ISOLATION AND CHARACTERIZATION OF GAMMA AND UV RADIATION RESISTANT SOIL MICROBES FROM WEST COASTAL REGIONS OF INDIA

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ABSTRACT:

Electro Magnetic Radiations like Gamma rays, UV rays and X rays etc are capable to cause a variety of mutagenic and cytotoxic DNA lesions in cells. Some microorganisms called "Extremophiles" are capable to survive high dose of radiation. At present such extremophiles are in sensation for their potential application in biotechnological, pharmaceutical and therapeutic industry. These radiation resistant microbes have developed defense mechanism to survive even in environments of high dose of radiation. These defense mechanisms involve protection of DNA from damage by participation of primary and secondary metabolic products called "extremolytes and extremozymes" which capable to absorb a wide spectrum of radiations. In addition to these, possess several cellular radio protective mechanisms including high activities of antioxidant enzymes catalase, SOD etc., unique cell structure and efficient DNA repair capacity. A limited number of studies reported the isolation followed by biochemical and phylogentical characterization such extremophiles because generally extremophiles are found in radionuclides contaminated environments and the restricted access to such contaminated environments limits study. Today, radiation resistant microbes are receiving worldwide attention for their potential in applications like bioremediation of radioactive waste, useful in anti-oxidant, anti-proliferating and anti-cancer agents etc., few studies reported the presence of radiation resistant microbes in sediments of Cauvery River and Karnataka. The present study was conducted to isolate and characterize gamma and UV radiation resistant soil microbes from west coastal regions of India. Twelve out of eighteen isolates from soil samples showed radiation resistance when subjected to 350Krads of gamma radiation using Co^{60} radiation source and 6 hours of UV light exposure. Among these twelve isolates, nine were found to be capable to with stand 6 hours of sustained radiation exposure. All the radiation resistant microbes were characterized by biochemical tests, antioxidant enzymatic assays and 16S rRNA sequence analysis.

KEY WORDS: Gamma radiation resistance, UV radiation resistance, West coastal regions of India, soil microbes.

INTRODUCTION:

"Extremophiles" are microorganisms that can endure harsh environmental conditions. Due to their unique chemicals, referred to as "extremolytes," extremophiles have recently made an influence on biotechnology and pharmaceutical research and industry. Microbial radiation resistance is an interesting phenomenon that most likely developed separately when their selection pressures (such as UV, X-ray, gamma radiation, etc.) evolved. Radiation causes various forms of oxidation and damage to proteins, nucleic acids, and other essential biological constituents. Acute γ-irradiation tests conducted in the lab form the basis of the majority of the literature on microbial radiation resistance. Other than bioremediation and the medicinal use of such promising bacteria, very little is known. Therefore, it is clear that studying the interaction of extremopliles with different radiations would be a fascinating phenomenon. Thus far, the benefits of radiation-resistant extremolytes and extremozymes in the fields of biotechnology and medicine have not been heavily implicated. By altering their physiological and biochemical characteristics according to their biological niches, organisms that live in harsh environments have evolved a variety of survival strategies. Interestingly, despite the lack of a naturally radiation-intensive environment, organisms with great radiation resistance have been found in the three domains of life (Bacteria, Archaea, and Eukarya).

Few studies reported the abundant presence of proteobacteria in highly contaminated (Uranium and other metals) sediments of Cauvery river, India (Suriya *et al.*, 2017). Karnataka's coastline called Karavali stretches 309.59km between Mangalore in Dakshina Kannada district and Karwar in Uttara Kannada district. The coast is drained by the Kali Nadi, Gangavali, Bedti, Tadri, Sharavati, and Netravati rivers, which have carved out narrow valleys with steep gradients and generally flow in a westerly direction. And no study was conducted to isolate and characterize radiation resistant bacteria from West coastal regions of India. Thus present study is aimed to isolate UV and Gamma radiation resistant soil microbes from west coastal regions of Karnataka, India. The study also intends to characterize the radiation resistant bacteria by gram staining, biochemical tests, antioxidant enzyme assays, membrane integrity assays and 16S rRNA sequencing.

METHODOLOGY:

West Coastal regions sample collection

The Soil samples used in this study were collected from the west coastal region, India. The study area was taken at GPS coordinates latitude, longitude 12.9384956,74.80397833333333. Samples were collected aseptically into sterile autoclaved glass containers and transported to laboratory with intense care.

