

FROM SPACE TO EARTH: *BACILLUS ARYABHATTAI* FOUND IN THE INDIAN SUB-CONTINENT

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ABSTRACT

An isolate from the rhizosphere region of *Lemna* sp. from the East Kolkata wetlands was cultured on Nutrient Agar media and characterized by carrying out several common tests, e.g. heat tolerance, cold tolerance, UV tolerance, urease, oxidase, gelatinase etc. This was followed by phylogenetic analysis based on partial 16S rRNA gene sequences, to establish the bacterial isolate as *Bacillus aryabhatai*. Further initiative to check for its antibiotic production and chromium uptake ability, was also undertaken. The isolate gave positive results for the Gram Stain, Oxidase, Urease, Gelatinase, Nitrate Reduction, Voges-Proskauer, Starch Hydrolysis assays and negative results for the Indole Production Assay. It showed cold tolerance to as low as 4°C but was intolerant to temperatures higher than 37°C. The isolate showed reduced but viable growth even under UV exposure for 30 minutes. However its Chromium uptake ability was low and gave negative results for the antibiotic production assay. Perhaps this is the first proof of this particular extra-terrestrial microorganism to be found in the Indian Sub-continent, evidence in support of the Theory of Panspermia.

Keywords: *Bacillus aryabhatai*, extra-terrestrial, Panspermia, UV resistant

INTRODUCTION

Cosmic Ancestry is a new theory pertaining to evolution and the origin of life on Earth. It proclaims that life on Earth was seeded from space, and that life's evolution to superior forms depends on genetic foundations that come from space. It is a completely scientific, testable theory for which evidence is increasing. That which deals with the origin of life on Earth, is known as *panspermia* — literally, "seeds everywhere". In the 1970s, British astronomers Fred Hoyle and Chandra Wickramasinghe rekindled interest in panspermia. By careful spectroscopic observation and analysis of light from distant stars they found new evidences for traces of life in the intervening dust. They also proposed that comets, made largely of water-ice, carry bacterial life across galaxies and protect it from radiation damage along the way. One aspect of this research program, that interstellar dust and comets contain organic compounds, has been pursued by others as well. It is now widely accepted that space contains the "ingredients" of life. (Arrhenius, 2009; Hoyle and Wickramasinghe, 2000; Wainwright, 2010; Wickramasinghe *et al.*, 2009) This development could be the first hint of a huge paradigm shift.

Evidence for Panspermia: Bacteria can survive harsh environment of space -

- a. Ultraviolet radiation
- b. Proton bombardments

c. Cold temperatures

Presence of bacteria and fungi in the stratosphere have been recently confirmed by Harris *et al.*, (2002), Wainwright *et al.*, (2003, 2004a,b), Narlikar, *et al.*, (2003), Griffin (2004, 2008), Yang *et al.* (2008), Shivaji *et al.* (2009). Most of the bacteria that find its ways into the stratosphere may not have come from Earth, but from elsewhere in space. This is because the tropopause acts a barrier to the free movement of particles above 17km thereby making such transfer from Earth to the stratosphere very difficult. For example, bacterial cell masses exceeding ten micron in diameter, found in stratosphere samples, seem unlikely to have been carried up from Earth even by monsoons, we assume that these are incoming from space and that they contribute to a mixed stratospheric biosphere made up of space and Earth-derived organisms (Wainwright *et al.*, 2004, Wainwright, 2008).

Shivaji *et al.* had undertaken a polyphasic taxonomic approach to characterize 12 bacterial strains isolated from cryotubes that were used to collect air at altitudes of between 27 and 41 km, during a balloon flight. Three of the 12 bacterial isolates, strains PVA5-1T, B3W22T and B8W22T, represent three novel species of the genera *Bacillus* (B3W22^T and B8W22^T) and *Janibacter* (PVA5-1^T).

B3W22^T was characterised to be *Bacillus isronensis* sp. nov., B8W22^T was *Bacillus aryabhatai* sp. nov., PVAS-1T was *Janibacter hoylei* sp. nov. *Bacillus aryabhatai* till date have not been evidenced in the Indian Sub-continent and is considered to be an extra-terrestrial survivor. However any instance of their existence on earth would to some extent assert the "Theory of Panspermia".

