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Genome Sequence Analysis of the Bacillus Cereus Isolated From Soil Sample

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ABSTRACT

In Recent decades researches investigated that groups of the *Bacillus cereus*will carry genes may have the capacity to cause gastrointestinaland somatic diseases. Despite the fact that most of the diseases caused by *B. cereus* group pathogens are relatively acute, scientist have identified from food and in the environment to investigate members of the B. cereus group. This current research was aimed to isolate and identify *Bacillu*s species from soil sample. The *Bacillu*s species isolated were identified byusing the classical method. The pure isolate was subjected to morphological tests, biochemical tests and gram staining. The antibiotic sensitivity test was studied using Cefotaxime/ clavulinic acid, Cefazolin, Ampicilin, Doxycycline, Methicilin, Gentamicin, Tobramycin, Rifampicin, Tetracycline and Erythromycin for the isolated bacteria from soil. The results showed high activity of zone of inhibition for Cefotaxime/ clavulinic acid, Doxycycline, GentamicinTobramycin,Tetracycline and Erythromycin, showed moderate zone of inhibition for Rifampicin and Methicilin.The same isolates were further identified by DNAsequencing method;Fragment of 16S rDNA gene was amplified by 27F and 1492R primers.The 16S rDNA gene sequence was used to carry out BLAST with the database of NCBI genbank database.PCR analyseswith DNA from *B. cereus*have been confirmed the specificity of the PCR assay. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with forward primer and reverse primers using BDT v3.1 Cycle sequencing kit.The study suggests that some of *Bacillus spp* from the study area have potential to produceantibiotics that may be used to control microbial growth.

Keywords: *Bacillus cereus,* morphological tests, Soil, PCR analyses.

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1. INTRODUCTION

Bacillus cereus has been identified asthe causative agent in a number of food poisoning outbreaks.The illness has been attributed to the presence of enterotoxinsand other toxins including haemolysins produced by strains of*B. cereus*. There is still confusion regarding how many differententerotoxins are produced by *B. cereus* in fact *B. Cereus*produces a high variety of toxins and enzymes that are activeon different cell tissues that has been recently associated withthe capability of this microorganism to induce non-gastrointestinalinfections as systemic and pulmonary infections.

This study was to isolate bacteria from soil samples and was subjected to morphological tests, biochemical tests, DNA isolation, DNA sequencing, and polymerase chain reaction amplification. The present study was also aimed to determine the *in vitro* antibiotic sensitivity test of the isolate

2. REVIEW OF LITERATURE

Recent investigations have shown that members of the Bacillus cereus group carry genes which have the potential to cause gastrointestinaland somatic diseases. Although most cases of diseases caused by the B. cereus group bacteria are relatively mild, it is desirable to be able todetect members of the B. cereus group in food and in the environment. Using 16S rDNA as target, a PCR assay for the detection of B. cereusgroup cells has been developed. Primers specific for the 16S rDNA of the B. cereus group bacteria were selected and used in combinationwith consensus primers for 16S rDNA as internal PCR procedure control. The PCR procedure was optimized with respect to annealingtemperature. When DNA from the B.

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cereus group bacteria was present, the PCR assay yielded a B. cereus specific fragment, while whennon-B. cereus prokaryotic DNA was present, the consensus 16S rDNA primers directed synthesis of the PCR products. The PCR analyseswith DNA from a number of non-*B. cereus* confirmed the specificity of the PCR assay.

3. MATERIALS AND METHOD

The soil samples were collected from different places in Chennai city. The collected soil samples were enriched in sterile distilled water and spread on nutrient agar plates for *Bacillus cereus* isolation. Incubated plates were observed for bacterial growth. Individual colonies were sub cultured to nutrient agar slant and stored at 4˚C. The colonies were confirmed as gram negative bacteria by gram staining and biochemical tests.

BIOCHEMICAL TEST

INDOLE TEST

Tryptone broth was inoculated with the test isolates and incubated at 37°C for 24-48 hours. About 0.2-0.3 ml of Kovac's reagent was then added to the test tube, shaken and allowed to stand. The formation of brown ring on the surface of the broth confirmed the production of indole.