Isolation of bacteria from soil

Bacteria were isolated from soil using serial dilution followed by agar plating technique. Simple nutrient agar (NA) medium was used for isolation and maintenance of bacterial cultures. Initial screening of bacterial isolates was done based on colony morphology such as shape, margin, elevation, surface, color, optical feature and Gram staining results. Out of 25 isolates, 18 morphologically different isolates were selected for the further study.

Irradiation of isolates

Selected isolates were suspended separately in 2ml of 0.05M sterile phosphate buffer. Aliquot of 1ml from each suspension was taken in sterile vial and vials were then exposed to350krads of Gamma radiation. 1ml of aliquot from each bacterial suspension was taken in small sterile tissue culture plate and were exposed to UV light (Philips UVC lamp - TUV – 15W/G15T8) for 6 hour in LAF Cabinet. Immediately after irradiation, the samples were covered with aluminum foil to prevent photo-reactivation. Assessment of viability was carried out using 0.1 ml inoculums from the irradiated or non-irradiated controls by plating them on NA plates at 37ºC for 24 hours. The reduction in survival rate after radiation exposure was assessed via comparing to non-irradiated control.

Characterization of radiation resistant isolates

Biochemical characterization

Viable colonies remaining after the exposure to radiation were considered to exhibit radiation resistantance and were identified using biochemical tests as per the standard protocols prescribed

Phylogenetic analysis

Radiation resistant colonies were subjected to DNA analysis and DNA samples were subjected to polymerase chain reaction using primer sequences as follows: Forward primer sequence (27F) - 5' AGAGTTTGATCCTGGCTCAG 3', Reverse Primer sequence (U1492R) - 5' GGTTACCTTGTTACGACTT 3'. The purified PCR products were sent for 16s RNA sequencing. Sequences were used to construct phylogenetic tree by using neighbor- joining method (Saitou N & Nei M, 1987) and MEGA4 software.

Analysis of membrane integrity

Membrane integrity was examined using 260-280 ratio analysis. 24hrs old bacterial culture was suspended in phosphate buffer (pH-7.8) and this suspension was centrifuged at 3000rpm for 10 minutes. The supernatant was collected and 260-280 ratios were analysed using UV-Visible spectrophotometer (control). The same procedure was repeated for irradiated samples. The test samples were compared with the control.

Analysis of SOD and Catalase enzyme activity

Superoxide dismutase enzyme activity in control and irradiated samples was estimated by NBT reduction method (Beaurchamp & Fridovich, 1971). The catalase enzyme activity was analysed using H_2O_2 as substrate (Aebi et al, 1984).

RESULT AND DISCUSSION:

Screening of Radiation resistant bacteria:

The number of bacteria (CFU) per gram of soil was found to be 2.5 x 10^6CFU. A total of 25 different colonies were selected from soil samples based on colony characteristics such as shape, margin, elevation, surface, color, optical feature, of which 18 different colonies were selected for irradiation. A drastic reduction in the bacterial colony count was observed after exposure to both Gamma and UV rays. The colonies that survived in the radiation treatment (Gamma or UV rays) were considered as radiation resistant bacteria. The colony count before and after UV and Gamma radiation treatment for each isolate is presented in a table 1 and The control and irradiated plates photo depicted in Fig 1.

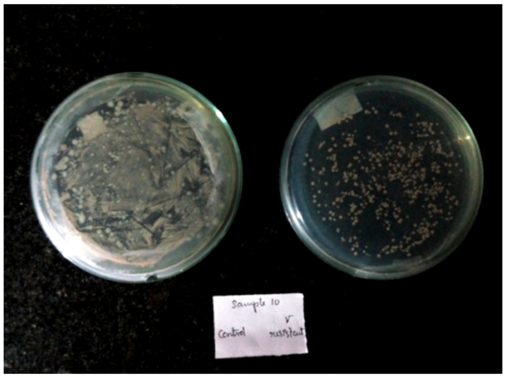


Fig 1: Control and irradiated plates

Table 1: Bacterial colony count before and after irradiation

Sample	Colony Count		Colony Count	
	Before Gamma Irradiation	**After Gamma Irradiation**	**Before UV Exposure**	**After UV Exposure**
A	2340	Nil	2340	894
B	2235	500	2235	779
C	4680	700	4680	941
D	3915	572	3915	Nil
E	3390	300	3390	974
F	5010	600	5010	Nil
G	2535	389	2535	Nil
H	1365	495	1365	1044
I	1560	Nil	1560	781
J	3825	141	3825	Nil
K	3345	10	3345	1126
L	2115	Nil	2115	928
M	1635	284	1635	Nil
N	1695	165	1695	713
O	2880	374	2880	Nil