MATERIALS AND METHODS

Subject: Isolate from the rhizosphere region of *Lemna* sp. Brown colonies were initially observed when streaked on Nutrient Agar, followed by a gradual disappearance of the characteristic colour of the colonies on repeated sub-culturing on Nutrient Agar plates.

Characterization:

Colony shape: Entire, round, flat.

Colony colour: Initially brown, later peach-coloured. (Refer to Figure 1.)

Gram staining: The heat-fixed smear of the culture is stained with Crystal-Violet; Iodine added to trap the dye and washed with alcohol to decolorize the smear and counter-stained with Safranin.

Oxidase test: Culture cultivated on trypticase soy agar plates with a single-line streak inoculation. The inoculated plates are incubated at 37°C for 24–48 hours to establish colonies. After colonies have grown on the media, two-to-three drops of the reagent DMPD are added.

Urease test: The broth contains two pH buffers, urea, a very small amount of nutrients for the bacteria, and the pH indicator phenol red.

Gelatinase test: The gelatin stab method employs nutrient gelatin deep tubes that contain 12% gelatin. A heavy inoculum from the culture is stabbed into the media. The gelatin media is incubated for at least 48 hours, and then placed into the refrigerator for approximately 30 minutes.

Indole Production: A tube of tryptone broth is inoculated with a loop full of culture and incubated overnight at 37°C. A few drops of Kovac's Reagent are added directly to the tube after incubation.

Voges-Proskauer test: The culture is inoculated in MR-VP broth and incubated at 37°C, overnight. After incubation 10 drops of Barritt's Reagent A and 4 drops of Barritt's Reagent B is added; the tube shaken and allowed to stand for 1 hour.

Nitrate Reduction test: Nitrate broth is inoculated with the culture and incubated for 48 hrs. After

incubation sulfanilic acid and N,N-dimethyl-1-naphthylamine is added.

Starch hydrolysis: A plate with starch agar is streaked with culture, incubated overnight at 37°C and then Iodine added.

Heat tolerance: Nutrient Broths were inoculated with a loop-full of the bacterial culture and incubated at

37°C, 50°C, 80°C

Cold tolerance: Nutrient Broths were inoculated with a loop-full of the bacterial culture and incubated at the following temperatures, overnight, 37°C, 4°C

UV tolerance: Nutrient Agar plates were streaked with the bacterial culture and then exposed to UV radiation (280-315 nm) for different time points as follows: Control (un-exposed), 5 minutes, 10 minutes, 15 minutes, 30 minutes

The plates were then transferred to a 37°C incubator for over-night incubation.

Chromium Uptake Assay: The East Kolkata Wetlands are used as the most preferred wastewater disposal reservoir for many tanneries in and around the area. So any microorganism thriving in such a water body would very probably be able to tolerate high levels of heavy metal ions such as Cr³⁺, Cd²⁺, Ni²⁺, Ti³⁺ etc. So a very logical approach would be to check for the level of such heavy metal tolerance of the *Bacillus* isolate.

Procedure: A Nutrient Agar slant of the pure *Bacillus* culture was sent to the Analytical and Environmental Engineering Laboratory, Jadavpur, Kolkata for hexavalent Chromium uptake assay.

Antibiotic Production Assay: The 16S rDNA sequencing and subsequent sequence alignment showed that *Bacillus aryabhatai* strain LSII is 99–100% homologous to *B. megaterium*. Since *B. megaterium* is known to produce antibiotic, the antibiotic production capability of *B. aryabhatai* was also tested for.

Procedure: A culture of *E. coli* (>200µl) [stock conc: 500µl in 1ml] was streaked on 4 Nutrient Agar plates. The *B. aryabhatai* culture was centrifuged and the supernatant collected. Dilutions of the supernatant were carried out - **Table 1**. Filter paper discs were blotted with each supernatant solution and placed on the *E. coli* streaked plates according to the nomenclature of the tubes. The plates were incubated at 37°C overnight and then checked for zone of clearance around the filter paper discs soaked in *B. aryabhatai* culture supernatant.

