



MediPharm

International Journal of MediPharm Research

ISSN: 2395-423X

www.medipharmsai.com
Vol.01, No.01, pp 27-36, 2015

DNA Sequencing by Targeting 16S rRNA Gene for Novel Strain Identification of *Bacillus* sp.

*Khusro A

Department of Plant Biology and Biotechnology, (PG. Biotechnology), Loyola College, Nungambakkam, Chennai (India)

Abstract : The present context was investigated to isolate and characterize novel strain of industrially important bacteria from poultry farm. Preliminary study of poultry faeces sample was done by serial dilution followed by microscopic analysis, morphological characteristics and biochemical tests of pure isolated culture. The bacterium was identified as *Bacillus* species. Further, the identification of bacterium as *Bacillus subtilis* strain KPA was confirmed by subjecting its amplicon (483 bp) to 16S rRNA gene sequence analysis and pairwise alignment through BLAST tool. Minimum Free Energy (-192.10 kcal/mol), Mountain plot, Entropy, Partition, Pseudo Knots and Folds of the sequence were determined under RNA Secondary structure prediction through RNAeval Web server and RNAfold Web server. Percentage content of G+C (55.1%) was calculated through FTG server tool. Pictogram was visualized to identify alignment of the sequence. Phylogenetic tree based on taxonomic positions was inferred using the Neighbor-joining (NJ) algorithm in Molecular Evolution Genetic Analysis (MEGA) software version 4.0.

The isolation of bacteria from different sources offers novel bacteria of unique functionality and their potential applications in different biotechnological processes. The present investigation clearly indicates the isolation, molecular characterization and sequence analysis (through Bioinformatics tools) of *Bacillus subtilis* strain KPA and its industrial applications.

Key words- *Bacillus subtilis*, Phylogenetic tree, RNA secondary structure, RNAeval Web server, RNAfold Web server.

Introduction

Livestock are major sources of fecal pollution that can introduce human pathogens, as well as chemical pollutants, into soil and water. Food borne diseases are major problems in developing countries. Bacteria, especially *Bacillus* species are one of the causative agents for the continuous increase in the illness around the developing countries. Phenotypically the genus *Bacillus* is a large, heterogeneous group of gram-positive, aerobic, endospore-forming, motile with peritrichous flagella and rod-shaped bacteria. The phenotypic diversity of the genus has been well documented, and nearly every imaginable phenotype is represented in one species or another¹. *Bacillus* sp. is widely distributed in the natural environments. They have unique ability to inhabit a variety of extreme and contaminated environments. Bacteria belonging to *Bacillus* sp. can be found mostly in alkaline environments such as soda soils, soda lakes, neutral environments and deep-sea sediments. Their tolerance to stresses are attributed to external shield, which is the cell membrane and internal enzymatic system, besides its spore coat which protects it against physical and chemical agents^{2,3,4}. The genus *Bacillus* encompasses a variety of phenotypically heterogeneous species exhibiting physiological and metabolic

diversity. Enzymes are biocatalysts that perform chemical reactions and are commercially exploited in the food, pharmaceutical and chemical industries. Enzymes produced by mesophilic organisms function in a narrow range of pH, temperature, and ionic strength. Moreover, the technological application of enzymes under demanding industrial conditions makes the currently known enzymes un recommendable. Thus, the search for new microbial sources for the enhanced production of enzymes is a developing area in the field of Biotechnology. Bacteria belonging to *Bacillus* sp. are by far the most important source of several commercial microbial enzymes. They can be cultivated under extreme temperature and pH conditions to give rise to products. There have been a number of reports on isolation, identification and characterization of microbial communities from different environmental sources. The historical method for identification of unknown bacteria is dependent on the comparison of an accurate morphologic and phenotypic description of new strains with the description of known isolates using Bergey's Manual of Systematic Bacteriology. Frequently, there would be no perfect match and a judgment would have to be made about the most probable identification which could vary among laboratories⁵. A new standard approach was discovered for identifying bacteria by comparing 16S rRNA gene^{6,7,8}. 16S rRNA gene analysis method has advantages over phenotypic identification due to its improved accuracy. Therefore, the present research was concerned to isolate a new strain of *Bacillus* species from poultry farm faeces soil sample by morphological, biochemical characteristics and 16S rRNA gene sequence analysis. The present study was also carried out to analyse 16S rRNA gene sequence of the novel strain using different Bioinformatics tools.

