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IDENTIFICATION OF UNKNOWN BACTERIA BY USING BIOCHEMICAL TESTS AND 16S RRNA SEQUENCING FROM DIFFERENT SOIL SAMPLES

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ABSTRACT

The identification of unknown bacteria produces benefits for many aspects of the research of microorganisms and helps physicians correctly treat patients. Multiple biochemical tests were performed to provide the fermentation abilities, presence of certain enzymes, and certain biochemical reactions. Qualitative observations were made on the tests, which were compared to unknown bacteria identification key to aid with the identification process. And 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, often existing as a multigene family, or operons; (ii) the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the 16S rRNA gene (1,500 bp) is large enough for informatics purposes. Finally the several amplified parts could be assembled together to have the entire sequence of the complete 16S rRNA. In addition to highly conserved primer binding sites, 16S rRNA gene sequences contain hypervariable regions that can provide species-specific signature sequences useful for bacterial identification. Species identification continues to be a challenge. After analyzing four unknown bacteria samples by using both Biochemical tests and 16S rDNA technique, I found to be Bacillus Cereus(S_1), Brevibacillus brevis(S_2 and S_4) and Pseudomona Putida(S_3).

Keywords: Biochemical Test, Bacillus Cereus, Brevibacillus brevis , PCR, Soil and Pseudomona Putida.

I. INTRODUCTION

Bacteria are present in most habitats on Earth, growing in soil, acidic, radioactive waste, (Fredrickson JK, et al.,2004). water, and deep in the Earth's crust, as well as in organic matter and the live bodies of plants and animals, providing outstanding examples of mutualism in the digestive tracts of humans, termites and cockroaches. There are typically 40 million bacterial cells in a gram of soil and a million bacterial cells in a milliliter of fresh water; in all, there are approximately five nonillion(5×10^{30}) bacteria on Earth. (Whitman WB,

1998) Most bacteria have not been characterized, and only about half of the phyla of bacteria have species that can be grown in the laboratory (Rappé MS, Giovannoni SJ, 2003).

If bacteria form a parasitic association with other organisms, they are classed as pathogens. Pathogenic bacteria are a major cause of human death and disease and cause infections such as tetanus, typhoid,fever, diphtheria, syphilis, cholera, foodborne,illness, leprosy and tuberculosis.Conditionally pathogenic bacteria are only pathogenic under certain conditions, such as a wound that allows for entry into the blood, or a decrease in immune function. For example, *Staphylococcus* or *Streptococcus* are also part of the normal human flora and usually exist on the skin or in the nose without causing disease, but can potentially cause skin infections, pneumonia, meningitis and even overwhelming sepsis, a systemic inflammatory response producing shock, massive vasodilation and death (Fish DN, 2002). Some species of bacteria, such as *Pseudomonas aeruginosa*, *Burkholderia cenocepacia*, and *Mycobacterium avium*, are opportunistic pathogens and cause disease mainly in people suffering from immunosuppression or cystic fibrosis (Heise E, 1982, Saiman L, 2004)

The 16SrRNA gene is used for phylogenetic studies (W G Weisburg et al., 1991) as it is highly conserved between different species of bacteria and archaea (Coenye T, Vandamme P 2003). Sequence analysis of the 16S rRNA sequences is done with the help of several primers, called "universal primers." These primers target the conserved region of 16S rRNA gene and amplify the target in parts. Finally the several amplified parts could be assembled together to have the entire sequence of the complete 16S rRNA. In addition to highly conserved primer binding sites, 16S rRNA gene sequences contain hypervariable regions that can provide species-specific signature sequences useful for bacterial identification. As a result, 16S rRNA gene sequencing has become prevalent in medical microbiology as a rapid, accurate alternative to phenotypic methods of bacterial identification (J. E. Clarridge III, 2004).Although it was originally used to identify bacteria, 16S sequencing was subsequently found to be capable of reclassifying bacteria into completely new species, or even genera. It has also been used to describe new species that have never been successfully cultured (Weisburg WG 1991, Brett P J et al., 1998, Gray JP, Herwig RP 1996). The introduction of comparative rRNA sequence analysis represents a major milestone in the history of microbiology. The current taxonomy of prokaryotes as well as modern probe and chip based identification methods are mainly based upon rRNA derived phylogenetic conclusions. Also of importance is single gene based phylogenetic inference and alternative global markers include elongation and initiation factors, RNA polymerase subunits, DNA gyrases, heat shock and recA proteins. Although the comparative analyses are hampered by the generally low phylogenetic information content, and different resolution power, and multiple copies of the individual markers, the domain and prokaryotic phyla concept is globally supported. A major innovation in laboratory sciences occurred in the late 1960s, when manual miniaturized identification systems were first introduced into the clinical microbiology laboratory.

