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Molecular identification and phylogenetic analysis of chemical resistant bacteria isolated from laboratory effluent

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Abstract - Antibiotics, disinfectants, drugs and even chemical resistant bacteria have been isolated from many sources like waste water, industrial effluents, contaminated soil, laboratory effluent etc. Chemicals are released directly to the environment from laboratories, pharmaceuticals and many industries. This may lead to many environment and health problems. The organisms grow in these effluents will be resistant to those chemicals which are present in it. In one hand, these organisms may cause harm to the environment, on the other hand, we can use these chemical resistant organisms for many purposes like bioremediation and fermentative production of variety of products. As they are resistant to chemicals, the by-products formed in bioremediation and fermentation may not affect the growth of these organisms. In this study some bacterial strains were isolated, which are resistant to chemicals in the laboratory effluent. Their molecular identification by using 16S rDNA sequencing and phylogenetic analysis were also done. It is difficult to have a detailed study on the resistance of organisms on all the chemicals in the effluent, therefore those chemicals which are easily available and common in laboratories are chosen such as hydrogen peroxide, phenol, hydrochloric acid, sodium hydroxide and acetone. The bacterial strains which are resistant to these chemicals are then undergone 16s rDNA Sequencing, molecular identification and phylogenetic analysis.

Key words: Bioremediation, Fermentation, BLAST, Phylogenetic analysis, PHYLIP, TreeView

1. INTRODUCTION

Microbes are having a wide range of applications in industries for the production of many types of pharmaceutical and medical compounds (e.g., antibiotics, hormones, transformed steroids, vaccines etc), solvents, organic acids, chemical feed stocks, amino acids, and enzymes that have high economic value. The microorganisms employed by industry have been isolated from nature, and in many cases, they were modified using classic mutation-selection procedures [1].

Resistance in microorganisms may be a boon or a curse. In this study, chemical resistant bacteria are isolated from laboratory effluent collected from M.E.S College, Marampally. The ability of resistance achieved by these bacteria can be explored for many useful processes which are among the important processes in this current world. Chemicals may be produced as a by-product in many processes and this will decrease the activity of microbes used in the process and thus the yield will be minimized. Application of chemical resistant microbial strains in such situation can overcome this problem.

Bioremediation is a method of degrading waste materials by using microorganisms. The failure in bioremediation of waste materials is due to the lack or decrease in number of microorganisms at the site. In this situation biostimulation or bioaugmentation method can be used. There is a chance to inhibit the activity of microbes on the waste materials by the presence of toxic chemicals in the environment. To overcome this problem, chemical resistant microbes can be used.

The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker used for a number of reasons. These reasons include: (i) its presence in almost all bacteria, (ii) the function of the 16S rRNA gene over time has not changed (iii) the 16S rRNA gene (1500bp) is large enough for informatics purpose [2]. One of the most attractive potential uses of 16S RNA gene sequence informatics is to provide genus and species identification for isolates that do not fit any recognized biochemical profiles, for strains generating only a "less likelihood" or "acceptance" identifications according to commercial systems, or for taxa that are rarely associated with human infectious diseases.

In bioinformatics, BLAST is an algorithm for comparing primary biological sequence information, such as the amino-acid sequences of different proteins or the nucleotides of DNA sequences. A BLAST search enables a researcher to compare a query sequence with a library or database of sequences, and identify library sequences that resemble the query sequence above a certain threshold. Blast is also providing space for the submission of our sequences [3].

In phylogenetic studies, the evolutionary relationships among a group of organisms are illustrated using a phylogenetic tree, a graph composed of nodes and branches, in which only one branch connect any two adjacent nodules. The nodes represent taxonomic units and the branches define relationship among the unit in terms of descent and

ancestry. The branching patter of a tree is called topology. The branch length usually represents the number of changes that have occurred in the branch. The taxonomic units represented by the nodes can be species, population, individual or genes. Clades in phylogenetic trees are monophyletic taxon. Clades can also be defined as group of organisms or genes that includes the most recent common ancestor. A taxon is any named group of organisms but not necessary a clade. In some analyses, branch lengths correspond to divergence. A node is a bifurcating branch point. Simply, a phylogenetic tree is specific type of cladogram where the branch lengths are proportional to the predicted or hypothetical evolutionary time between organisms or sequences [4].