Materials and Method

Collection, Isolation and Screening of sample

Poultry faeces sample was collected from poultry farm of Guduvanchery, Tamil Nadu (India). Faeces were brought to the laboratory in aseptic condition. One gram of sample was suspended in 9 ml of saline and mixed vigorously to make uniform suspension. After that soil samples were serially diluted up to 10^{-5} and 0.1ml of aliquots were spread over nutrient agar plates from 10^{-5} dilution. The plate was incubated at 37°C for 24 h. Pure strain was picked out and purified by repeated streaking on nutrient agar slants. The culture was streaked on slants and kept in incubator at 37°C for 24 h and were preserved in slants at $4\pm 2^\circ\text{C}$.

Organism identification

Purified isolate was characterized by Biochemical analysis using Indole test, Methyl Red test, Voges Proskauer test, Citrate utilization test, Catalase test, Urease test, Oxidase test and Amylase test (according to the Bergey's Manual of Systemic Bacteriology). Gram staining and Motility test were performed under Morphological test

Genomic DNA isolation

Two ml of bacterial culture were centrifuged at 6000 rpm for 5 minutes. The supernatant was discarded. One ml of UniFlex™ Buffer 1 and 10 µl of RNase were added to the pellet obtained. Mixed well by pipetting and incubated for 30 minutes at 37°C in a water bath. To the lysed samples 1 ml of 1:1 phenol: chloroform was added and mixed well. The samples were centrifuged at 10,000 rpm for 15 minutes at room temperature. The aqueous layers were separated in a fresh 1.5 ml vial. To the aqueous layer 1 ml of UniFlex™ Buffer 2 were added and mixed well by pipetting. The mixture was centrifuged at 12,000 rpm for 15 minutes at room temperature. The supernatant was discarded. To the pellet 500 µl of 70% ethanol were mixed. Again it was centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was discarded. The pellet was air dried for about 10-15 minutes till the ethanol evaporate. The pellet was resuspended in 50-100 µl of UniFlex™ Elution Buffer. DNA was stored at -20°C .

PCR amplification and sequence of 16S rRNA

The 16S ribosomal RNA was amplified by using the PCR (ependorfep.Gradient) with *Taq* DNA polymerase and primers 27F (5' AGTTTGATCCTGGCTCAG 3') and 1492R (5' ACGGCTACC TTGTT ACGACTT 3'). The conditions for thermal cycling were as follows: denaturation of the target DNA at 94°C for 4 min followed by 30 cycles at 94°C for 1 min, primer annealing at 52°C for 1 min and primer extension at 72°C for 1 min. At the end of the cycling, the reaction mixture was held at 72°C for 10 min and then cooled to

4°C. PCR amplification was detected by agarose gel electrophoresis and visualized by alpha image gel doc after ethidium bromide staining.

Purification of amplified product

PCR sample is taken in fresh vial and 5 µl of 3M Sodium acetate solution (pH-4.6) and 100 µl of absolute ethanol were added into it. The vial was mixed thoroughly. The vial was kept at -20°C for 30-40 minutes to precipitate the PCR product. Then it was centrifuged at 10,000 rpm for 5 minutes. 300 µl of 70% ethanol were added to the pellet, without mixing, and the centrifugation was repeated at same rpm. The pellet was air dried until the ethanol effervescence is removed. The pellet is suspended in 10 µl of sterile distilled water⁹.

Sequencing of PCR product

The PCR product obtained was sequenced by an automated sequencer (Genetic Analyzer 3130, Applied Biosystems, and USA). The same primers as above were used for sequencing. The sequence was compared for similarity with the reference species of bacteria contained in genomic database banks, using the NCBI BLAST available at [http:// www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/).

RNA Secondary structure prediction

RNA secondary structure prediction at 37°C was performed to determine the stability of chemical or biological molecules or entities of the isolate. Folds, ProbKnot, Mountain Plot, Entropy, Partition and Minimum Free Energy (MFE) of the sequence were calculated by RNAeval Web server and RNAfold Web server. FTG server tool was used to determine the % content of G+C. Pictogram was visualized to identify sequence alignment.

Neighbor-joining tree analyses of *Bacillus* 16S rRNA gene for sequence comparisons

Phylogenetic relationship of the isolate with other *Bacillus* species were inferred from phylogenetic comparison of the 16S rRNA sequences using Clustal W 1.8¹⁰. Phylogenetic trees were inferred using the neighbor-joining (NJ) algorithm in Molecular Evolution Genetic Analysis (MEGA) software version 4.0¹¹.