Identification of Resistant strains

All the isolates were identified as Gram positive bacilli. Isolates A, B, C, E, H, I, J, L, M, N were motile and the others were non motile. Strain/isolates A, C, D, F, G, H, L, M, N, O were spore formers. The results of staining, colony morphology, fermentation tests and biochemical characterization tests for 18 isolates were summarized in Table 2 & 3.In biochemical characterization, all the isolates were shown to be negative for fermentations of sucrose, lactose and mannitol and negative for production of Indole, urease and H_2S. With the exception of I, L, N the rest of the isolates were positive for MR. Only isolate K has shown slight positive result for glucose fermentation.

All the isolates tested negative for fermentation of sucrose, lactose, mannitol, Indole , as well as for urease and H_2S production . t. Isolate I, L, N were MR negative and the rest showed positive results. Only isolate H showed positive result for VP test. IsolateA, G, I, K, M, N, O showed negative result for starch hydrolysis test. Only IsolateJ was catalase negative. Isolate A, H, J, K, L were tolerant to 6.5% NaCl. Isolate C, H, J, L, M could reduce nitrate. Isolate A and L showed positive result for citrate utilization test. Only isolateB, J, O showed negative result for oxidase test. Only isolates A, C, F, J, L, M, O were acid fast positive. (Table 2& 3)

Table 2: Results of gram staining, colony morphology and Fermentation tests of isolates.

Sample	Staining Result				Fermentation Test			
	Gram staining		**Acid Fast staining**	**Endospore Staining**	**Glucose**	**Sucrose**	**Lactose**	**Mannitol**
A	+	Rod	+	+	-	-	-	-
B	+	Rod	-	-	-	-	-	-
C	+	Rod	+	+	-	-	-	-
D	+	Rod	-	+	-	-	-	-
E	+	Rod	-	-	-	-	-	-
F	+	Rod	+	+	-	-	-	-
G	+	Rod	-	+	-	-	-	-
H	+	Rod	-	+	-	-	-	-
I	+	Rod	-	-	-	-	-	-

J	+	Rod	+	-	-	-	-	-
K	+	Rod	-	-	+	-	-	-
L	+	Rod	+	+	-	-	-	-
M	+	Rod	+	+	-	-	-	-
N	+	Rod	-	+	-	-	-	-
O	+	Rod	+	+	-	-	-	-

Table 3: Results of the Biochemical characterization of isolates.

Sample	Indole test	Methyl Red test	VP test	H_2S production test	Starch Hydrolysis test	Catalase test	Urease test	Nitrate reduction test	6.5 % NaCl tolerance test	Citrate utilization test	Motility test	Oxidase test
A	-	+	-	-	-	+	-	-	+	+	+	+
B	-	+	-	-	+	+	-	-	-	-	+	-
C	-	+	-	-	+	+	-	+	-	-	+	+
D	-	+	-	-	+	+	-	-	-	-	-	+
E	-	+	-	-	+	+	-	-	-	-	+	+
F	-	+	-	-	+	+	-	-	-	-	-	+
G	-	+	-	-	-	+	-	-	-	-	-	+
H	-	+	+	-	+	+	-	+	+	-	+	+
I	-	-	-	-	-	+	-	-	-	-	+	+
J	-	+	-	-	+	-	-	+	+	-	+	-
K	-	+	-	-	-	+	-	-	+	-	-	+
L	-	-	-	-	+	+	-	+	+	+	+	+
M	-	+	-	-	-	+	-	+	-	-	+	+
N	-	-	-	-	-	+	-	-	-	-	+	+
O	-	+	-	-	-	+	-	-	-	-	-	-

‘+’ = Positive result ‘-’ = Negative

Phylogenetic Analysis

The evolutionary history for the isolates (strains) was inferred using the Neighbor-Joining method and the Phylogenetic analyses were conducted using MEGA4 software. Based on this analysis, isolate A shares 63% similarity with *B. aerius* and 100% similarity with *B. pumilus*.

