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BACTERIAL TOLERANCE TO HEAVY METALS UNDER THE INFLUENCE OF pH, TEMPERATURE AND SALINITY

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ABSTRACT

Microorganisms are ubiquitous in nature and are involved in almost all biological processes of life. Bioremediation of metal pollutants from industrial waste water using metal resistant bacteria is a very important aspect of environmental biotechnology. In the present study three bacterial isolates were isolated from BHEL effluent, Trichy.. A total of three bacteria isolates in the samples were identified by Morphological and Biochemical tests. The identified bacterial isolates were allowed to grow on acidic pH, salt and higher temperatures supplemented with copper metal. The statistical data showed that there was significant increase in the growth of bacterial isolates at different stress conditions. Among the three bacterial isolates Enterobacter asburiae and Pseudomonas aeruginosa were able to tolerate the different stress conditions. The results showed that the bacteria could able to grow in heavy metals and could able to grow in different stress conditions.

Key words: *Effluent, heavy metals, bacterial resistance, bioremediation.*

1. INTRODUCTION

Heavy metals from Industrial process are of special concern because they produce water for chronic poisoning in aquatic animals **(Ellis 1989).** Environmental pollution due to chemicals including heavy metals is a problem that may have negative consequences on the biosphere. In recent years, ground soil and other materials polluted with heavy metal have become a serious environmental problem throughout the world due to their use in many manufacturing processes, and up as waste in industrial effluent, through which heavy metals can enter water cycle, and then in the food chain where they are concentrated ultimately reaching the toxic levels (**Stillman *et al*., 2000).** Use of industrial waste water for irrigation is a common practice in most of the third world countries which could alter the fertility of soil (**Bouwer et al., 2002).**

Bacteria tolerance to heavy metals has been reported in both Gram positive and Gram negative bacteria (Foster, 1983). It is generally believed that Gram positive bacteria are less tolerant to heavy metal stress than Gram-negative bacteria. Some bacterial species like *Bacillus* spp may be resistant owing to their ability to sporulate, *Corynebacterium* sp have unique membrane lipids that can protect the cells from environmental stress (**Beverigde and Doyle, 1989).**

The mechanisms of resistance include metal reduction or transformation to more volatile or less toxic forms. Some bacteria including *Pseudomonas*, *E.coli* and *Clostridium* enzymatically reduce Hg^{2+} to Hg^{0} which is highly volatile and diffuses away from the bacterial cell. Others have specific metal efflux systems, which are the most commonly found mechanism of plasmid mediated metal resistance (**Silver, 1992**). Chelation and complexion of metal species with the media components and organism induced pH changes can also contribute to metal tolerance **.**

1.1 MATERIALS AND METHODS

The effluent sample was collected from the Bharat Heavy Electricals Limited (BHEL) Industry at Tiruchirappalli District, Tamil Nadu, India. The collected sample was transferred to a sterile plastic container and taken to the laboratory and maintained at 37^{0}C for further studies. The samples were further serially diluted and colonies were then sub cultured to isolate the pure culture colonies.

1.2 IDENTIFICATION OF BACTERIA PRESENT IN THE EFFLUENT

The isolated bacterium was subjected to identification by staining and biochemical testes. Gram staining procedure was carried out to identify the gram reaction of the organism. The biochemical test such as Indole, Methyl red, Voges proskauer, Citrate, Urease test, Starch hydrolysis, Catalase test, Mannitol fermentation etc.

The molecular techniques like 16srRNA sequencing were forther done to identify the bacteria at the species level. 16S rRNA gene sequences were assembled and edited using the **Big Dye Terminator version 3.1″Cycle sequencing kit**. The software **60** was used to assemble each forward and reverse sequence into a consensus sequence, which was edited to resolve base pair ambiguities between the two strands by evaluation of electropherograms. Then comparisons were made between the consensus sequence and the entries in the MicroSeq database. The sequencing was carried out at **ABI 3130 Genetic Analyzer**. The results were taken as concordant when the biochemical and the DNA sequencing result matched and discordant when they differed.

