

Molecular Characterization of Biodegrading Bacteria from Soil Sample

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ABSTRACT

In the present study, a total of four species of biodegrading bacteria namely *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas putida* were isolated from soil samples collected from the sites where lead and mercury were discharged. 16s RNA is amplified using Polymerase Chain Reaction and the amplified product was confirmed by agarose gel electrophoresis and it has been subjected to sequencing and the sequence obtained was compared with the sequence obtained from the nucleotide database of NCBI

Key words: Biodegradation, Bacteria, Soil Sample.

INTRODUCTION

Biodegradation technology uses microorganisms to reduce, eliminate or transform the benign contaminants and their products which are present in soil, sediments, water and air. Evidence of kitchen middens (ancient household garbage dumps) and compost piles dates back to 6000 B.C. demonstrating that some form of biodegradation and bioremediation was practiced by humans since the beginning of recorded history. Biodegradation was used over 100 years ago with the opening of the first biological sewage treatment plant in Sussex, UK, in 1891 however, the word "Biodegradation" is fairly new; first appeared in a peer-reviewed scientific literature in 1987.

Biodegradation can be a cost efficient and reliable method for removing hazardous waste from heavily contaminated sites with organic compounds. For the last decade researchers have also discovered that biodegradation technology can be used at sites contaminated with solvents, explosives, polycyclic aromatic hydrocarbons (PAHs), and polychlorinated biphenyls (PCBs). More recently, researchers have discovered that microbial

processes can be used to cleanup radioactive and metallic compounds.

Staphylococcus, *Bacillus*, *Pseudomonas*, *Citrobactera*, *Klebsilla*, and *Rhodococcus* are organisms that are commonly used in biodegradation and bioremediation mechanisms. (Al-Maghrabi *et al.*, 1998). These mechanisms include bioaugmentation, in which microbes and nutrients are added to the contaminated site, and biostimulation, in which nutrients and enzymes are added to supplement the intrinsic microbes of the site. For example *Alcaligenes* and *Pseudomonas* have been used in the biodegradation of chromium (Barkay and Colwell, 1983). Likewise, there are records that organisms like *Escherichia* and *Pseudomonas* are used in the biodegradation of copper (Barkay and Cowell, 1983). Currently, more research is being performed on the use of microbes to degrade metals. It is anticipated that government as well as private industries will invest heavily to clean up polluted environments. This investment validates the need to research the utilization of microbial processes to clean up contaminated sites. In the present paper efforts have been made to isolate and identify the biodegrading bacteria from soil.

MATERIAL AND METHODS

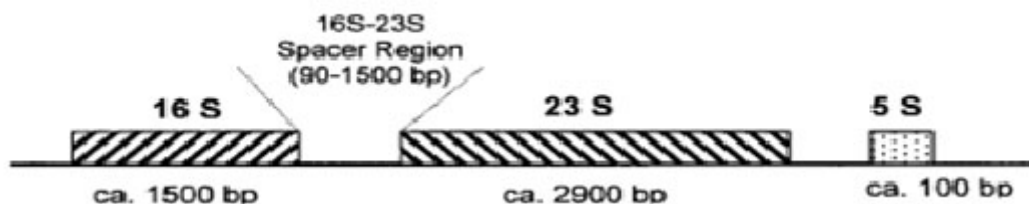
Identification of the Isolated Bacteria by Molecular Methods

The most powerful tool to identify the unknown bacteria is to sequence the DNA coding for 16s rRNA, since the 16s rRNA is encoded by the gene in the chromosome of the bacteria. So the gene coding for the 16s rRNA is amplified using the Polymerase Chain Reaction and the amplified product was confirmed by agarose gel electrophoresis and it has been subjected to sequencing and the sequence obtained has been compared with the sequence obtained from the Nucleotide Database of National Center For Biotechnology Information (NCBI)..