Closest phylogenetic relative for isolate B was found to be *Fictibacillusbarbaricus* with 97% sequence similarity. Isolate C has 99% sequence similarity with *Bacillus pseudomycoides, Bacillus cereus, and Bacillus anthracis.*Strain/isolate D

*B. qingshengii*has shown 95% similarity with isolate D. Isolate E was identified as *Bacillus kochii.Bacillus aryabhattai*was the closest phylogenetic relative of isolate F. *Bacillus infantis* was the closest phylogenetic relative of isolate G with 99% sequence similarity. IsolateH has 99% sequence resemblance with *Bacillus pseudomycoides, Bacillus cereus, and Bacillus anthracis.*IsolateI was identified as *Paenibacilluslautus.*isolateJ did show 96% similarity with *Bacillus tequilensis*and 91% similarity with *Bacillus subtilis.* Closest phylogenetic relative for isolate K was found to be *Exigobacteriumprofundum.*Isolate L shares 96% similarity with *Bacillus tequilensis*and 91% similarity with *Bacillus subtilis.Bacillus firmus* has shown 96% sequence similarity with isolate M. Isolate N was identified as *Lysinibacillus sp. Xfchu4.Bacillus nealsonii*was the closest phylogenetic relative of isolate O with 77% similarity.

Table 4: Predicted Organisms

Bacterial Sample	Name of the Predicted Organism	% of sequence similarity	Accession Number
A	*Bacillus aerius*	63	NR_118439
	Bacillus pumilus	100	NR_043242
B	*Fictibacillusbarbaricus*	97	NR_028967
C	*Bacillus pseudomycoides*	99	NR_074540
	Bacillus cereus	99	NR_113991
	Bacillus anthracis	99	NR_074453
D	*Bacillus qingshengii*	95	NR_133978

E	*Bacillus kochii*	100	NR_117050
F	*Bacillus aryabhattai*	63	NR_115953
G	*Bacillus infantis*	99	NR_043267
H	*Bacillus cereus*	99	NR_074540
	Bacillus pseudomycoides	99	NR_113991
	Bacillus anthracis	99	NR_074453
I	*Paenibacilluslautus*	100	NR_113991
J	*Bacillus tequilensis*	96	NR_104919
	Bacillus subtilis	91	NR_113265
K	*Exigobacteriumprofundum*	86	NR_043204
L	*Bacillus subtilis*	91	NR_113265
	Bacillus tequilensis	96	NR_104919
M	*Bacillus firmus*	96	NR_112635
N	*Lysinibacillus sp. Xfchu4*	100	GQ_480496
O	*Bacillus nealsonii*	77	NR_044546