2. GROWTH STUDIES OF BACTERIA ISOLATED FROM EFFLUENT

Growth sutides of pseudomonas, enterobacter, and protues, were carried out in Nutrient broth medium

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supplemented with peptone and yeast extract etc. The different parameters like pH, salt and temperature were carried out to find the tolerance level of the bacterial isolates. 10 µl of culture were innoculated in to the nutrient broth medium supplemented with copper metal analysed for growth studies.

2.1 Effect of temperature on Pseudomonas sps, Enterobater sps , Bacillus sps , isolate.

1.10 µl of culture were innoculated in to the nutrient broth medium supplemented with copper at 3mmol/L, 5mmol/L and 6 mmol/L concentration. Pseudomonas sps, and Enterobacter sps, Proteus sps isolate was inoculated in to 50ml of nutrient broth and incubated at different temperature 37^0C, 41^0C, 45^0C for 24 hours, 48 hours 72 hours. The baterial growth was measured at 525 nm for 24, 48, 72 hrs.

2.2 Effect of pH on Pseudomonas sps, Enterobacter sps, Bacillus sps, Isolate

10 µl 0f culture where inoculated in to the nutrient both medium. supplemented with copper at 3mmol/L, 5mmol/L and 6 mmol/L concentration. The acidic pH of the medium were prepared using Hcl and NaoH .100 ul Pseudomonas sps Enterobacter sps Proteus sps isolate was incubated in to 50ml of nutrient broth .They were prepared at different pH values of 3, 3.4, 4 and incubated at 37^0C for the bacterial growth was measured at 525 nm for 24, 48, 72, 96 hrs.

2.3 Effect of salinity concentrations on Pseudomonas sps, Enterobacter sps, Bacillus sps isolate.

10 ul of culture were inoculated in to the nutrient broth medium supplemented with copper at 3mmol/L, 5mmol/L and 6 mmol/L concentration. Pseudomonas, Enterobacter, Proteus, isolate were inoculated in to 50ml of nutrient broth that prepared at different sodium chloride salt concentrations, of 0.2g, 0.6g, 1g, 4g, 8g, for the bacterial growth was measured at 24, 48, 72, 96 hrs.

3. RESULTS AND DISCUSSION

In this study we have isolated three bacterial isolates from the BHEL effluent. The bacterial isolates were characterizes based on the morphological and biochemical tests (Table-1). Sequence analysis of the 16S rRNA gene has been considered a fast and accurate method to identify the phylogenic position of bacteria. The 16S rDNA sequence analyses of bacterial isolates indicated that they belong to/or are closely related to *Enterobacter asburiae, Bacillus cereus*, *Pseudomonas aeruginosa* and *Alcaligenes faecalis* respectively (Fig. 1, Fig. 2, Fig. 3 and Fig. 4). The16S rDNA of the bacteria were then submitted in the Genbank (NCBI) with the accession number (Table-3). The three bacterial isolates were grown in a nutrient medium supplemented with copper metal at in the nutrient broth (Table-2).These bacteria also showed copper resistance in the pour plate methods. They showed growth at 3mM, 5mM, 6mM of $CuCl_2$

Table 1 shows the biochemical tests for the isolated bacterial strains from 3 sites

Tests	**For IJ1**	**For IJ2**	**For IJ3**
Gram Stain	G $^{-ve}$ rod	G $^{-ve}$ rod	G $^{+ve}$ rod
Motility			Positive
Indole	Negative	Negative	Negative
Methyl red	Negative	Negative	Negative
Vogesproskauer	Negative	Positive	Negative
Citrate	Positive	Positive	Negative
Urease	Positive	Negative	Negative
Starch	Negative	Negative	Positive
Oxidase	Positive	Negative	Negative
Catalase	Positive	Positive	Positive
EMB Agar	Negative	Negative	Negative
Mannitol SaltAgar	Negative	Negative	Negative
Blood Agar	Negative	Negative	Negative
Organism	Pseudomonas sp	Enterobacter sp	Bacillus sp

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Table-2 16s rRNA sequencing of organisms

S.No	Name of the Organism	Accession Number in Genbank
1	Bacillus cereus	KC920741
2	Enterobacter asburiae	KF984153
3	Pseudomonas aeruginosa	KF984154