VOGES - PROSKAUER TEST

MR - VP medium inoculated with culture was incubated at 37°C for 24-48 hrs. After incubation, 3 ml of Barrit's reagent A and one ml of Barrit's reagent B was added. The tubes were shaken and allowed to stand for 15 minutes and observed for colour change. The development of red colour was considered as positive.

CITRATE UTILIZATION TEST

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Citrate utilization test was used to detect the ability of an organism to utilize citrate as the sole carbon source for its growth, lightly inoculate Simmon Citrate Agar (Hi media, Mumbai) with a loop (pure culture) of culture. Record the visible growth as positive and no growth as negative. Incubated at 37°C for 24hrs. Record a blue colour growth on slant as positive and absence of growth as negative.

TRIPLE SUGAR IRON TEST

The Triple Sugar Iron (TSI) test is to find ability of a microorganism to ferment sugars and produce hydrogen sulfide. Acidic byproduct gives yellow color where as alkaline by products gives red color. Black color indicated hydrogen sulphide formation. Gas production is indicated by lifting of butt from surface of test tube. TSI medium inoculated with the culture was incubated for 24 hrs at 37°C.

UREASE TEST

When urea is utilized, ammonia is formed during incubation which makes the medium alkaline, showing a pink-red colour. Urease medium inoculated with the culture was incubated for 24 hrs at 37°C.

CATALASE TEST

A small amount of culture was placed over a clean slide. A drop of 3% hydrogen peroxide was placed over the culture and observed for effervescence. The production of effervescence showed the ability to produce the enzyme catalase.

OXIDASE TEST

The organism was spotted on oxidase disc, blue or purple colour change was observed within 10 seconds.

GRAM STAINING

Bacterial smears of 16-18 hrs old cultures were made on clean grease free slides, heat fixed and stained as follows. The slide was flooded with crystal violet solution for a minute, drained and rinsed with water; followed by Grams iodine solution for one minute, drained and rinsed with water. Decolourised with ethyl alcohol for 30 Sec and later

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counterstained with safranin for one minute and observed under an oil immersion microscope.

ANTIBIOTIC SENSITIVITY

Antibiotic sensitivity was determined by disc diffusion method on Muller Hinton agar (MHA) medium. The Muller Hinton Agar medium was weighed as 3.8gms and dissolved in 100ml of distilled water and add 1gm of agar. Then the medium is kept for sterilization. After sterilization the media was poured in to sterile petriplates and were allowed to solidify for 1hr. After the medium was solidified, the inoculums were spread on the solid plates with sterile swab moistened with the bacterial suspension and antibiotic discs were placed. These plates were incubated for 24 hrs at 37ºc .Then the microbial growth was determined by measuring the diameter of zone of inhibition

Isolation of DNA

Isolation of DNA was carried out by alkaline lysis procedure (Sadasivam and Manickam, 2008) with some modification. One ml of an overnight culture was transferred into an effendorf tube. The cells were sediment by centrifuging briefly (5000 rpm) in the microfuge and the supernatant was drained off. The pellet was resuspended by adding 100 µl of 1X TE buffer and the contents were mixed by a vortex. Then 100µl of 10 % SDS was added and mixed well by inverting the content (4 -5 times). To the above viscous content 100 µl of Solution C was added and the content was inverted 4 -5 times to get mixed, the bulk of genomic DNA and other cell debris will precipitate into a viscous clump. It was centrifuged at 12000 rpm in micro centrifuge and the clump was removed. The clear lysate (supernatant) was transferred to another eppendroff tube. 150µl of 100% Isopropanal was added and mixed well and centrifuged the content at 12000 rpm for 30 minutes. The supernatant was drained off and 150µl of absolute alcohol was added and centrifuged the content at 10000 rpm for 20 minutes. The supernatant was drained off and dissolved the DNA pellets with 20 µl TE buffer. The extracted Plasmid DNA was confirmed by running of agarose gel electrophoresis