2. MATERIALS AND METHODS:

2.1 Sample Collection

The effluent sample was collected from Bioscience laboratory, M.E.S College, Marampally, Aluva, Kerala. The sample was aseptically collected in an air tight polythene bag, from where the laboratory effluent is disposed.

2.2 Isolation of bacteria from soil sample

A 100 ml of nutrient agar media was prepared and autoclaved along with seven test tubes containing 9 ml distilled water and Petri dishes. The sterile media was transferred to Petri dishes in aseptic condition and left for solidification. The sterilized tubes were labelled as 10^{-1} ,10^{-2} ,10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} respectively. 1 g of the soil sample was transferred into 9 ml of sterile distilled water in 10^{-1} labelled test tube. 1 ml of sample from the 10^{-1} dilution was then serially diluted to other tubes. Spread plate technique was used to isolate the colonies from serially diluted samples. The grown colonies were picked from plates which are not TNTC or TFTC. The colonies were subcultured in nutrient agar slants and maintained at 4^{o}C for further studies [5].

2.3 Testing of chemical resistance of isolated bacteria

2.3.1 Chemicals used

Effect of various chemicals on the growth of bacteria was evaluated by growth the bacteria in peptone water along with selected chemicals for 12hrs at room temperature on a shaker under vigorous shaking. The chemicals studied included:

i. Hydrogen peroxide 2%
ii. Sodium hydroxide 2%
iii. Hydrochloric acid 2%
iv. Phenol 2%
v. Acetone 2%

2.3.2 Procedure

Into sterile test tube, 5ml peptone water was transferred and chemicals were weighed and added to these tubes to prepare 2% concentration. The stains were inoculated in to the prepared media and incubated for 12-14 hrs at room temperature on a shaker under vigorous shaking. The turbidity of the medium was inferred as growth of the organism [6].

2.3.3 Confirmation of chemical resistance

Into sterile test tube, 5ml sterile distilled water was transferred and chemicals were weighed and added to these tubes to prepare 2% concentration. Th isolated strains which show resistance to all the chemicals were inoculated in to the test tubes and incubated for 12-14 hrs at room temperature on a shaker under vigorous shaking. The turbidity of the sterile distilled water with chemicals was inferred as growth of the organism.

2.4 Extraction of nucleic acid

DNA extraction was performed employing Phenol-chloroform method [7] and agarose gel electrophoresis is performed [8].

2.5 Polymerase chain reaction (PCR)

PCR was performed using the genomic DNA (100 ng/ml) as template and 16s rDNA specific primer. The sequence of 16s rRNA gene was separated by PCR using a thermal cycler and automated sequencing. Finally, PCR product was undergone agarose gel electrophoresis.

Table – 1: Primer sequence

Primer	Sequence (5'-3')	Reference
16SF	AGTTTGATCCTGGCTCA	[9]
16SR	ACGGCTACCTTGTTACGACTT	[10]

2.6 Molecular identification of bacterial strains

The sequence of 16S rRNA gene was separated by PCR and automated sequencing. For the identification, the sequence was submitted in the nucleotide BLAST program in FASTA format.

Using a heuristic method, BLAST finds homologous sequences, not by comparing either sequence in its entirety, but rather by locating short matches between the two sequences. After making the words for the sequence of interest, neighbourhood words are also assembled. These words must satisfy a requirement of having a score of at least the threshold, T, when compared by using a scoring matrix. Once both words and neighbourhood words are assembled and complied, they are compared to the

sequences in the database in order to find matches. Note that increasing the T

score limits the amount of space available to search, decreasing the number of neighbourhood words, while at the same time speeding up the process of BLAST. [11]

2.7 Phylogenetic analysis

16S rRNA gene sequences of different bacteria were collected from NCBI databases. Retrieved sequences of 16S rRNA genes were aligned by CLUSTALX multiple sequence alignment program. The resulting file was edited and saved as PHYLIP format. This file is then used for running PHYLIP programs. PHYLIP program is a series of programs which finally gives the result in a tree form but it cannot be directly visualized. The final tree form file will be able to view using TreeView software. Some of the PHYLIP programs which we used in construction of phylogenetic tree: SEQBOOT will generate random samples, DNADIST, which will calculate the DNA distance matrix, NEIGHBOR, will transform matrix in to a tree, CONSENSUS, draws consensus trees from multiple trees and finally the out tree from CONSENSUS will be imported to TreeView, a tree drawing program which can be accessed as free program same as of PHYLIP.