Table-3 Growth of bacterial isolates supplemented with copper metal of 3mmol/L5mmol/L and 6mmol/L concentration

Psuedomonas sps with Copper metal (OD at 595 nm)					**Bacillus sps with Copper metal (OD at 595 nm)**					**Enterobacter sps with Copper metal (OD at 595 nm)**				
Hours	**Control**	**3mM**	**5mM**	**6mM**	**Hours**	**Control**	**3mM**	**5mM**	**6mM**	**Hours**	**Control**	**3mM**	**5mM**	**6mM**
0 Hour	0	0.01	0	0	0 Hour	0	0.01	0.01	0	0 hour	0	0.01	0	0
24 Hours	0.06	0.06	0.2	0.19	24 Hours	0.23	0.25	0.21	0.19	24 Hours	0.1	0.16	0.21	0.09
48 Hours	0.21	0.25	0.24	0.08	48 Hours	0.3 6	0.23	0.21	0.08	48 Hours	0.25	0.38	0.47	0.16
72 Hours	0.51	0.89	0.65	0.99	72 Hours	0.52	0.68	0.61	0.79	72 Hours	0.54	0.54	0.5	0.71
96 Hours	0.67	0.89	0.68	1.12	96 Hours	0.54	0.67	0.61	1.03	96 Hours	0.61	0.73	0.69	0.99