Agarose Gel Electrophoresis

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Agarose gel electrophoresis was carried out in a horizontal submarine electrophoresis unit. Sixty ml of 1 % Agarose gel was prepared with 1X TBE buffer (do not mix) and heated the content to get up to clear solution for casting Agarose gel. After cooling the solution, 5 µl of staining dye solution was added into the casting system. The gel was allowed to solidify, and then carefully disassembled from the casting system without disturbing the wells and placed in 1X TBE buffer filled electrophoresis tank (the buffer level should be above gel). The 10µl of plasmid DNA was added and mixed with 2 µl of gel loading dye and then loaded to gel. The power card terminals were connected at respective positions, run the gel at 50 V, till the gel loading dye migrate more than half the length of the gel. Then switched off the unit and visualized the isolated DNA under UV Transilluminator.

4. RESULTS AND DISCUSSION

The soil samples were collected from three different places in Chennai city. The collected soil samples were serially diluted and spread on nutrient agar plates for Bacillus cereus isolation. The isolated bacteria were identified based on morphological and biochemical characterization. The soil samples were identified by gram staining, rod-shaped gram-positive bacteria was observed under microscope and biochemical test results showed that indole negative, MR, VP, catalase, oxidase, urease, citrate were positive and TSI showed A/A no gas and H2S production. The predominantly grown bacterial species were identified as Bacillus cereus.

The antibiotic sensitivity test was studied using Cefotaxime/ clavulinic acid, Cefazolin, Ampicilin, Doxycycline, Methicilin, Gentamicin, Tobramycin, Rifampicin, Tetracycline and Erythromycin for the isolated bacteria from soil. The results showed high activity of zone of inhibition for Cefotaxime/ clavulinic acid, Doxycycline, Gentamicin

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Tobramycin, Tetracycline and Erythromycin, showed moderate zone of inhibition for Rifampicin and Methicilin. No zone of inhibition was seen for Cefazolin and Ampicilin.

The isolated culture was further identified Genomic DNA of the isolate was visualized under UV. A single band of high-molecular weight DNA has been observed. Fragment of 16S rDNA gene was amplified by 27F and 1492R primers. A single discrete PCR amplicon band of 1500 bp was observed when resolved on Agarose gel. In the present study, 16S rDNA gene sequencing of the isolate was investigated. Fragment of 16S rDNA gene was amplified by 27F and 1492R primers. The isolate was identified as B. cereus strain. The 16S rDNA gene sequence was studied using BLAST with the database of NCBI genbank database. PCR analyses with DNA from B. cereus have been confirmed the specificity of the PCR assay. Forward and reverse DNA sequencing reaction of PCR amplicon was examined with forward primer and reverse primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 16S rDNA gene was generated from forward and reverse sequence data using aligner software. The 16S rDNA gene sequence was used to carry out BLAST with the database of NCBI genbank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated and the phylogenetic tree was constructed using MEGA 7.

Molecular Phylogenetic tree analysis by Maximum Likelihood method:

The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2- parameter model. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms toa matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1434 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

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The organism was identified as Bacillus cereus and it was confirmed by PCR amplification and DNA sequencing.

Sanger Sequence Chromatogram File Data:

>Forward Seq data

AGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGA
ATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACC
AGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTCTCCTTCGGGAGCATAGTGACATGTGGTG
CATGCTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAACTCCCGCAACGAGCGCAACCCTTGATCT
TAATTGCCATCATTCGGTTGGGCACTCTAACGTCACTGCCGGTGACAAACCGAAGGAACGTCGCGAT
GACATTGATGCAGGCTTTCAGGTCGTACCTCTCTGTTGTTAGGGAAGAACAAGTGCTAATTAAATACT
CTGGCACCTTGACGGTACCTTACCATAATGCCACGGTTAACTACGTGGCATCAACCGCGGTAATACCT
ACGTGGCGAGCGTTATCCGGAATTATTGTGCATAGATTGCGCGCACGTGGGTTCTTTACTTTTGATGT
TAAAGCCCTGCGGCTCAAACTCCGAAGGGTCATTGCAAACTGCGATAACTTTCAGTGACTAGAAGGA
GGATGCACTCTCATGTGTAGCGCTCAACGGTTACGGAGATGTACTAGAAGCGCTATCTGACTCATGGT
TAGCTTCCCGCGCTGTACGCGCCTCAGTGGGCAGTTAACGCACCGGAAAGTCCACCTTCATCACATGG
CGGTGCTACTTCATCTCTGAACAA