RESULT AND DISCUSSION:

2.8 Isolation of bacteria from soil sample

In this study 12 chemical resistant bacteria were successfully isolated and they were named as MES KR 1 to MES KR 12.

2.9 Testing of chemical resistance of isolated bacteria

Isolates	Chemicals (2%)				
	H_2O_2	NaOH	HCL	Phenol	Acetone
MES KR 1	R	S	S	S	R
MES KR 2	S	S	S	S	S
MES KR 3	S	S	S	S	S
MES KR 4	R	R	R	R	R
MES KR 5	R	S	S	S	R
MES KR 6	S	S	S	S	S
MES KR 7	R	R	R	S	R
MES KR 8	S	S	S	S	S
MES KR 9	R	R	R	R	R
MES KR 10	S	S	S	S	S
MES KR 11	S	S	S	S	S
MES KR 12	R	S	R	S	R

Two isolated bacteria showed resistance to all the five chemicals after incubation for 12-14hrs at room temperature.

Table – 2: Resistance

*R – Resistant S- Sensitive

2.10 Confirmation of chemical resistance

Confirmation test is done only to those who showed resistance to all the chemicals i.e. MES KR 4 and MES KR 9. Same results were shown and let's confirm ability to degrade and utilize the chemicals in their metabolism.

2.11Extraction of nucleic acid and PCR

The genomic DNA of those bacteria which shows resistance were extracted and 16S rDNA were successfully isolated with the help of universal primers.

2.12Molecular identification of bacteria

The sequence were submitted in to nuclieotide BLAST and which optimized for megaBLAST and BLASTn separately. The sequences were identified as:

MES KR 1 *: Stenotrophomonas maltophilia*

MES KR 4 : *Bacillus subtilis*

MES KR 9 : *Bacillus megaterium*

MES KR 12 : *Brevibacillus agri*

2.13Phylogenetic analysis

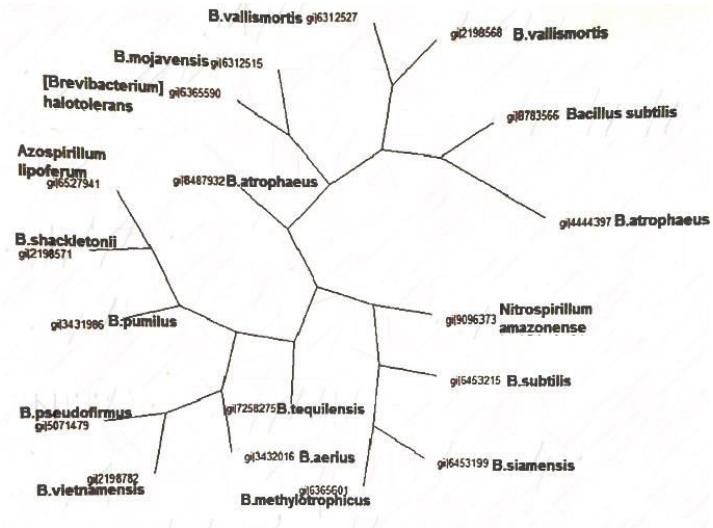

Fig – 1 : MES KR 4 : *Bacillus subtilis*

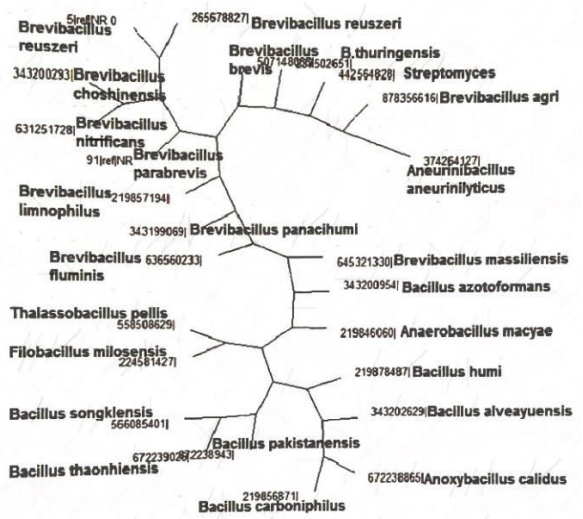

Fig – 2: MES KR 12 : *Brevibacillus agri*

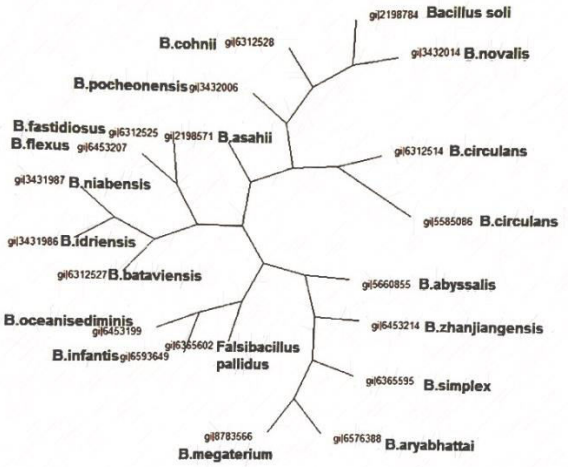

Fig – 3: MES KR 9 : *Bacillus megaterium*

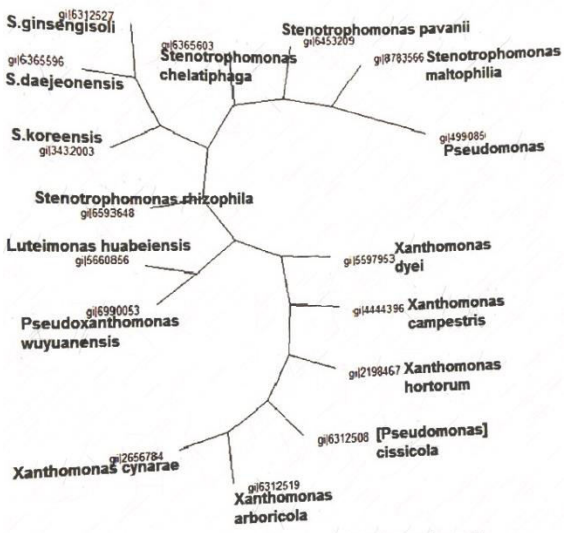

Fig – 4: MES KR 1 *: Stenotrophomonas maltophilia*

3. CONCLUSION:

The bacterial cultures MES KR 4 and MES KR 9 shows resistance to all the five chemicals. They were identified as Bacillus subtilis and Bacillus megaterium.

In many countries, genetically modified organisms are used in bioremediation. By using chemical resistant bacteria at the site of pollution, the chance of their survival will be high and as a result, the rate of degradation can be raised. In the other way, these chemical resistant bacteria are useful in fermentation. Currently, most of the pharmaceutical products and other products are produced by the means of fermentation technique. In many of these fermentation processes, chemicals may be produced as a by-product. There is a chance of inhibition of organism/ activity of organism which enhance fermentation, by those chemical by-products. This problem can be avoided by using chemical resistant bacteria in such fermentation processes.

For many years, sequencing of the 16S DNA has served as an important tool for determining phylogenetic relationships between bacteria. The features of this molecular target that make it a useful phylogenetic tool also make it useful for bacterial detection and identification in the clinical laboratory. Sequence analysis of the 16S rRNA gene is a powerful mechanism for identifying new pathogens in patients with suspected bacterial disease, and more recently this technology is being applied in the clinical laboratory for routine identification of bacterial isolates.

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