RESULTS AND DISCUSSION

Experimental results of Gram Staining showed that the microbe was Gram Positive. The biochemical tests, namely Oxidase test, Urease test, Gelatinase test, Voges-Proskauer test, Nitrate Reduction test and Starch Hydrolysis test yielded positive results. However the Indole Production assay gave negative results.

Heat Tolerance: The results were observed after overnight incubation at the respective temperatures. There was considerable growth at 37°C but no visible growth was seen at 50°C and 80°C.

Cold Tolerance: The results were observed after overnight incubation at the respective temperatures. There was considerable growth at both 4°C and 37°C.

UV Tolerance: The observations were made after overnight incubation of the experimental and control plates at 37°C. There was profuse growth in the control plate. The growth was profuse but reduced in the 5 minute exposure plate, further reduced in the 10 minute exposure plate. Growth was drastically reduced in the 15 minute exposure plate and there were just 2-3 colonies in the 30 minute exposure plate.

Fig. 2. For results at the experimental temperatures.

Chromium Uptake Assay: Under the test method of APHA (20th Edition), 3500 – Cr B, the microorganism was capable of up to 0.44 mg/L hexavalent chromium uptake.

Antibiotic Production Assay: There was no zone of clearance in any of the plates (Fig. 3).

Table 1: Dilution of an isolate from the rhizosphere region

Sample No.	Dilution
1	Undiluted
2	10 folds
3	100 fold
4	1000 d

Table 2: Biochemical Test of an isolate from the rhizosphere region

Biochemical Test	Result
Gram Stain	Positive
Oxidase Test	Positive
Urease Test	Positive
Gelatinase Test	Positive
Indole Production	Negative
Voges-Proskauer Test	Positive
Nitrate Reduction Test	Positive
Starch Hydrolysis	Positive

Table 3: Temperature of an isolate from the rhizosphere region

Temperature (°C)	No. of tubes incubated	Growth
37	2	Yes
50	3	No
80	3	No
4	2	Yes
37	2	Yes

Table 5: Exposure time and growth of an isolate from the rhizosphere region

Exposure Time (Min)	Growth
Control (0)	Yes (profuse)
5	Yes (reduced in number)
10	Yes (further reduced, but countable)
15	Yes (drastically reduced)
30	Yes (2-3 colonies)

Table: Sequence Producing Significant Alignments

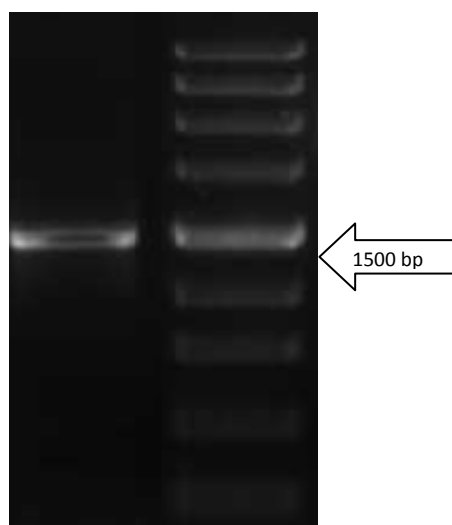
Accession	Description	Max score	Total score	Query coverage	E value	Max ident
GU563347.1	Bacillus aryabhatai strain LS11	2649	2649	100%	0.0	100%
GQ199722.1	Bacillus sp. 210_20	2638	2638	100%	0.0	99%
GQ870260.1	Bacillus megaterium strain O-2	2634	2634	100%	0.0	99%
DQ323079.1	Bacillus sp. M9H	2634	2634	100%	0.0	99%
FN692034.1	Bacillus sp. HY, strain HY 1.1	2632	2632	99%	0.0	99%
GQ927173.1	Bacillus megaterium strain Z3-2	2630	2630	99%	0.0	100%
GQ169805.1	Bacillus sp. B1408	2630	2630	99%	0.0	100%
FJ386540.1	Bacillus megaterium strain ST53	2630	2630	99%	0.0	99%
EU070405.1	Bacillus sp. B261Ydz-hn	2630	2630	99%	0.0	100%
HM371417.1	Bacillus megaterium strain RB-05	2628	2628	99%	0.0	99%

Identification of the Isolate by 16S rRNA sequencing:

The microbial culture was sent to the Xcelris Labs Ltd., Ahmedabad, India for identification.