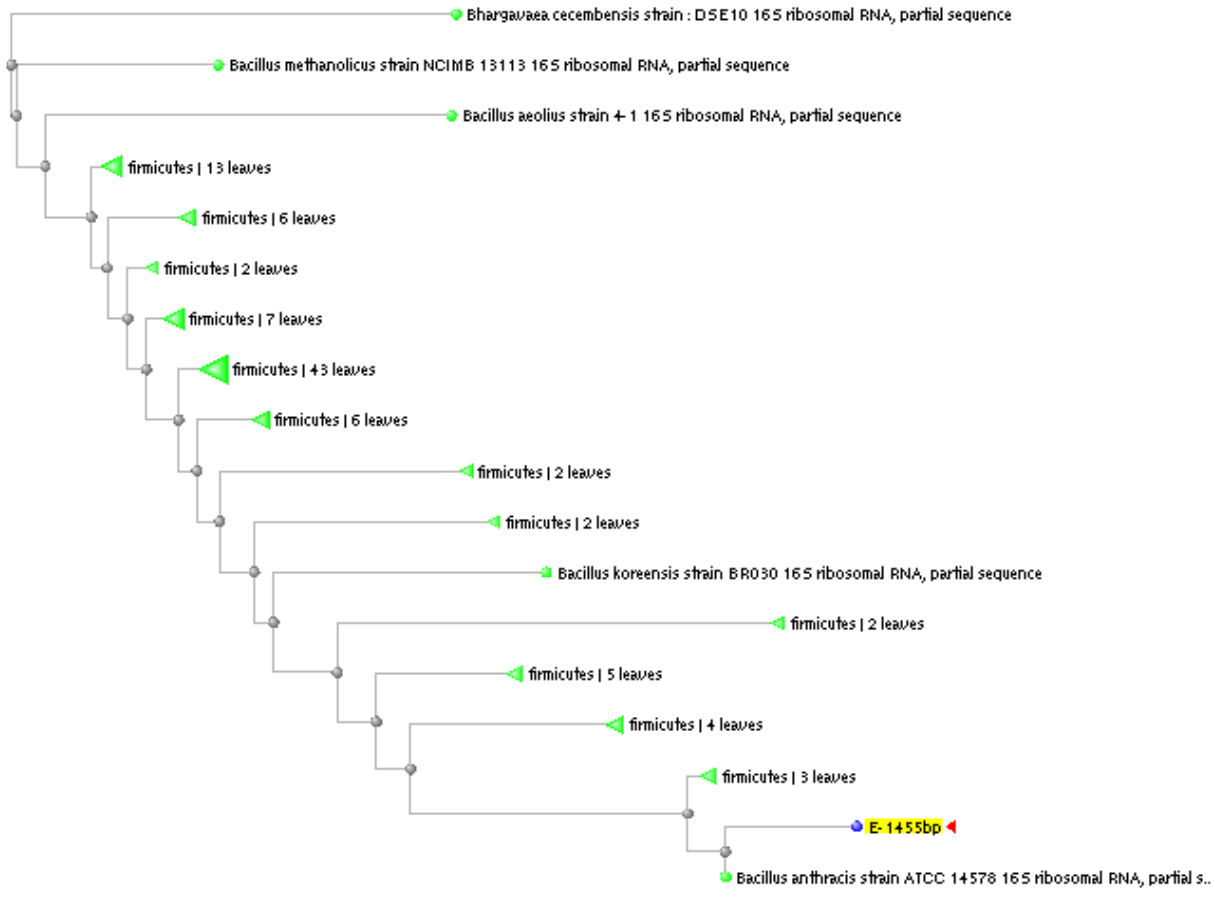

Figure 1: shows the Dendrogram of Bacillus cereus which shows 99% similarity