Results

Morphological and Biochemical test analysis

The morphological and biochemical characteristics of the isolate was studied (Table 1 and Fig.- a, b and c). The isolated bacterial strain was identified as *Bacillus* sp. based on the taxonomical characteristics.

Table 1: Shows the Morphological and Biochemical test report

S.No.	Tests	Result
1	Morphology	Rod shaped
2	Gram Staining	Positive
3	Motility	Positive
4	Indole	Negative
5	Methyl Red	Negative
6	Voges –Proskauer	Positive
7	Citrate utilization	Positive
8	Urease	Negative
9	Catalase	Positive
10	Amylase	Positive
11	Oxidase	Negative

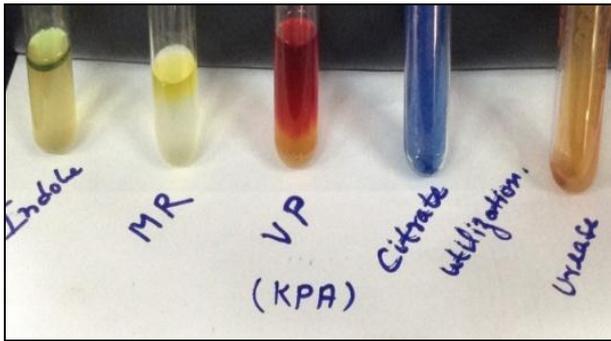


Fig-a: Shows few Biochemical

Test results

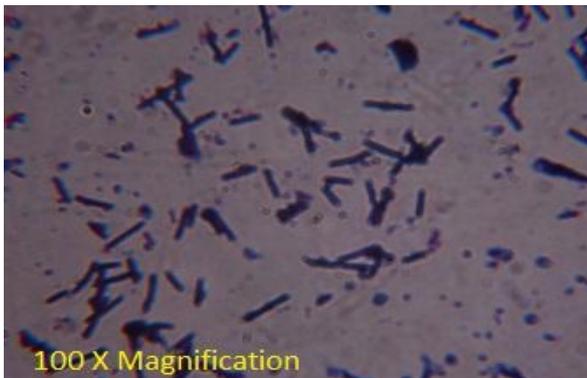


Fig-b: Shows Gram Staining



Fig-c: Shows Starch hydrolysis

PCR amplification and sequencing of 16SrRNA

Genomic DNA of the isolate was visualized under UV. The amplicon of 483 bp was observed using PCR amplification. In the present study, 16S rRNA gene sequencing of the isolate was investigated. The isolate was identified as *Bacillus subtilis* strain KPA by comparing the similarity with the reference species of bacteria contained in genomic database banks, using the NCBI BLAST. The comparison showed that the similarities of 16S rRNA gene sequences were 99%. The identities of strain KPA were determined by comparing them with the available sequences of the strains and with high scored rRNA sequences in BLAST search. The novel isolated sequence was deposited in GenBank (Accession number-KC918878), maintained by NCBI, USA.

RNA Secondary structure prediction

The optimal secondary structure with a minimum free energy (MFE) of -192.10 kcal/mol was represented in Fig-d. Mountain plot representation and entropy for each position were also determined (Fig-e). A mountain plot represents a secondary structure in a plot of height versus position, where the height is given by the number of base pairs enclosing the base at given position i.e. loops correspond to plateaus (hairpin loops are peaks), helices no slopes. Fig-f, g, h, i and j represent Folds, ProbKnot, Partition, % G+C (55.1%) content and Pictogram of the sequence respectively. Folds predict the lowest free energy structure in a set of low free energy structures for a sequence. ProbKnot predict a secondary structure of base pairs including pseudo knots. Partition performs a partition function calculation on a single sequence to calculate base pair probabilities.

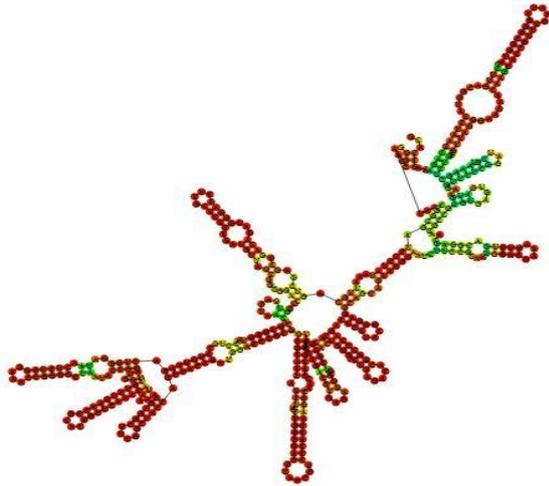


Fig-d: Shows RNA Secondary structure of *Bacillus subtilis* strain KPA with minimum free energy of -192.10 kcal/mol.