Nucleic Acid Sequencing

Genome structures can be directly compared by sequencing DNA and RNA. Since the

the 16s rRNA is the most conserved (least variable) gene in all cells. The ribosomes that are the work benches of the protein synthesis present in bacteria are of 70s ribosomes composed of two subunits 30s is the smaller subunit and 50s is the larger subunit. These two subunits bind only during the protein synthesis. The small ribosomal subunit (30s) contains the 16S rRNA (s in 16S represents Svedberg units). The large ribosomal subunit contains two rRNA species (the 5S and 23S rRNAs). Bacterial 16S, 23S, and 5S rRNA genes are typically organized as a co-transcribed operon. There may be one or more copies of the operon dispersed in the genome. To infer relationships that span the diversity of known life, it is necessary to look at genes conserved through the billions of years of evolutionary divergence. An example of genes in this category is those that define the ribosomal RNAs (rRNAs). Most prokaryotes have three rRNAs, called the 5S, 16S and 23S rRNA.



Ribosomal RNAs in Prokaryotes

5S located in the large subunit of ribosome (50s) contains around 120 nucleotides, 16S located in the small subunit of ribosome (30s) contains around 1500 nucleotides and 23S located in the large subunit of ribosome contains around 2900 nucleotides.

The 5S has been extensively studied, but it is usually too small for reliable phylogenetic inference. The 16S and 23S rRNAs are sufficiently large to be useful. The 16s rDNA sequence has hyper variable regions, where sequences have diverged over evolutionary time. These are often flanked by strongly-conserved regions. Primers are designed to bind to conserved regions and amplify variable regions. The DNA sequence of the 16S rDNA gene has been determined for an extremely large number of species. In fact, there is no other gene that has been as well characterized in as many

species. Sequences from tens of thousands of clinical and environmental isolates are available over the internet through the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) and the Ribosomal Database Project (www.cme.msu.edu/RDP/html/index.html). These sites also provide search algorithms to compare new sequences to their database.

Sequencing of the 16s rRNA Sequence

One of the most powerful approaches to identify the unknown bacteria is the study of nucleic acids as it is the genes that are specific for specified protein. Comparisons of nucleic acids yield considerable information about true relatedness. These more recent molecular approaches have become increasingly important in prokaryotic taxonomy. Hence sequencing of the DNA coding for 16s rRNA can enable the identification of bacteria.

Isolation of DNA from Lead and Mercury Degrading Isolates

The DNA from Mercury and Lead degrading isolates were isolated as per the protocol:

- 1.5ml of overnight culture of each isolate was taken into different 1.5ml centrifuge tubes and centrifuged at 8000rpm for 5min.
- Supernatant was discarded and briefly vortex pellet to dislodge.
- 400µl of Lysis buffer [1.5M NaCl, 10mM Tris-HCl (pH8.0), 5% SDS] was added to the dislodge pellet and incubated in water bath for 15min at 65°C.
- The solution was cooled to room temperature and 130µl of potassium acetate (pH5.2) was added and mixed. The solution was incubated for 5 minutes in room temperature.
- The sample was centrifuged at 10000rpm for 10min. Clear supernatant was transferred into fresh 1.5ml tube. Equal volume of isopropanol was added, gently inverted for 5 times and incubated 10min in room temperature.
- The sample was centrifuged at 10000rpm for 10min. Supernatant was discarded and pellet is washed with 75% ethanol.
- The pellet was dried in room temperature for another 15 minutes. Pellet was dissolved in 100µl of TE buffer. To remove RNA from the preparation, 10µl of RNase A (10mg/ml) was added and incubated at 37°C for 1 hour.

Purification of Isolates' DNA

Solutions

RNase A

RNase A of 10 mg/ml was dissolved in 10 mM Tris-HCl (pH 7.5), 15 mM NaCl. The contents were heated at 100°C for 15 minutes and allowed to cool slowly at room temperature. Dispensed into aliquot and stored at -20°C.

Procedure

5 µl/ml RNase solution (the amount of RNase depends upon the RNA contamination) was added to the crude DNA and incubated at 37°C for 45 minutes.

To the above, 1 ml of TE saturated phenol was added, mixed the contents thoroughly and then centrifuged at 15,000 x g at 4°C.

The upper aqueous phase was transferred

to a fresh tube and added with equal volume of a mixture of phenol: chloroform: isoamylalcohol (25:24:1) to the solution and mixed thoroughly without vortexing.