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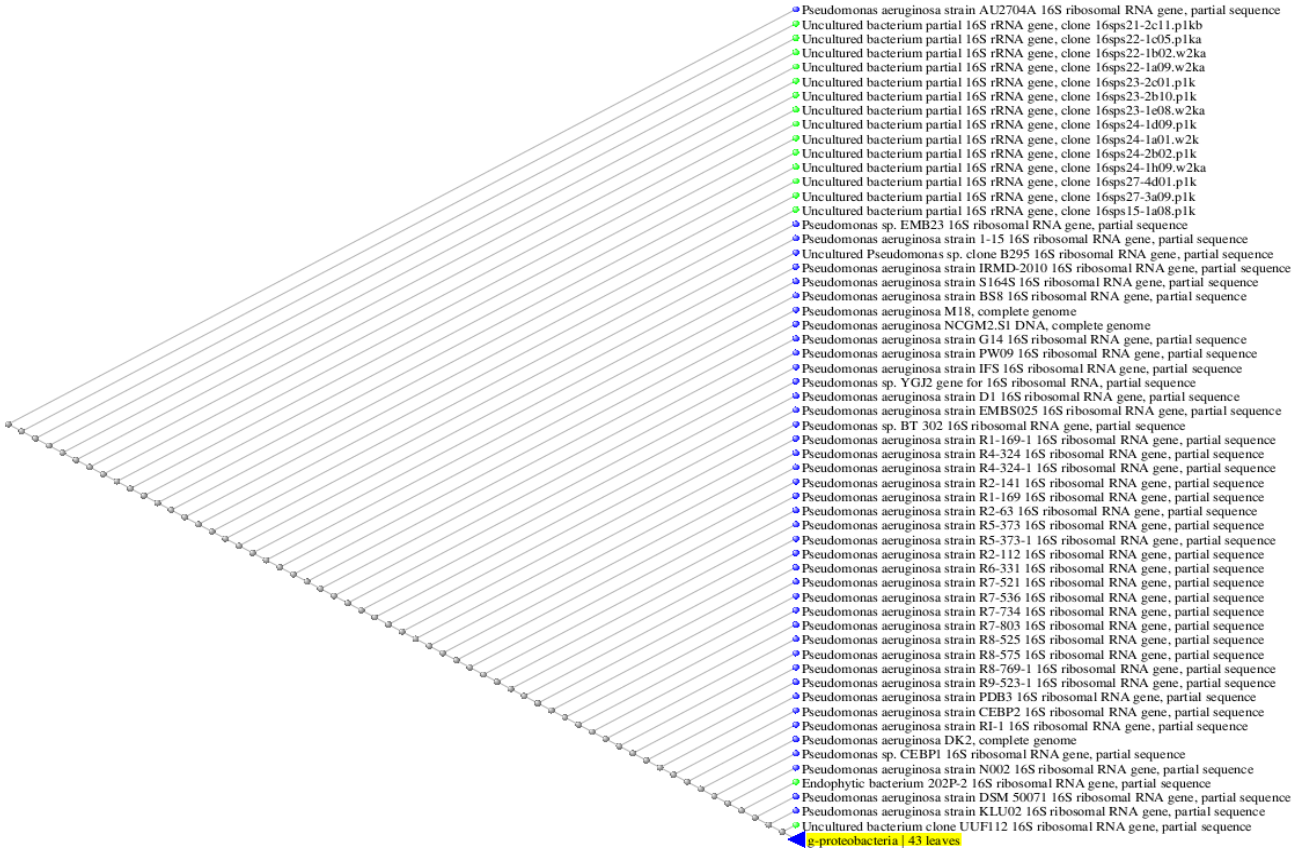