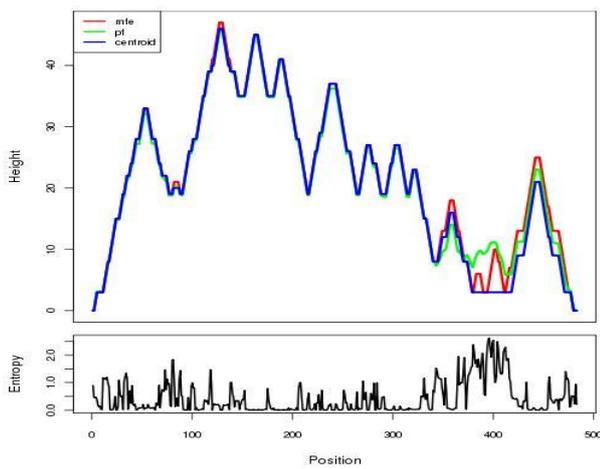


Fig-e: Shows Mountain plot and Entropy for each position

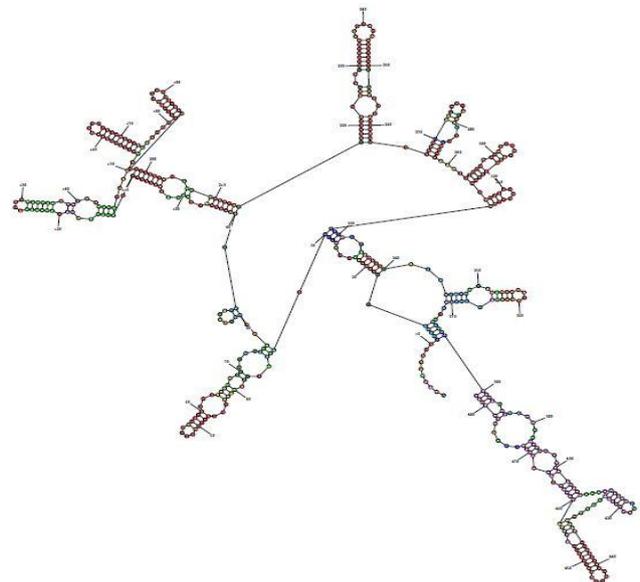


Fig-f: Shows Folding of the 16S rRNA sequence

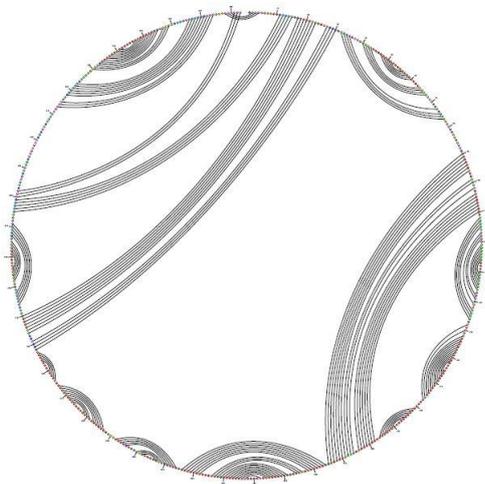


Fig-g: Shows Secondary structure of base pairs, including pseudo knots

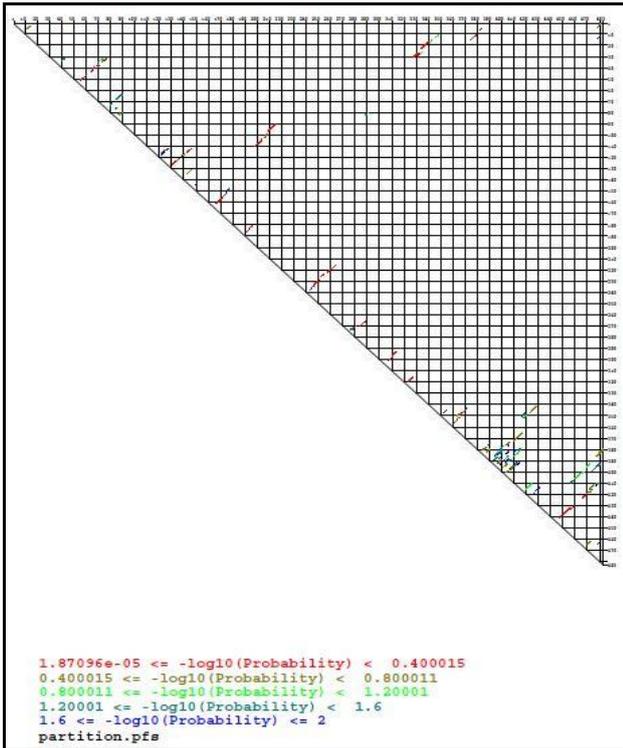


Fig-h: Shows Secondary structure Partition