The contents were centrifuged at 15,000 x g at 4°C for 5 minutes and transferred the upper aqueous phase to a fresh tube.

And to the contents, an equal volume of Chloroform: Isoamylalcohol (24:1) was added and centrifuge at 15,000 x g at 4°C for minutes. This step is repeated until no precipitate is seen.

To the 1/10th volume (of the aqueous phase) of 3M Sodium acetate (pH 5.2) was added, mixed the contents it and then added with twice its volume of chilled absolute ethanol. Mixed the contents thoroughly by inverting the tubes and incubate at -20°C for one hour to get precipitate of the DNA.

The contents were centrifuged at 15,000 x g for 5 minutes at 4°C. The DNA was collected discarding the supernatant and the pellet was air dried to remove ethanol and dissolved in appropriate volume of TE buffer.

Quantification of DNA

In this procedure, the DNA was diluted in water and absorbance was measured at 230, 260, 280 and 300 nm. From this method an approximate idea about the amount of protein contamination can be known but the drawback is that if there is RNA or small nucleic acid contamination, quantification of DNA would be wrong since absorption maxima of all nucleic acids are at 260 nm. This method requires microgram amounts of DNA to ensure reliable readings.

Calculating the purity and yield of DNA

One absorbance unit at 260 nm of the double stranded DNA is equal to 50 µg/ml of the double stranded DNA and one absorbance unit at 260 nm of the single stranded DNA is equal to 40 µg/ml of the single stranded DNA.

Total A_{260} Units = (A_{260}) v dilution factor

Concentration (ig/ml) = total A_{260} units × (50 µg/ml)

Yeild (µg) = Volume × Concentration

Pure DNA will exhibit an absorbance ratio (A_{260}/A_{280}) of 1.8 to 2.0.

If the DNA exhibit an absorbance ratio ($A_{260}/$

A_{280}) of less than 1.7, the sample is contaminated by protein.

Procedure

The isolated DNA was amplified the region of the 16S rDNA gene of the isolated bacteria using Polymerase Chain Reaction (PCR). The amplified DNA was subjected to 1.5% of Agarose gel electrophoresis and basing on the size of the amplified fragment rDNA gene was confirmed. The DNA band of the amplified product was cut from gel, eluted and subjected for sequencing. The sequence so obtained was compared with the reported results with the public databases (NCBI) and the sequence of the unknown bacteria was determined.

The objective of this rDNA sequencing is to determine a new bacterial strain has been isolated basing on its taxonomy: whether it belongs to a known genus, and if not, to identify the new species within known genera and also to determine (as much as possible) if it is a new species or a new strain of an already known species.

Amplification of the 16s rRNA Gene of the Bacterial Chromosome

The polymerase chain reaction is an enzyme catalyzed biochemical reaction in which small amount of the specific DNA sequences are amplified into large amounts of linear double stranded DNA (Millis, 1990). PCR is used to amplify the DNA sequence in between two known sequences.

The 16s rRNA gene of the bacteria was carried out in our laboratory in the Thermocycler (Eppendorf). In PCR the specific primers (Forward and reverse primers) complementary to the known sequences were added along with the master mix (HELINI Biomolecules, Chennai) to the DNA and the mixture was placed in a thermocycler heated at 94°C for denaturation of the DNA. The mixture is then allowed to cool enabling the primers to anneal to the complementary sequences. A heat stable DNA polymerase was used to make the copies of the DNA from the original DNA sequence About 30 cycles of the DNA amplification was performed that resulted in a very large amplification of the DNA .

The 1542 bp rRNA gene was amplified using two primers. Two primers annealing at the 5' and 3' end of the 16S rDNA were (Forward Primer) 5' - GAGTTTGATCCTGGCTCAG-3' (positions 9–27 [*Escherichia coli* 16S rDNA numbering]) and (Reverse primer) 5'-AGAAA GGAGG TGATC CAGCC-3' (positions 1542–1525 [*E. coli* 16S rDNA numbering]) were used. The master mix containing 10X *Taq* buffer, 10 mM dNTPs, 25 mM of $MgCl_2$, 1 U of *Taq* DNA polymerase, 1.5 μ l of forward primer, 1.5 μ l of Reverse primer, 100 ng of Genomic DNA and PCR grade molecular water to make the final volume to 20 μ l was used. *Taq* DNA polymerase initiates the replication of DNA fragments by using nucleotide base from dNTP mixture (A, T, G, C).