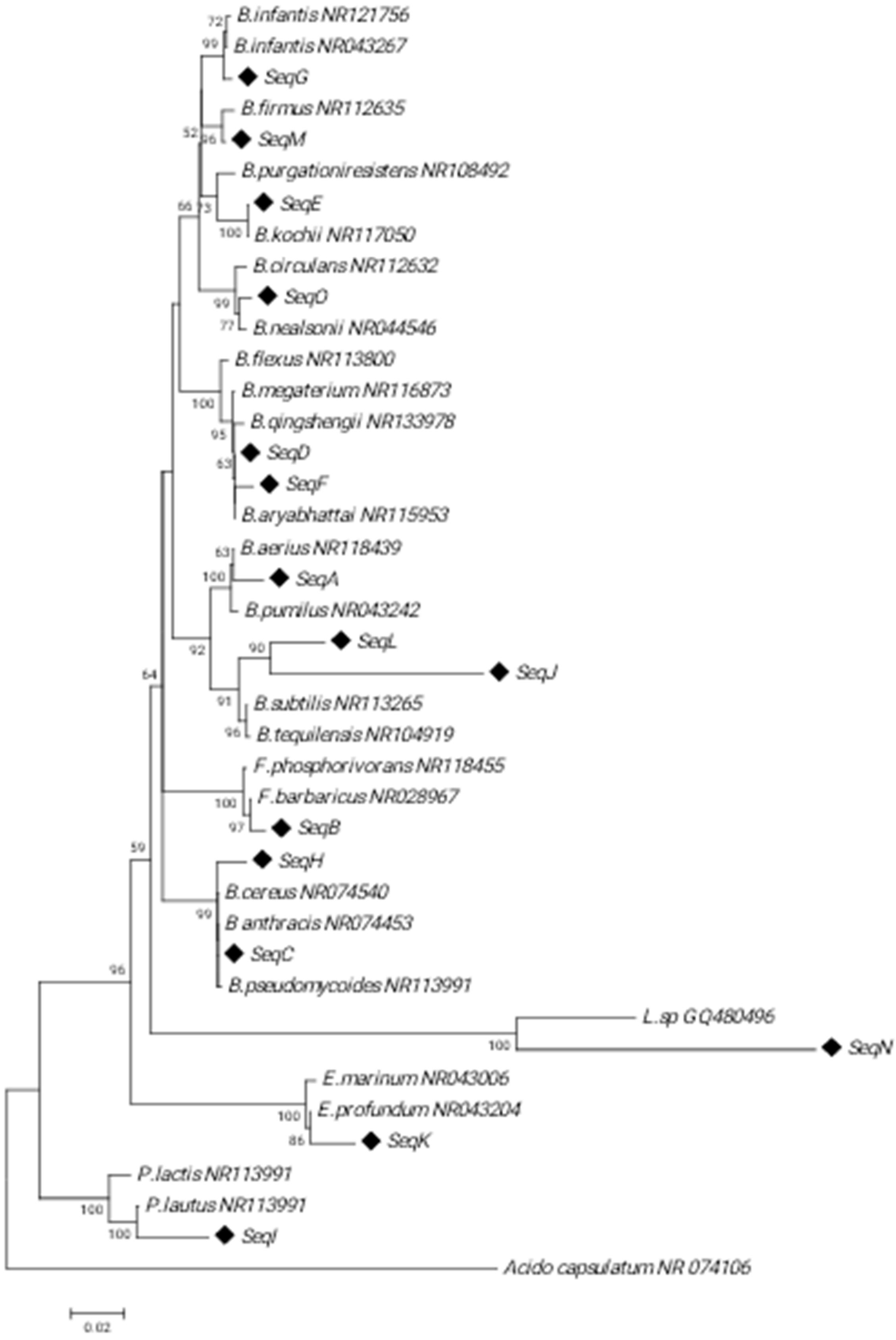
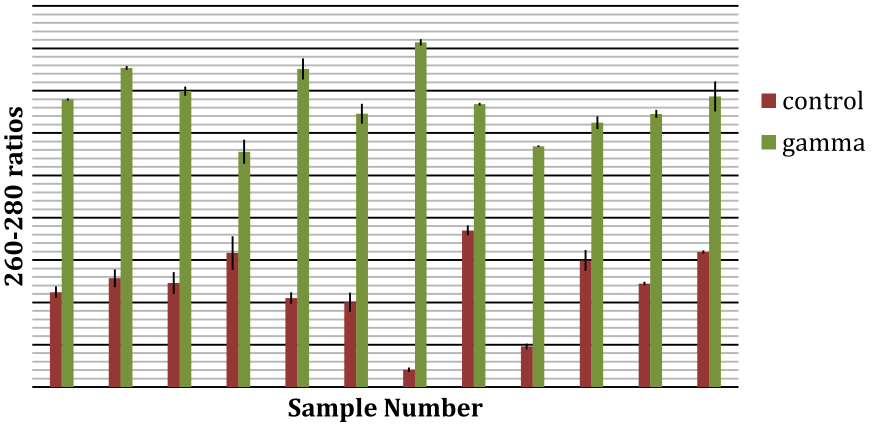


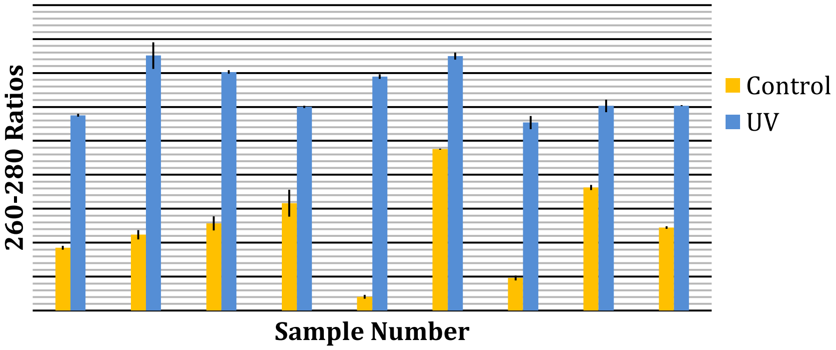
Fig 2: Phylogenetic tree showing the evolutionary relationships of bacteria isolated from soil