Gel Image of 16S rDNA amplicon (Sample: Brown colony R2)

Lane 1 2



Lane 1: 16S rDNA amplicon band Lane 2: DNA marker

Consensus Sequence of Brown colony R2 (1434 bp)

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GGGGGGCCAATACATGCAGTCGAGCGAACTGATTAGAGCTTGCTTCTATGACGTTAGCGGCGGACGGGTGAGTAACACGTGGG
CAACTGCCTGTAAGACTGGGATAACTTCGGGAAACCGAAGCTAATACCGGATAGGATCTTCTCCTTCATGGGAGATGATTGAAAGAT
GGTTTCGGCTATCACTTACAGATGGGCCCGCGGTGCATTAGCTAGTTGGTGAGGTAACCGGCTACCAAGGCAACGATGCATAGCCGA
CCTGAGAGGGTGATCGGCCCACTGGGACTGAGACACGGCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTCCGCAATGGA
CGAAAGTCTGACGGAGCAACGCCGCTGAGTGATGAAGCTTTCGGGTCGTAACCTCTGTTGTTAGGGAAGAACAAGTACGAGA
GTAAGTCTGACCTTGACGGTACCTAACAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCG
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GGAAACTGGGGAACCTGAGTGCAAGAGAAAAGCGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCA
GTGGCGAAGGCGGCTTTTGGTCTGTAAGTACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCC
ACGCCGTAACGATGAGTGCTAAGTGTAGAGGGTTTCCGCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTA
CGGTGCAAGACTGAACTCAAAGGAATTGACGGGGGCCGACAAGCGGTGGAGCATGTGGTTAATTCGAAGCAACGCGAAGA
ACCTTACCAGGTCTTGACATCCTCTGACAACCTAGAGATAGAGCGTTCCCTTCGGGGGACAGAGTGACAGGTGGTGCATGGTTGT
CGTCAGCTCGTGTGATGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCTTGATCTTAGTTGCCAGCATTAGTTGGGCACTCTA
AGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCTTATGACCTGGGCTACACAGTGTAC
AATGGATGGTACAAAGGGCTGCAAGACCGCGAGGTCAAGCCAATCCATAAAACCATTCTCAGTTCGGATTGTAGGCTGCAACTCGC
CTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGTGAATACGTTCCCGGGCCTTGACACACCGCCGTCACACC
ACGAGAGTTTGTAAACCCGAAGTCGGTGGAGTAACCGTAAGGAGCTAGCCG

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BLAST DATA: (Alignment view using combination of NCBI GenBank)