Procedure

Setting up the PCR reaction

A fresh master mix tube which contains the dNTP and *Taq* DNA polymerase was taken and the following components are added

Master Mix vial	10 μ l
Forward Primer	1.5 μ l
Reverse primer	1.5 μ l
Template DNA	2 μ l
Nuclease free water	5 μ l
Distilled water	20 μ l

(Master Mix contains 1U *Taq* DNA polymerase, 10X *Taq* buffer, 10 mM dNTPs and PCR grade water. Primer dye mix contains 10 μ M forward primer, 10 μ M reverse primer and PCR compatible dye with glycerol).

After addition of all the components the PCR tube is gently spun down in centrifuge briefly and is placed in the Thermal cycler. The thermal cycler was programmed as follows.

Program 1 (one cycle) (Initial denaturation)

94°C for 2 minutes

Program 2 (30 cycles) (Amplification)

Step one (denaturation) 94°C for 45 seconds

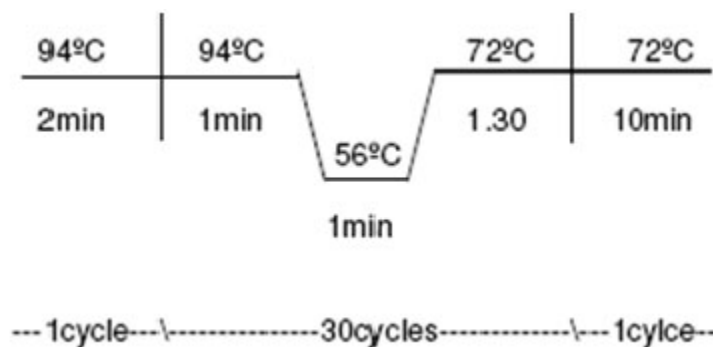
Step two (annealing) 56°C for 1 minute

Step three (extension) 72°C for 1 minute 30 seconds

Program 3 (one cycle - final extension)

72°C for 5 minutes, then hold at 4°C

The programmed temperatures of the PCR reaction is represented in the diagram shown below



Agarose Gel Electrophoresis

Gel electrophoresis is a technique used for the separation of nucleic acid and protein, where electrically charged molecules migrate at a rate proportional to their charge in mass ration when place under an electric field. It is undoubtedly the routine technique to monitor the success of the nucleic acid isolation procedures and analyze enzymatic manipulations such as restriction enzyme digestion. About 1.8 g of agarose was weighted and taken into 100 ml reagent bottle. To it 100 ml of 1X TBE buffer was added and it was heated in a microwave oven till all agarose gets melted up. The agarose solution was then poured in to gel casting unit assembled with appropriate comb and it was allowed to polymerize. After the polymerization the comb was removed and the gel was placed in an electrophoretic tank consisting of 1X TBE buffer. About 12 μ l of the 16s rRNA amplified DNA was mixed with 2 μ l of the gel loading dye (Bromophenol blue 6x) and it was loaded in 1.8 % agarose gel. The gel was electrophoresed at 90 volts for about 30 minutes and it was observed in a gel documentation system.

Eluting DNA from agarose gel fragments

- ' Ethidium bromide stained agarose gel was visualized under a transilluminator on low setting. The fragment of interest was excised with a clean razor blade. After removing the excess liquid, the agarose fragment was placed in the spin column.
- ' The tube was centrifuged at 5500 rpm for not more than 45 seconds for the elution of DNA.
- ' The eluent was checked using a transilluminator for the presence of ethidium

bromide stained DNA.

