

Isolation, screening and characterization of uranium microremediable actinomycetes from fallen leaves of *Azadirachta indica* in Western Ghats

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Abstract Microremediation of harmful radioactive waste such as uranium has been carried out by the endophytic actinomycetes strains isolated from the unnoticed fallen leaves of commonly available medicinal plant *Azadirachta indica*, which are considered as unique source. Among six actinobacteria isolates, one microbe (A5) effectively removed uranium in 12 h at temperature 30 °C and pH 8–9. Molecular characterization and phylogenetic analysis support the classification of the isolate A5 as a new strain which was named as *Streptomyces* sp. MINYYAA7 (Genbank accession number KF909129).

Keywords Microremediation · Uranium · *Streptomyces* sp. MINYYAA7 · *Azadirachta indica*

Introduction

Uranium and its decay products are the major radioactive, polyvalent heavy metal contaminant which affects the environment with its increased utilization in human activities and hence its degradation has been under prime focus. The main source of uranium are mining and milling operations and nuclear waste disposal sites. Additional contamination resulted from trace amounts of uranium being released from the combustion of coal as well as from the manufacture and application of phosphate fertilizers [1]. Depleted Uranium oxides formed at high temperature are not the same as its metallic form. Once non-degradable uranous ion is absorbed, it gets distributed throughout the

internal organs and produces toxicological effect by inhibiting the enzyme production which leads to improper carbohydrate metabolism. The overall mechanism of organs like liver, kidney, lungs and bones gets affected which in turn trigger cancerous growth.

To solve this difficulty, eco-friendly microbes with high biodegradability were explored in and around nature. With the proper microbe or combination of microbes, the latter generally can be degraded to harmless compounds or inorganic constituents like carbon dioxide, minerals, and water [2]. Lovley et al. [3] reported the capability of dissimilatory metal reducing microbes to remove uranium from aqueous solutions. Shumate et al. [4] reported rapid uptake of uranium from solution by resting *Saccharomyces cerevisiae* and *Pseudomonas aeruginosa* cells. Takehiko Tsuruta [5] reported the capability of metal reducing microbe like *Streptomyces levoris* that selectively adsorb uranium from acidic solutions. He also reported that actinomycetes are more capable in removing uranium compared to bacteria, fungi and yeast.

Hence, the present aim of this study was to isolate and screen the rare actinomycetes from fallen leaves of *Azadirachta indica* (Neem) in Western Ghats, India that has the capacity to microremediate radioactive waste Uranium. The influence of different temperature and pH on Uranium removal was also studied.

Experimental

Sample collection

Fallen leaves of common medicinal plant Neem (*A. indica*) with high medicinal value were gathered from the garden of Nirmala College for Women, Coimbatore (11.0183°N,

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76.9725°E) belonging to Western Ghats of Southern India during the period of December 2011 to January 2012. The collected plant materials were subjected to surface sterilization followed by inoculation work within 96 h.

Isolation of endophytic actinobacteria

Neem leaves were cut into small pieces (2*2 cm) and washed by running tap water for 1–2 min to remove the soil particles completely. The resultant were subjected to a five-step surface sterilization procedure as per the method of Sheng et al. [6]: a 4–10 min wash in 5 % sodium hypochlorite, followed by a 10 min wash in 2.5 % sodium sulphite, a 5 min wash in 75 % ethanol, a wash in sterile water, and a final rinse in 10 % sodium bicarbonate for 10 min to disrupt the plant tissues and inhibit the fungal growth. At this point, the final washed solution was spread onto ISP 2 (International *Streptomyces* Project 2) agar and observed for microbial growth to validate the surface sterilized protocol. After the sterility check, the surface sterilized tissues were subjected to continuous drying at 100 °C for 15 min.

For the isolation of rare actinomycetes, the surface treated leaf samples were aseptically transferred on Humic acid Vitamin agar supplemented with nalidixic acid (50 mg/liter) and nystatin (100 mg/liter) and incubated at 28 °C for 2–8 weeks.

The isolates picked up from Humic acid Vitamin agar plates were purified on ISP 2 (International *Streptomyces* Project 2) agar and incubated at room temperature (28 ± 2 °C) for 24–72 h. The pure isolates were then used for the following bioremediation studies.