II. MATERIALS AND METHODS

In this research paper we are generally used to two method for identification of unknown bacteria from soil samples with different cities which are far about 60-70 km each other.(1) Biochemical tests and (2)16S rRNA technology.

Firstly, we collected the soil samples from four different cities and then applied the $1/10^{th}$ rule to the serial dilution for decrease the concentration of micro organisms up to 10^{-4} at 5 test tubes. After this we applied spread plate technique for growth of the micro organisms, at last we are focused a single colony of each plates to the single colony isolation technique (Streak plate method).

Gram's staining and Biochemical tests like- Voges Proskauer, MR Test, Catalase Test, Urease Test, Mannitol Test, Citrate Test, Starch Utilization Test, 6.5% NaCl Tolerance Test etc. (Pelczar/Chan/Krieg Fifth edition, K.R. Aneja Fourth edition)

16S rRNA sequencing method:- Isolation of genomic DNA from the targeted bacteria then amplified each samples by using universal primer with the help of PCR, to send the samples for sequencing in the Bio serve laboratory Hyderabad then used Insilco laboratory.

III. RESULTS

Gram Staining

	S_1	S_2	S_3	S_4
Gram Staining	-ve	-ve	-ve	-ve

Following results have been obtained after the Biochemical test by using Bergey's manual analysis. Obtained results were showing similarity with the closely associated bacteria Bacillus Cereus (S_1), Brevibacillus brevis (S_2 and S_4) and Pseudomona Putida (S_3).

Biochemical tests

Tests	S_1	S_2	S_3	S_4
VP Test	+ve	+ve	+ve	+ve
MR Test	+ve	+ve	-ve	+ve
Catalase Test	+ve	+ve	+ve	+ve
Urease Test	+ve	+ve	+ve	+ve
Mannitol Test	-ve	-ve	-ve	-ve
Citrate Test	+ve	-ve	+ve	-ve
Starch Utilization Test	-ve	-ve	-ve	-ve
6.5% NaCl Test	+ve	-ve	+ve	-ve

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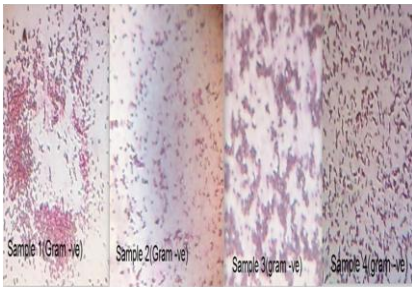
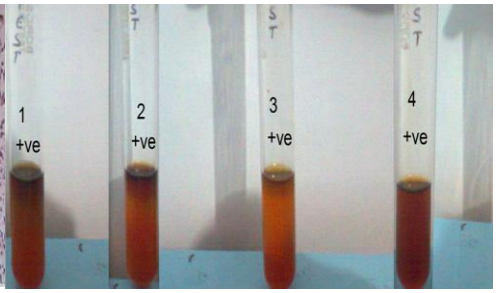
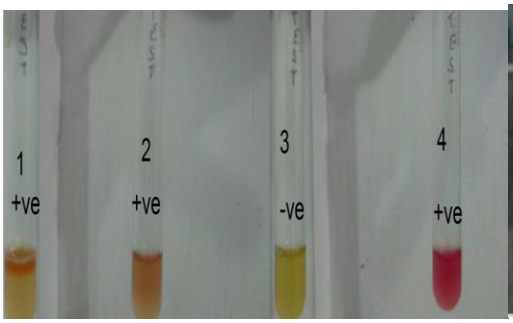
Light Microscopic view of gram staining