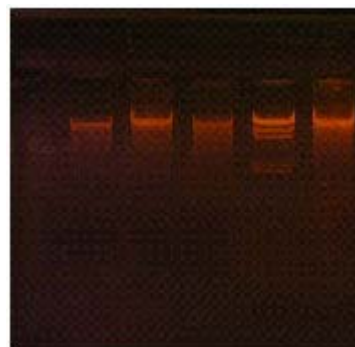
The eluted DNA was used directly in enzymatic reactions.

This DNA fraction was now subjected for sequencing.

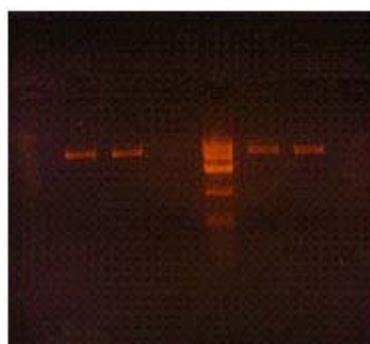
RESULTS AND DISCUSSION

Genomic DNA Isolation of the four test bacteria

Genomic DNA with Lambda Hind III digests



Above gel represents Genomic DNA isolated from four organisms along with Hind III digested lambda DNA as ladder.



PCR amplified 16S r DNA of the 4 test bacteria

Well 1	:	16s r RNA amplified product of <i>Escherichia coli</i>
Well 2	:	16s r RNA amplified product of <i>Pseudomonas putida</i>
Well 4	:	100 bp ladder
Well 5	:	16s r RNA amplified product of <i>Bacillus subtilis</i>
Well 6	:	16s r RNA amplified product of <i>Staphylococcus aureus</i>

16S rDNA sequencing

16S rDNA sequence amplified from each isolates was subjected to sequencing. Applied Biosystem Corporation Auto Sequencer was used to sequence. The results of sequencing obtained in the form of color peaks. The color peak represents one particular nucleotide. Black peaks represent Guanine, Red color represents Thiamine, Green colors represent Adenine and Blue colors represent Cytosine.

Sequencing of Purified Product

The purified product was subjected for DNA sequencing and it was carried out at Biotranics, Chennai, India.

Tracing the Phylogenetic history of each sequence

Once the four sequences of the 16S r DNA was obtained for each bacteria then for each sequence the related sequences of 7 different sub species was retrieved from NCBI. This was followed by the use of SDSC Biology Workbench and ClustalW to do the Multiple Sequence Alignment for obtaining four different Dendrogram (Phylogenetic Tree) from which the evolutionary divergence of each sequence can be traced and the closest species to that of the query can be found out.

Over the past one decade, process industries have increased many folds. Industrial uses of metals such as metal plating, tanneries,

industrial process utilization metal as catalysts, have generated large amounts of aqueous effluents that contain high levels of heavy metals, these heavy metals include Cadmium, Chromium, Cobalt, Copper, iron, Mercury, Nickel, Silver and Zinc. Metal polluted industrial effluents discharged into sewage treatment plants could lead to high metal concentrations in the activated sludge.

Microbial population in metal polluted environment contain microorganisms which have adapted to toxic concentrations of heavy metals and become metal-resistance. At present, metal polluted industrial effluents are mostly treated by the chemical method such as chemical precipitation, electrochemical treatment and ion exchange. These methods provide only partially effective treatment and are costly to implement, especially when the metal concentration is low. The biosorption (which is removal using absorption into living/dead organisms) for the removal and recovery of toxic metals from industrial effluents can be economical and effective methods for metal removal. The metal removal ability of microorganisms including bacteria (Bizily *et al.*, 2000), microalgae and fungi (Bizily *et al.*, 1999) has been studied extensively. The heavy metal removal capacity is higher than those conventional methods. And uptake of heavy metals can be selective (Furukawa and Tonomura., 1972). Microbial cells can also be supplied inexpensively as waste in industrial fermentation processes as well as biological waste water treatment plants (Bizily *et al.*, 1999).

In the present investigation it has been found that different physico-chemical parameters such as temperature, pH and NaCl etc have their influence in the growth of bacteria. Good growth observed at 37°C temperature, while in case of pH 6.5 is optimum for the four species of bacteria. In case of NaCl for the four species of bacteria the concentration is 0 to 0.5 percentage of NaCl was favorable for the good growth of four species of bacteria.

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