Uranium bioremediation study

Uranium bioremediation study was carried out as per the procedure by Golab et al. [7]. Briefly, 1 % (v/v) freshly prepared inoculum of isolated actinomycetes strains from Neem leaves was pure cultured in Yeast extract malt extract broth containing 4 g Yeast extract, 10 g Malt extract, 4 g Dextrose in 1,000 ml distilled water at pH 7–8. The culture was shaker incubated for 120 rpm at 30 ± 2 °C temperature and then harvested by centrifugation at 6,000 rpm for 10 min. The pellet was washed three times with 0.85 % sodium chloride solution followed by three washings with double distilled water. The yielded biomass was used for the following experiment.

1 % (v/v) of biomass was suspended in a solution containing 100 mg/L uranyl nitrate as uranium source which was shaker incubated for 120 rpm at 30 ± 2 °C temperature. Samples for spectrophotometric analysis to estimate the uranium contents in the filtrate were withdrawn at

predetermined intervals. Cells were removed from aqueous phase by centrifugation at 10,000 rpm for 10 min.

The amount of residual uranium ions in the solutions was determined by Arsenazo III method [8] using UV–Vis spectrophotometer. Briefly 0.5 ml sample was mixed with 0.1 ml of oxalic acid (4 %) and 0.1 ml of Arsenazo (III) (0.05 %) and diluted with hydrochloric acid (4 M) to a total volume of 2.5 ml before analyzing at a wavelength of 650 nm. Solution without biomass was used as control.

The screened isolate with better uranium removal in shortest duration was selected to study the influence of temperature and pH on uranium removal.

Effect of temperature and pH on Uranium removal

The effect of pH on uranium removal by strain A5 was studied in the range of 3.0–9.0 at regular time intervals (12, 24, 48, 72 h). Same way, the effect of temperature on metal removal was studied in the range of 10–60 °C, maintaining pH as 9.

Identification of actinomycetes

The screened isolate with better uranium removal in shortest duration was identified according to morphological criteria, including characteristics of colonies on plate, morphology of substrate and aerial hyphae, morphology of spores and pigment production [9, 10]. The isolate was preserved in 20 % (v/v) glycerol for subsequent investigation. Molecular identification has been carried out to identify the microbe up to species level in order to check its novelty in preliminary level.

Molecular identification

The genomic DNA from actinomycetes used for PCR was prepared from the single colony grown on ISP 2 broth for three days. The total genomic DNA from the potent strain was isolated employing Chromous DNA isolation kit (Chromous Biotech, Bengaluru, India) according to the manufacturer protocol. The 16S rRNA gene fragment was amplified by using universal primers corresponding to positions 8–27 for the forward primer and 1492–1510 for the reverse primer mentioned below.

Forward primer: 5'-AGAGTTTGATCMTGGCTCAG-3'.

Reverse primer: 5'-TACGGYTACCTTGTTACGACTT-3'.

	94°C	94°C	55°C	72°C	72°C
	5 min	30 s	30 s	1 min	5 min
Initial denaturation	Denaturation	Annealing	Extension	Final extension	
	35 cycles				

A positive control (*E.coli* genomic DNA) and a negative control in the PCR were also included. Sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an Applied Biosystems model 3500XL automated DNA sequencing system (Applied BioSystems, USA).

Nucleotide sequence of 16S rRNA gene from actinomycetes strain was determined and compared for similarity level with the reference species present in genomic database bank. The NCBI BLAST program was employed in order to assess the degree of DNA similarity. Multiple sequence alignment and the phylogenetic tree was constructed using MEGA software [11].