VP test



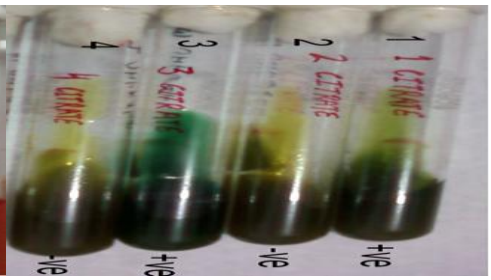
MR test



Catalase Test

Mannitol test

Citrate test



Sequencing results

S_1-

gctcaggatgaacgctggcggcgtgcctaatacatgcaagtcgagcgaatggattaagagcttgctcttatgaagttagcggcggacgggtgagtaacacgtggg
taacctgcccataagactgggataactccgggaaaccggggctaataccggataacattttgaaccgcatggttcgaaattgaaaggcggcttcggctgtcacttat
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acggcccagactcctacgggaggcagcagtagggaatcttccgcaatggacgaaagtctgacggagcaacgccgcgtgagtgatgaaggctttcgggtcgtaa
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gtaggtggcaagcgttatccggaattattgggcgtaaagcgcgcgcaggtggtttcttaagtctgatgtgaaagcccacggctcaaccgtggagggtcattggaaa
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agtgctgaagttaacgcattaagcactccgcctggggagtacggccgcaaggctgaaactcaaaggaattgacgggggcccgcacaagcggtggagcatgtgg
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ccggaggaaggtggggatgacgtcaaatcatcatgccccttatgacctgggctacacacgtgctacaatggacggtacaaagagctgcaagaccgcgaggtgg
agctaatctcataaaaccgttctcagttcggattgtaggctgcaactcgcctacatgaagctggaatcgctagtaatcgcggatcagcatgccgcggtgaatacgttc

ccgggccttgtacacaccgcccgtcacaccacgagagtgtaacacccgaagtcggtggggtaacctttttggagccagccgcctaaggtgggacagatgattgg
ggtgaagtcgtaacaaggtagccg

S_2 and S_4-

gcggctggctccaaaaggttacctcaccgacttcgggtgttacaaactctcgtggtgtgacgggcggtgtgtacaaggcccgggaacgtattcaccgcggcatgc
tgatccgcgattactagcgattcggcttcatgtaggcgagttgcagcctacaatccgaactgagaacgactttatcggattagctccctctcgcgagttggcaaccgtt
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agccgttacctcaccaactagctaatgcgccgcgggcccatcctatagcgacagccgaaaccgtctttcagtctttcacctgaagcaaaagagattattcggtattag
ccccggtttcccggagttatcccaaactatagggtaggttgcccacgtgttactcacccgtccgccgctaacgtcaaaggagcaagctccttttctgttgctcgacttg
catgtattaggcacgccgccagcgttcgtcctgagccaggatcaaactct

S_3-

GAATTCGTAATCATGGCCATGGATTACCTCGTTCTTATTGTGTTGTGGGTATGAGCCGGCCAGATCGACC
CGCCTCGTGCAGTTGTTCGGGGCAGGCCCTATATCTGCGATATATTGAGCATATTGGCGAGAAGGAGTGT
GGTCAACATCTTCGTTTAAAAACGGCGCAAATGTTGGCTTGCGGTTTACCCTCAGGCTTGAGCTGGCATT
GTGCGGATCGGTATCTGCAGGGGGCTCGGAGCATCGAGGTTCTCGAGGCGCCACGAACTGAATCAGCAGT
GAGGACTTACGTTGACGATAGCCAGCCCTTTGAAGGGAAAGGCTCGGATCGAGCCGGGCAACGTGGACAT
CAAGCAGAGCTTGCTGCACGCAGCTATGCGCCTGCTGAGTGCCAAGGGGCGTGACGGTGCGACCATGCGA
GCGATCTGTGCGGAGGTGGGTGTCACGCCGCCAACCCTGTACCACCATTACGGAGATCTGCAAGGGCTGC
ACAAGGCTGCGATTGACGAAACCTGCCGGCAGGTTGCCGAGGCTTATCATGGCGGCACCGAAGAAAGAGG
GCCGCTCAAGGGGATACGTGATGGATGGGCAACCTTTCTTCAGTTCGCCTATTCAGAGCCGAATATGTGT
CGCATGCTGGTTCAGCACATCATGGCGGGAGAGCCACCGAGTATGGTGGCCGATACCCTGCGAGGCGTGG
CTGACGATTTAGCCCAGTTCCATGCACAGGGGCGATTGACGTTCCCTCCGAGAGAGGCCGCGCAGTTGCT
GTGGATGGGGGCCTTGGGTGCGCTGACCTACGCACTCAGCCGGGAAGGAGCAGGGTACACACAAGACTTG
GCCCTGCAAAAAGCCAAACTCGACATTACCTTGGTCGCGCTCTTCAATATCGAGGAAGAGTAGGCGGGTT
CGTGCCGAGTTTGTTTCAGGGGTGGTCTCCAACTGGGCCAAACTCAATGGGTACCCAGACGTAGGCGCCA
TCCGTCGCGCGAACACGGCCAAGCCCGGGAAAGGGTAGATGTGCACCCGCTACCCAAAAATGCTCCTTTG
CAGCGTTCGTTAGCATGCTCTGCCGTGAGCTCCTGGCTTGCGCAGAGTCTGCGTCAAACTCGATAACCAC
TTCCGGCCGGGCGAACTGAATGGCATGGTTATGCACCAGATCCTCCCAGACCATCAGGCGTTCATCGCCA
GAGGTAAAGAGGTAGGCTGAGTGACCAGGTGTATGCCCGTAGGTAGGCACGCTTTCCACGCCCGGCAGCA
ATGCGGCGTCAGGCTCATAGCGCAGCAGGCGCCCCTGCGCATAGGGTGCGACTGCTGCCCTGGCCATCAG
GAAAAACGCTTGGCTGGGTTCCGGCATGGCGGCGATATCCTGATCCAGCCAGAATTC

