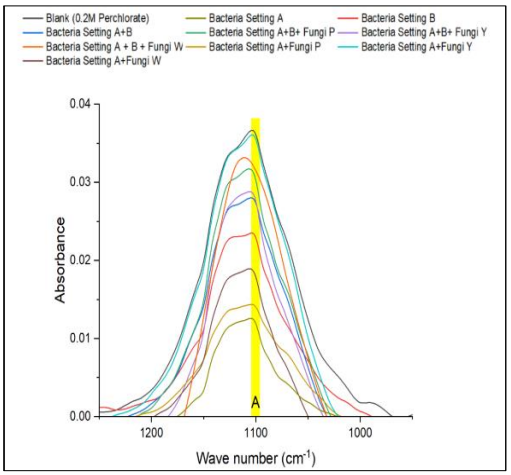

Figure 3. FTIR absorbance peaks of different treatments for sodium perchlorate

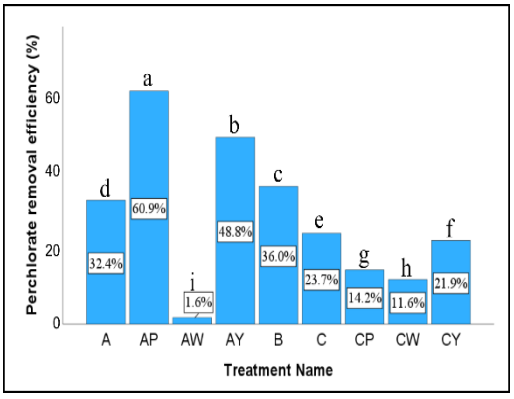

Figure 4. Perchlorate reduction efficiency (%) of different microbial combinations

A recent study investigating perchlorate reduction in methane-based biofilm reported a maximum perchlorate removal percentage of 48.3 % at an initial perchlorate concentration of 10 mg/L (Lv *et al.*, 2024). In contrast, the findings of the current study revealed a significantly higher percentage of perchlorate reduction, even at a concentration 2000 times higher, with an initial concentration of 19,890 mg/L. Another study has discovered perchlorate

reduction of up to 25 % (Acevedo-Barrios *et al*., 2019) and the maximum perchlorate tolerance of the bacteria was reported to be 10,000 mg/L KClO4 (equivalent to a perchlorate ion concentration of 0.072 M).

CONCLUSIONS

This study aimed to isolate microorganisms from soils in the Ussangoda area and explore their perchlorate reduction ability for future applications, including bioremediation in the context of colonizing Mars. Three fungal isolates with perchlorate tolerance ranging from 0.2 M to 0.5 M and a bacterial isolate with perchlorate tolerance of 0.5 M were identified. Mixed cultures of these isolates were tested in a 0.2 M perchlorate-containing medium, showing a reduction ranging from 1.63 % to 60.9 %. With high perchlorate tolerance, the ability to survive under extreme environments, and high efficiency in perchlorate reduction, these mixed cultures have potential applications in bioremediation of Martian soil and water.

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