>Reverse Seq Data

GTTAACTTCAGCACTAAAGGGCGGAAACCCTCTAACACTTAGCACTCATCGTTTACGGCGTGGACTAC
CAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCGCCTCAGTGTCAGTTACAGACCAGAAAGTCG
CCTTCGCCACTGGTGTTCCTCCATATCTCTACGCATTTCACCGCTACACATGGAATTCCACTTTCCTCT
TCTGCACTCAAGTCTCCCAGTTTCCAATGACCCTCCACGGTTGAGCCGTGGGCTTTCACATCAGACTT
AAGAAACCACCTGCGCGCGCTTTACGCCCAATAATTCCGGATAACGCTTGCCACCTACGTATTACCGC
GGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGGTACCGTCAAGGTGCCAGCTTATTCAACTA
GCACTTGTTCTTCCCTAACAACAGAGTTTTACGACCCGAAAGCCTTCATCACTCACGCGGCGTTGCTC
CGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTC
AGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTACACATCGTTGCCTTGGTGAGCCGTTACCTCA
CCAACTAGCTAATGCGACGCGGGTCCATCCATAAGTGACAGCCGAAGCCGCCTTTCAATTTCGAACC
ATGCGGTTCATAATGTTATCCGGTATTAGCCCCGGTTTCACGGAGTTATCCCAGTCTTATGGGCAGGT

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TACCCACGTGTTACTCACCCGTCCGCCGCTAACTTCATAATAGCAAGCTCGTAGTCCATTCGCTCGAC
TTGCATGTATTACGCACGACGGCCGGCGTTCATCCTGAACCATGATCAGACACTAAGGCCGCCCGCA CA

>Reverse complement

TGTGCGGGCGGCCTTAGTGTCTGATCATGGTTCAGGATGAACGCCGGCCGTCGTGCGTAATACATGCA
AGTCGAGCGAATGGACTACGAGCTTGCTATTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGG
TAACCTGCCCATAAGACTGGGATAACTCCGTGAAACCGGGGCTAATACCGGATAACATTATGAACCG
CATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTT
GGTGAGGTAACGGCTCACCAAGGCAACGATGTGTAGCCGACCTGAGAGGGTGATCGGCCACACTGG
GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGT
CTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAA
CAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGC
CAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGG
TGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACT
TGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACA
CCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAG
GATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTA
GTGCTGAAGTTAAC

>3 consensus seq

GTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGG
GGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGA
CATCCTCTGACAACCCTAGAGATAGGGCTTCTCCTTCGGGAGCATAGTGACATGTGGTGCATGCTTGT
CGTCAGCTCGTGTCGTGAGATGTTGGGTTAACTCCCGCAACGAGCGCAACCCTTGATCTTAATTGCCA
TCATTCGGTTGGGCACTCTAACGTCACTGCCGGTGACAAACCGAAGGAACGTCGCGATGACATTGAT

GCAGGCTTTCAGGTCGTACCTCTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCT
TGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCA
AGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCC
ACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTC
CATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTG
TAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGT
AAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGC

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5. CONCLUSION

In this present study, Bacillus cereus was isolated from soil were identified as Bacillus species based on their morphological and biochemical properties as well as its 16S rDNA genes with the forward and reverse primers. The results showed the potential of PCR technique as a tool for investigating relationships among our Bacillus species.

The study showed that the isolated bacteria with the ability to produce antibiotic are present in the soil. The antibiotics are commercially available the search for the most effective one is still on, and this study may contribute in providing information on the antibiotic producing microorganisms.

Despite that, by using multiplex PCR is known that samples contained virulent strain of bacterium, it is described by the formation of bands. Furthermore, research should be continued to study the improved methods of molecular characterization of this strain other than 16S rDNA gene sequencing.

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