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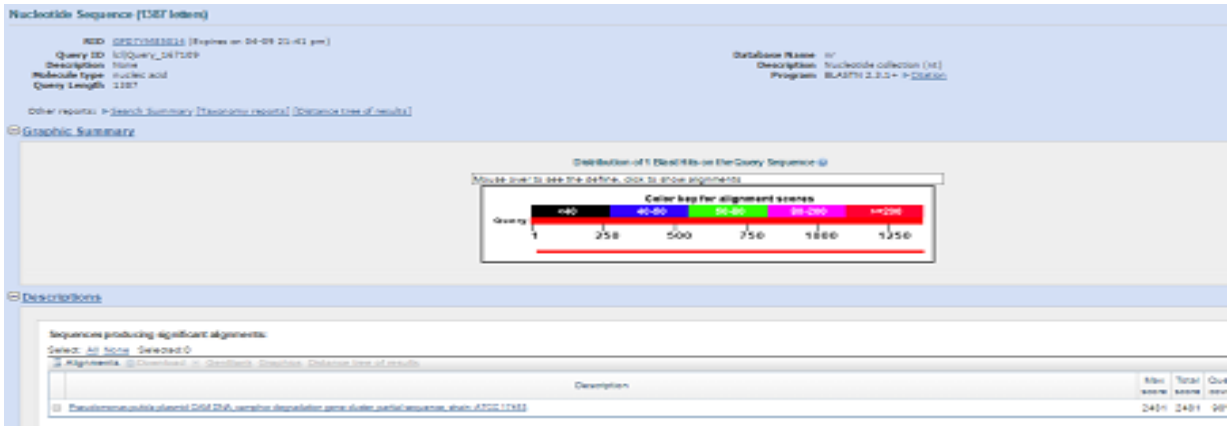
Blast

S_1 **S_2 and S_4**

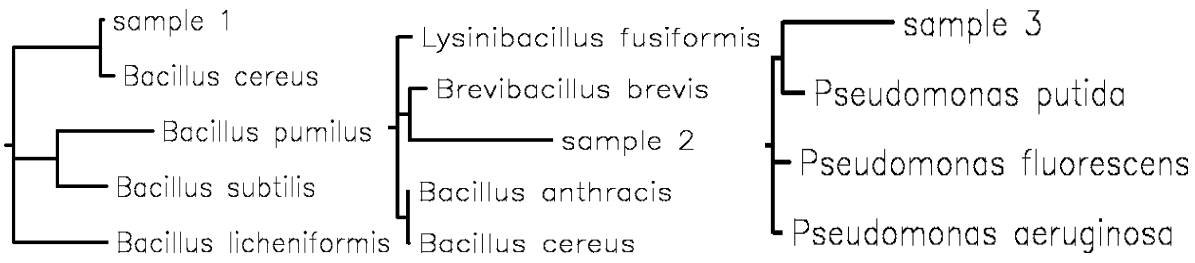
S_3



After the alignment of sequence by ClustalW of unknown bacteria with some related strains following results have been obtained-

The phylogenetic rooted trees

S_1 **S_2 and S_4** **S_3**



IV. CONCLUSION

After analyzing four unknown bacteria samples by using both Biochemical tests and 16s rDNA technique, we came to the conclusion that the bacteria samples which I was analyzing, found to be Bacillus Cereus(S_1), Brevibacillus brevis(S_2 and S_4) and Pseudomona Putida(S_3).

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