Analysis of Membrane integrity before and after irradiation

Both Gamma and UV radiations show significant effect on membrane integrity. Alteration in membrane integrity results in increased elution of cellular protein and nucleic acid content in to the surrounding medium. In order to find out the extent of change in membrane integrity after irradiation, 260-280 ratio of the surrounding buffer system was analyzed. This did show significant increases in the protein and nucleic acid content outside the cellular system indicating the leakage of cellular contents after radiation exposure



Graph 1: Showing 260-280 ratios before and after UV

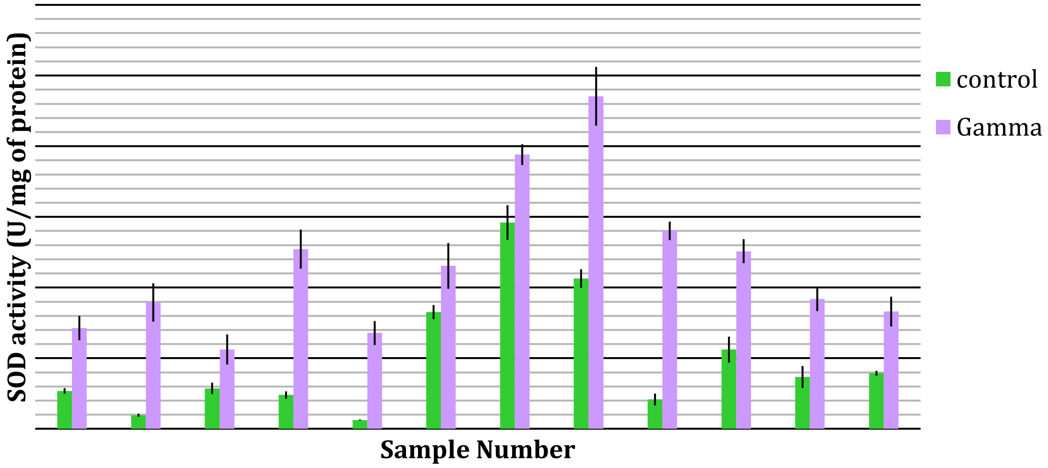


Graph 2: Showing 260-280 ratios before and after UV

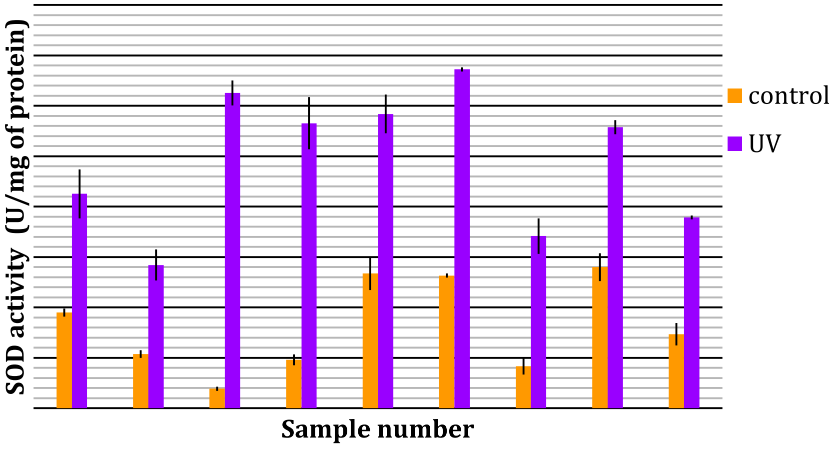
Analysis of antioxidant enzyme activity:

Both Gamma and UV radiations induce oxidative stress. This oxidative stress results in the generation of free radicals. Generation of free radicals is injurious to cell. There are many antioxidant enzymes which act as free radical scavengers. SOD is one such enzyme which prevents oxidative stress by scavenging Superoxide radicals. Oxidative stress induced by radiations also leads to the accumulation of H_2O_2 inside the cell. Catalase enzyme converts this H_2O_2 into H_2O and O_2 and thereby reduces its toxicity.