Figure 2: shows the Neighbor joining tree of Pseudomonas aeruginosa.

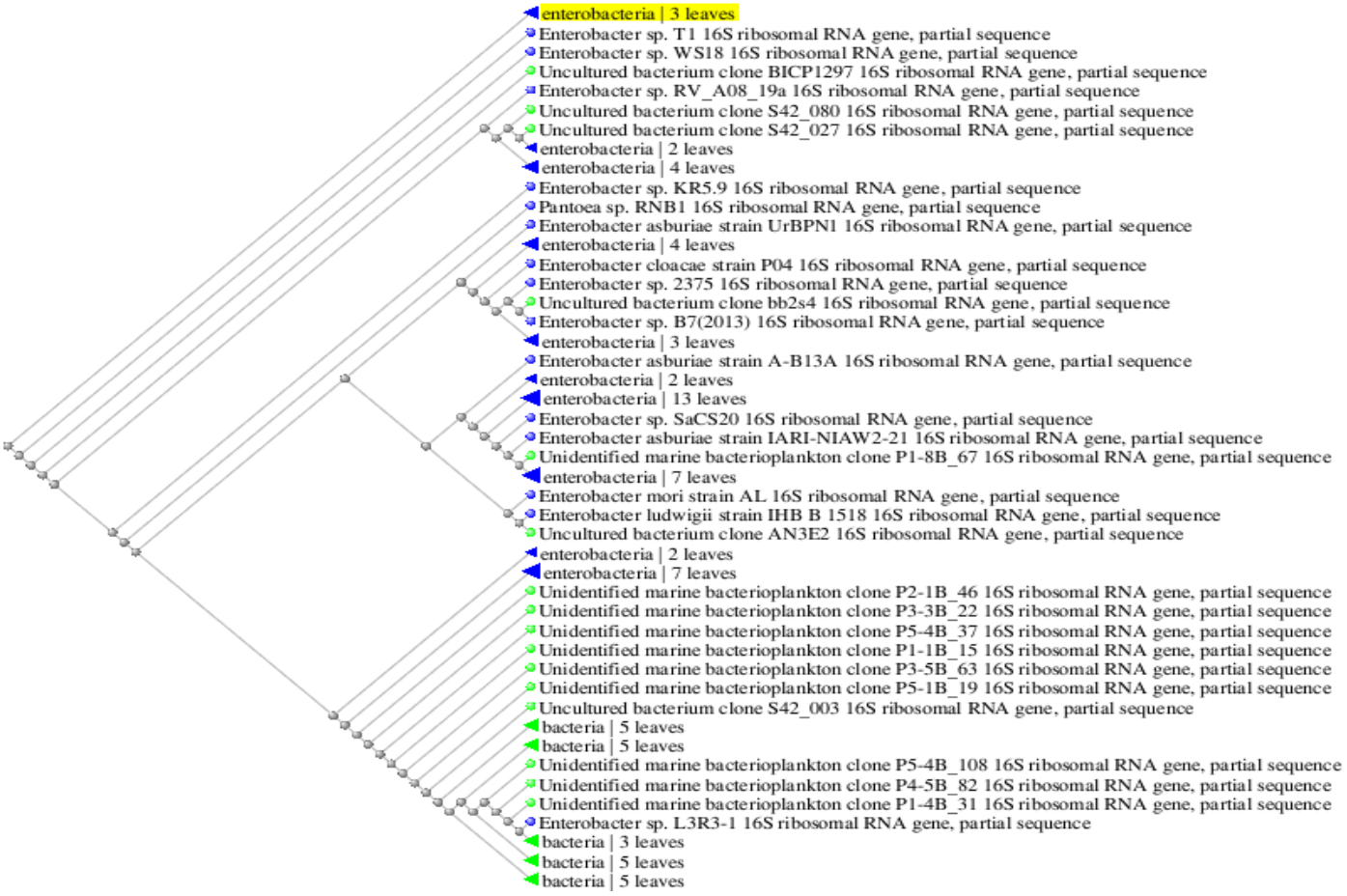

Figure 3: shows the Neighbor joining tree of Enterobacter asburiae

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Figure 4: Copper Resistance of Pseudomonas aeruginosa. The growth of Pseudomonas aeruginosa in 3 µm, 5 µm and 6 µm of Copper was observed

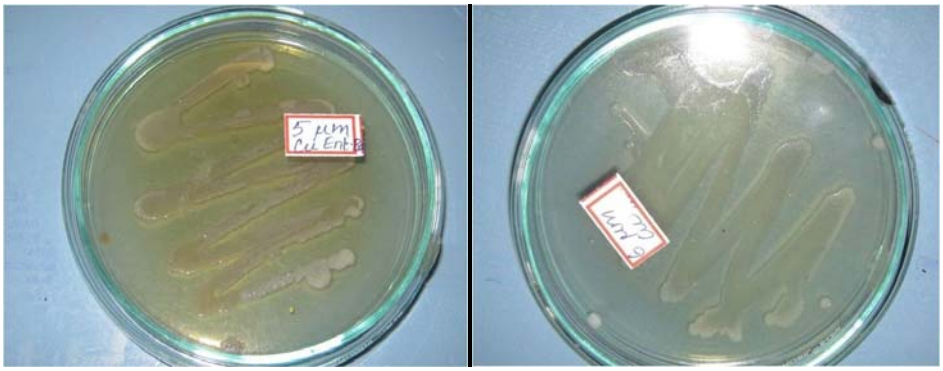

Figure 5: Copper Resistance of Enterobacter asburiae. The growth of Enterobacter asburiae in 3 µm, 5 µm and 6 µm of Copper was observed

Figure 6: Copper Resistance of Bacillus cereus. The growth of Bacillus cereus in 3 µm, 5 µm and 6 µm of Copper was observed

Table-4 Estimated resistance of Pseudomonas sp, Enterobacter sp and Bacillus sp to different pH

Organism	**Mean±Std.Deviation**
Pseudomonas	0.04 ± 0.0739
Enterobactor	0.05 ± 0.0090.01
Bacillus	0.0367 ± 0.011

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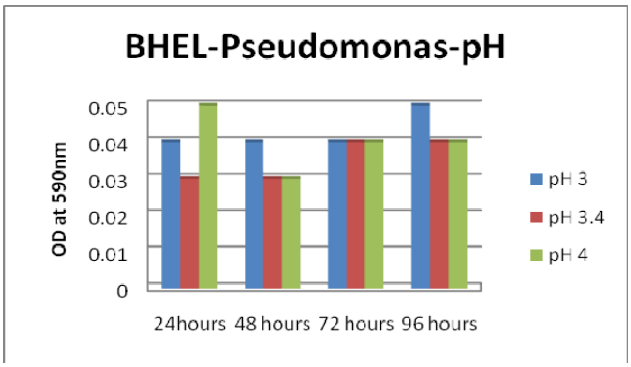
Table-5 Estimated resistance of Pseudomonas sp, Enterobacter sp and Bacillus sp to different salt concentrations.