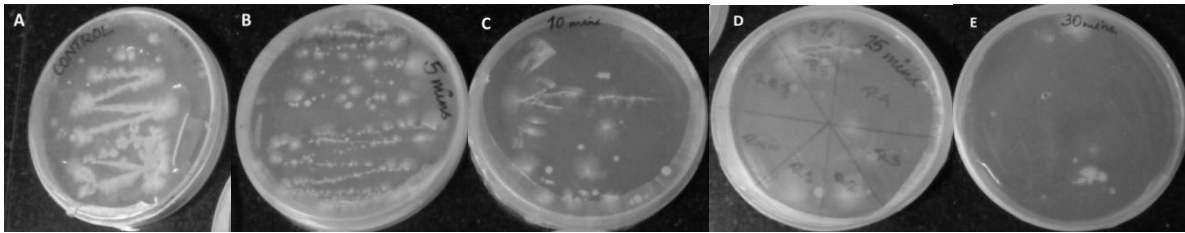


Fig 2: UV exposed plates observed after overnight incubation at 37°C

A: CONTROL B: 5MINS EXPOSURE C: 10MINS EXPOSURE D: 15 MINS EXPOSURE E: 30MINS EXPOSURE

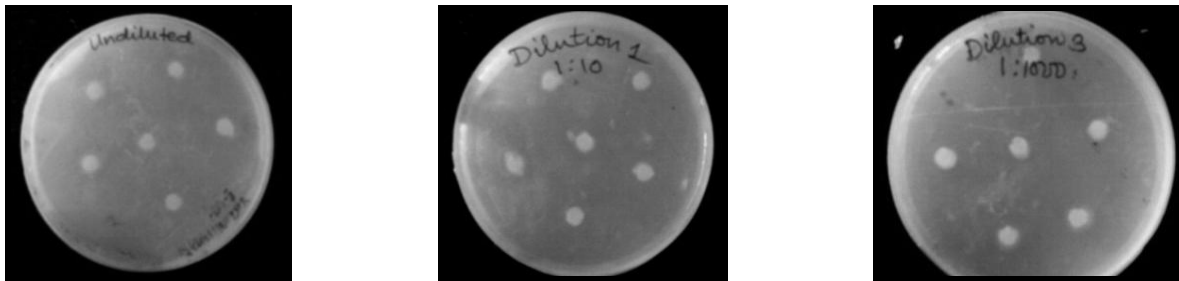
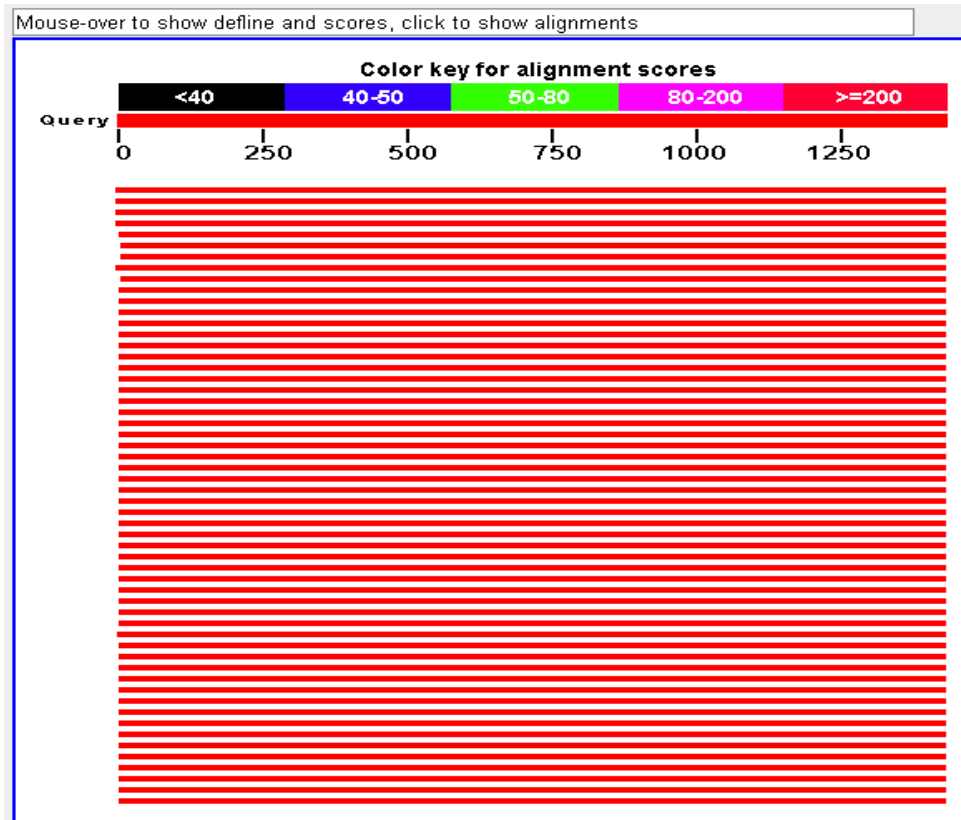


Fig 3: undiluted

1:10 dilution

1:1000 dilution

Distribution of 120 Blast Hits on the Query Sequence



Information about other close homologs for the microbe can be found from the Alignment View table.