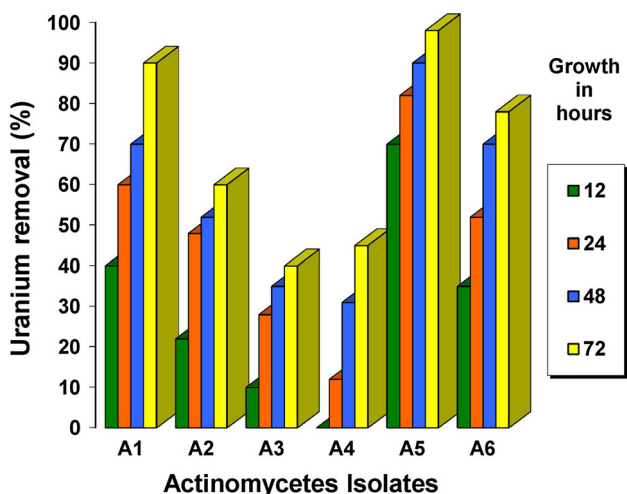


Fig. 1 Uranium bioremediation study

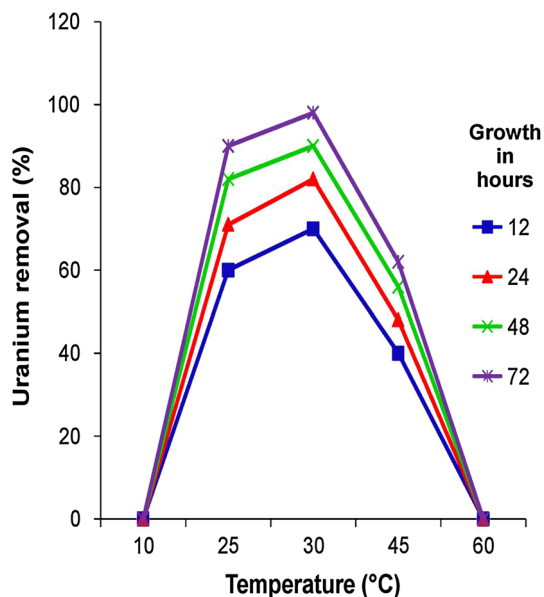


Fig. 2 Effect of temperature in Uranium removal

Result and discussion

Totally six actinomycetes (A1–A6) isolates were isolated from surface sterilized fallen leaves of *A. indica*. Gradual increase in uranium removal has been observed in all six isolates. Generally, they showed 40–98 % uranium removal in 72 h under the tested conditions (Fig. 1). In particular, strain A5 was selectively screened due to its remarkable microremediation of radioactive waste uranium since it could remove 70 % uranium in 12 h of growth compared to other isolates.

Effect of temperature on uranium removal was studied using initial uranium concentration of 100 mg/L. Range of temperature studied was 10, 25, 30, 45 and 60 °C. Growth range of microbial culture from 12 h to 72 h was taken into study. No uranium uptake was observed at 10 °C. Around

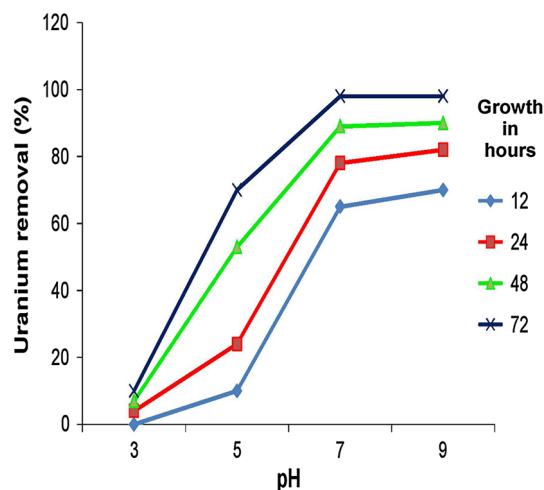


Fig. 3 Effect of pH in Uranium removal



Fig. 4 *Streptomyces* MINIYAA7 on ISP2 agar

Table 1 Morphological and metabolic profile of *Streptomyces* A5 isolate

Characteristics	Characterization of <i>Streptomyces</i> A5
Morphology (ISP2 agar)	Aerial mycelium (Greyish white); Basal mycelium (Light yellow)
Nitrate reduction	+
Urease	–
Triple sugar iron	Alk/Alk
Catalase	+
Oxidase	+
Indole	–
Methyl red	–
Voges proskauer	–
Citrate utilization	–

Alk alkaline, + positive; – negative

70 % removal of uranium was noted at 30 °C (Fig. 2). The microbe A5 does not show any influence on uranium removal at temperature 60 °C since it dies at this high temperature.

Effect of pH on uranium removal was studied using initial uranium concentration of 100 mg/L. Range of pH studied was 3, 5, 7 and 9. Growth range of microbial culture from 12 h to 72 h was taken into study. The depletion of uranium goes higher around pH 8–9 and decreases with increasing acidity (Fig. 3). Thus, the removal of uranium by strain A5 cells is markedly affected by factors like temperature and pH.