Organism	**Mean±Std.Deviation**
Pseudomonas	0.20 ± 0.138
Enterobacter	0.19 ± 0.166
Bacillus	0.226 ± 0.23080

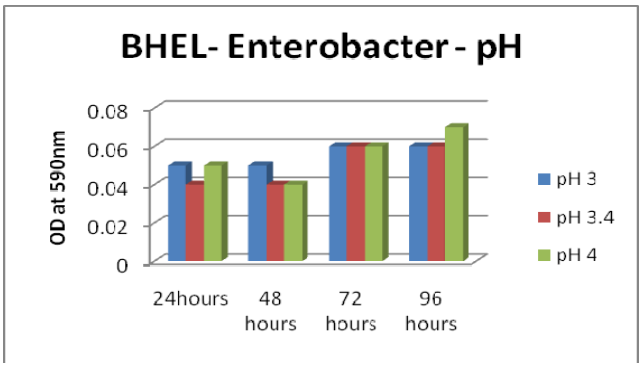
Table-6 Estimated resistance of Pseudomonas sp, Enterobacter sp and Bacillus sp to different temperatures.

Organism	**Mean±Std.Deviation**
Pseudomonas	0.38 ± 0.131
Enterobacter	0.482 ± 0.126
Bacillus	0.36 ± 0.1546

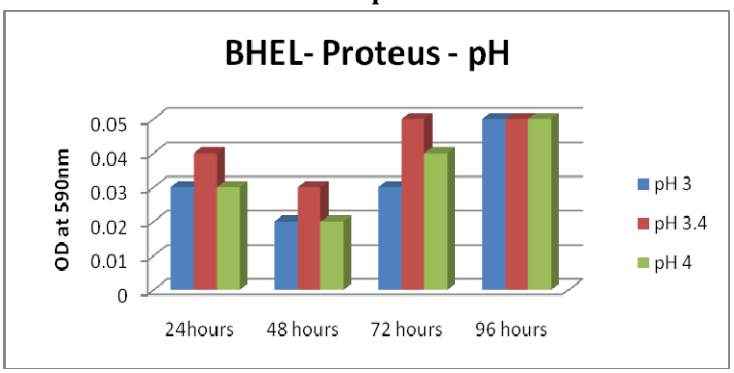
Graph-1



Graph-2

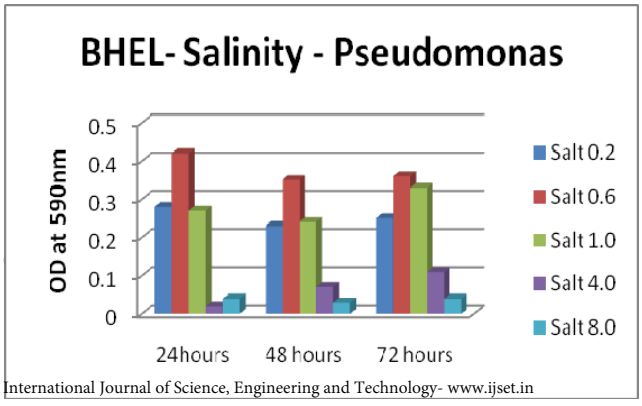


Graph-3

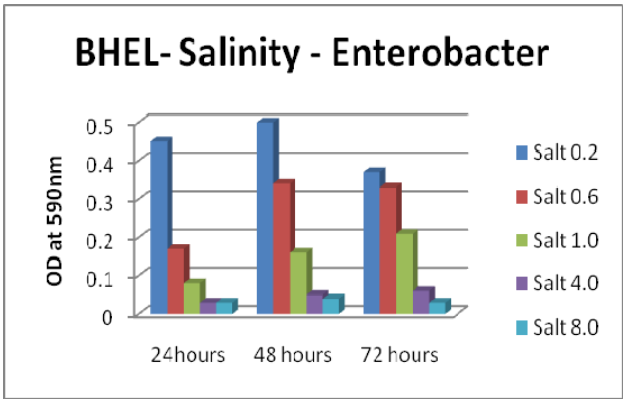


Graph-1, Graph-2 and Graph-3 shows the growth of Pseudomonas sp, Enterobacter sp and Proteus sp at different acidic conditions (pH 3, 3.4 and 4)