Phylogenetic Tree:

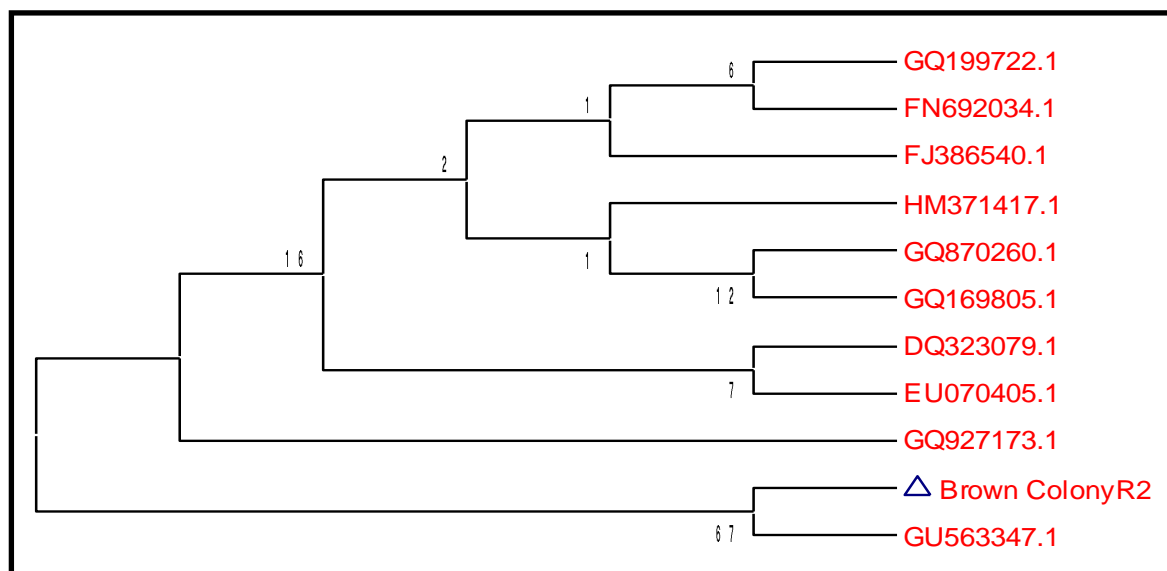


Fig. Evolutionary relationships of 11 taxa

The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 1425 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4.

DISCUSSION

The case for Cosmic Ancestry is not yet proven, of course. The Theory of Panspermia stands doubted till date, a proper confirmation for its existence has still not been achieved. In the 1920s, Russian biochemist Alexander Oparin and English geneticist J.B.S. Haldane, writing independently, revived the doctrine of spontaneous generation in a more sophisticated form. Supporting this theory, in 1953, American chemists Stanley Miller and Harold Urey showed that some amino acids can be chemically produced from ammonia and methane.

Hoyle and Wickramasinghe also broadened or generalized panspermia to include a new

understanding of evolution. But mainstream science has not accepted the hard core of modern panspermia, that whole cells seeded life on Earth. While accepting the fact that life on Earth evolved over the course of about four billion years, they say that the genetic programs for higher evolution cannot be explained by random mutation and recombination among genes for single-celled organisms, even in that long a time: the programs must come from somewhere beyond Earth. In a nutshell, their theory holds that all of life comes from space.

Until recently most of the work on panspermia has been theoretical. However, there is now laboratory evidence to support the view that microbes can be transferred across the cosmos, simply by sampling space at a height above the Earth where there is no possibility of contamination from below, which suggests that, at this moment; life is entering the Earth's atmosphere from space.

The organism *Bacillus aryabhatai* strain LS11 is a dweller of the East Kolkata Wetlands, but its source is not proved. It has somehow survived the Cr³⁺, salinity, and stress without any major physiological disability. The colony colour which was initially brown was also lost in the process of sub-culturing, indicating that the secondary metabolite which was responsible for the characteristic colour was also lost or was not necessary for its survival on the Nutrient Agar media. It has not produced any secondary metabolite, to survive in the extreme conditions.

The UV tolerance assay proves that the microbe is capable of growing in regions with high UV exposure, as is the case in space. From the temperature tolerance assays we can clearly see that the microbe is better suited to growth at colder temperatures than at high temperatures, a further proof to the assumption that it is indeed an extra-terrestrial survivor. Hence, we can prove that life form can adjust themselves to alien environment without much time lag. Further insight into the aspect of such a dual survival is required to establish the existence of extra-terrestrial microbe on Earth.

CONCLUSION

The culture, which was labeled as **Brown colony**

R2 was found to be *Bacillus aryabhatai strain LS11* (GenBank Accession Number:GU563347.1) based on nucleotide homology and phylogenetic analysis.

It does not have a considerable uptake of Cr³⁺, is majorly resistant to UV light and does not produce any antibiotic.

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