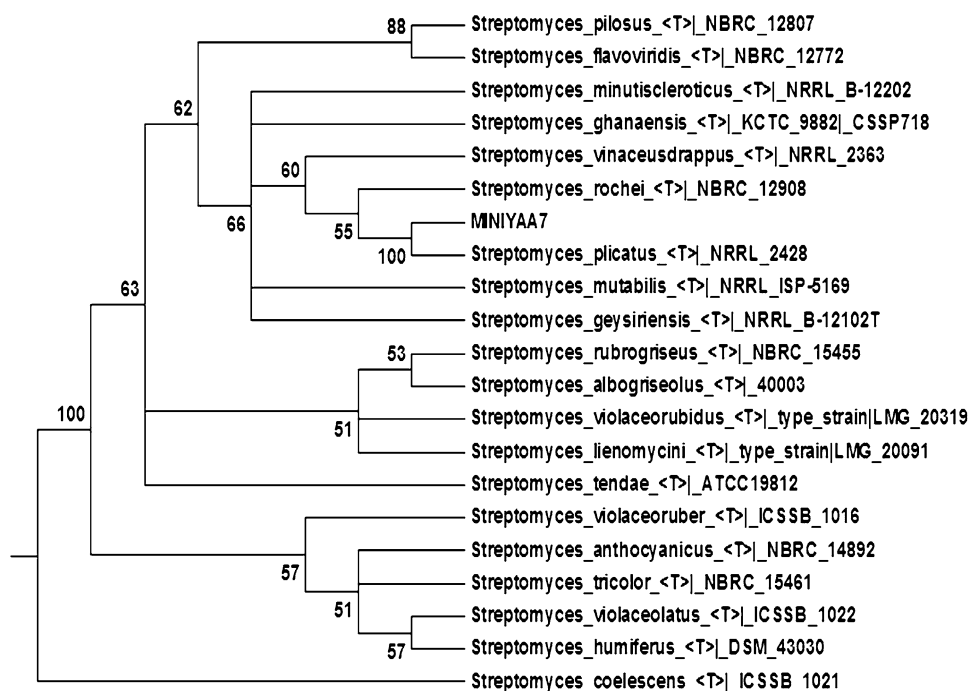
Morphological and biochemical characteristics showed that A5 belonged to the *Streptomyces* sp. The isolate produced irregular, raised, grey white colonies with light yellow substrate mycelium on ISP2 agar surface (Fig. 4). Dark brown melanin pigment was produced by the isolate. By viewing under 1,000× magnification, gram positive rods with rounded ends; branched hyphae with non-motile spores were found. Therefore, based on actinomycetes identification key [9, 10], the isolate was assigned to the genus *Streptomyces* (Table 1).

Molecular characterization by 16S rRNA ribotyping and phylogeny analysis was carried out in order to identify *Streptomyces* A5 up to species level.

Phylogenetic analysis and species identification

Using BLAST search in the NCBI data bank, sequences homologous to 16S rRNA gene of the isolate A5 were collected. The DNA sequences were aligned and phylogenetic tree was constructed using MEGA software (bootstrap method) [11] (Fig. 5). The evolutionary history was inferred using the Neighbor joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) was shown next to the branches. Only values greater than 50 % were shown. Comparison of 16S rRNA nucleotide gene sequence of strain A5 with corresponding *Streptomyces* sequences clearly showed that the organism form a distinct phyletic line with *Streptomyces* genera. The isolate was closely related to the type strain of *S. plicatus* sharing a

Fig. 5 Phylogram depicting the taxonomic position of *Streptomyces* sp. MINYAA7



16S rRNA gene sequence similarity of 96 %. Hence the molecular results support the classification of the isolate A5 as a new strain which was named as *Streptomyces* sp. MINIYAA7. The partial 16S rRNA sequence of isolate A5 (840 bp) was determined and deposited in Genebank under the accession number KF909129. Still, complete sequence analysis, DNA–DNA hybridizations, phenotypic comparisons, and chemotaxonomic analysis need to be performed to confirm its novelty.

Conclusion

There was no scientific report before on actinomycetes from medicinal plant leaves that can microremediate uranium. Hence, isolation and screening of actinomycetes from medicinal plants in optimum condition could be the promising resource of potential eco-friendly radionuclide microremediating agent. Even, its microremediation capacity with other radionuclides and heavy metals may also be investigated. If the research proves to be successful, the radioactive wastes can also be decomposed in areas where trees with good micro remediation property are present.

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