Graph-4



Graph-5



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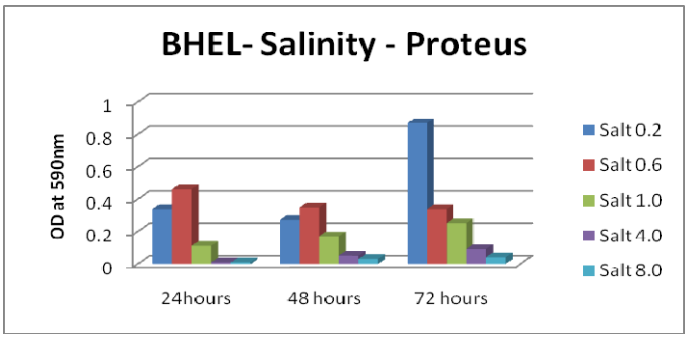
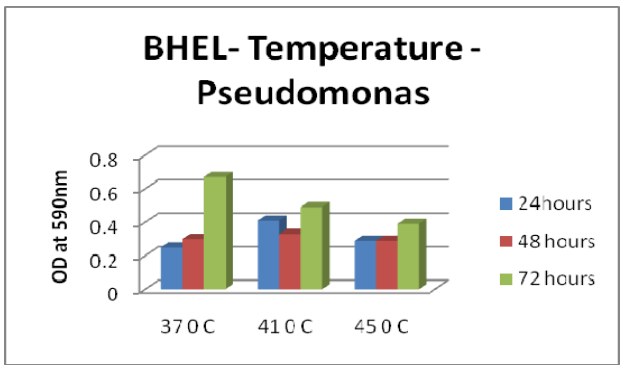
Graph- 6

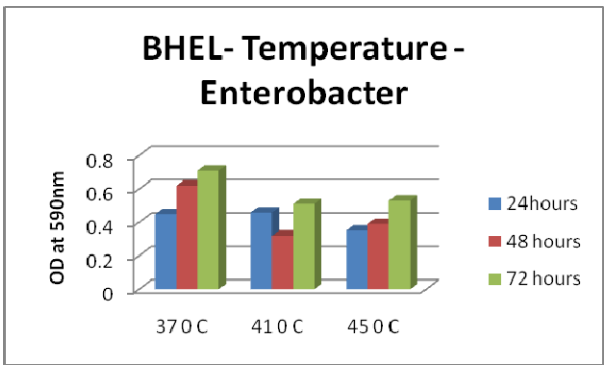


Graph-4, Graph-5 and Graph-6 shows the growth of Pseudomonas sp, Enterobacter sp and Proteus sp at different salt conditions (0.2-8.0gram/100ml)

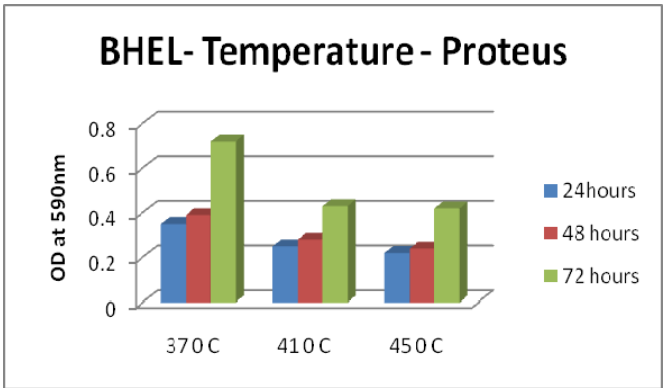
Graph-7



Graph-8



Graph-9



Graph-7, Graph-8 and Graph-9 shows the growth of Pseudomonas sp, Enterobacter sp and Proteus sp at different temperatures (37°c, 41°c and 45°c)

The three bacterial isolates were then subjected to different stress conditions (acidic pH , high temperature and high salinity) (Table-4,5,6). These bacterial isolates among the three bacterial **Enterobacter asburiae> Pseudomons aeruginosa>Bacillus cereus** showed tolerance to heavy metals and the growth of the bacteria was observed under stress conditions. The heavy metal resistant genes identified in these bacteria could be cloned into a suitable vector. These bacteria can be further applied for metal degradation process. *Pseudomonas aeruginosa*, *Enterobacter asb*uriae and *Bacillus cereus* were found to be highly resistant to heavy metals and they can be further used to clean up the heavy metal polluted environment

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