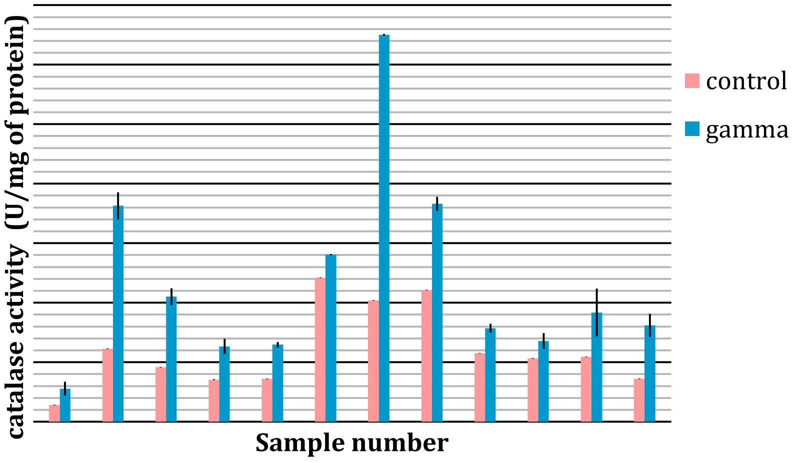
In order to find out the role of antioxidant enzymes in radiation resistance, difference in SOD and Catalase activities before and after irradiation were analyzed. A significant increase in SOD and catalase activities was observed after both Gamma and UV irradiation.



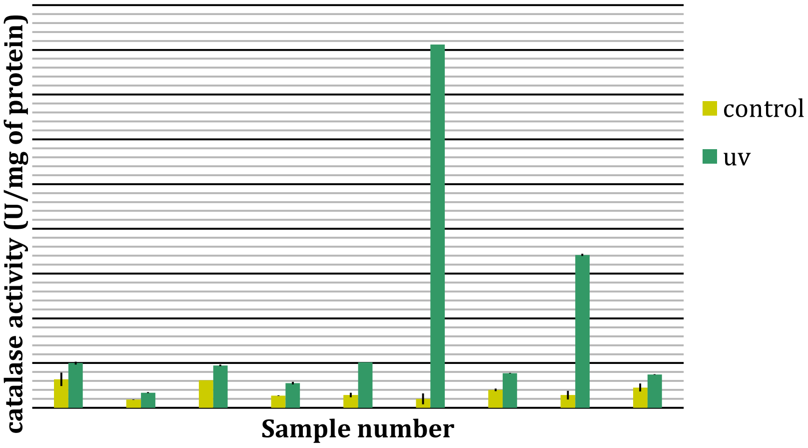
Graph 3: Showing SOD activity before and after UV



Graph 4: Showing SOD activity before and after UV



Graph 5: Showing Catalase activity before and after UV



Graph 6: Showing Catalase activity before and after UV

CONCLUSION:

18 different Bacterial samples were isolated from soil sample of Industrial area, Mangaluru,Karnataka. Out of 18 samples, 12 samples were found to be Gamma radiation resistant at dose of 3.5KGray and 9 samples were found to be UV tolerant at the dosage level of 6 hours.

All the 15 isolates of bacteria were identified then by using biochemical and 16S rRNA gene sequence analysis. Majority of the test isolates which we identified are rare and least studied for their biochemical and radiation tolerant properties.Out of 15 varieties of isolates, only 4 are already known for their radiation tolerance,the radiation resistant nature of the remaining isolates was revealed in the current study.

Soon after irradiation, all the resistant bacterial samples were tested for the antioxidant enzyme activities. The result obtained showed the significant increase in SOD and Catalase activities. Effect of radiation on bacterial membrane integrity was tested by 260-280 ratio analysis. Comparison of the results with that of the control samples showed significant difference.

Further understanding of the protective biochemical mechanisms employed by proteins responsible for UV resistance holds potential for their use in sun protection lotions.

The respective Gamma radiation resistant bacteria can be utilized for the Bioremediation of radioactive pollutants. The proteins which are responsible for Gamma resistant property in the bacteria can be harvested, and used as precautionary protectants for the workers working at radiation hazardous areas.

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