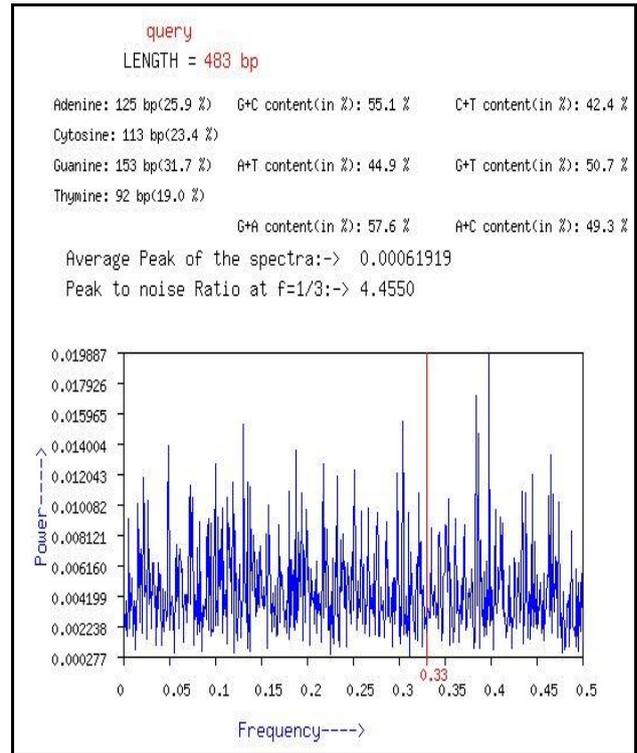


Fig-i: Shows total % G+C content

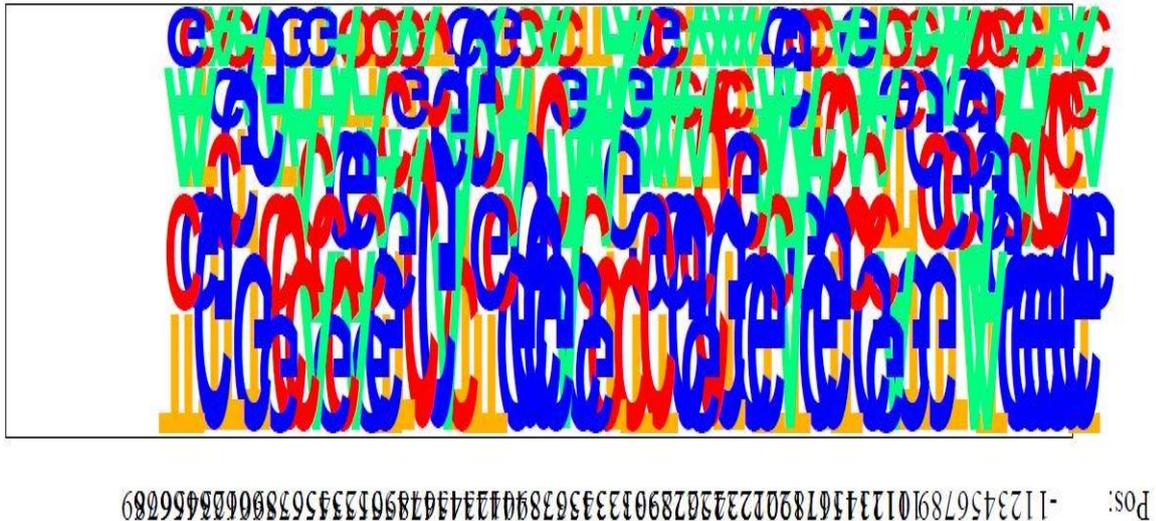


Fig-j: Shows Pictogram analysis

Phylogenetic tree of strain KPA 16S rRNA

A Neighbor- joining tree of *Bacillus* 16S rRNA sequences, including different strains of *Bacillus* species, clustered all the isolates belonging to the previously identified species to the corresponding species (Fig-k).

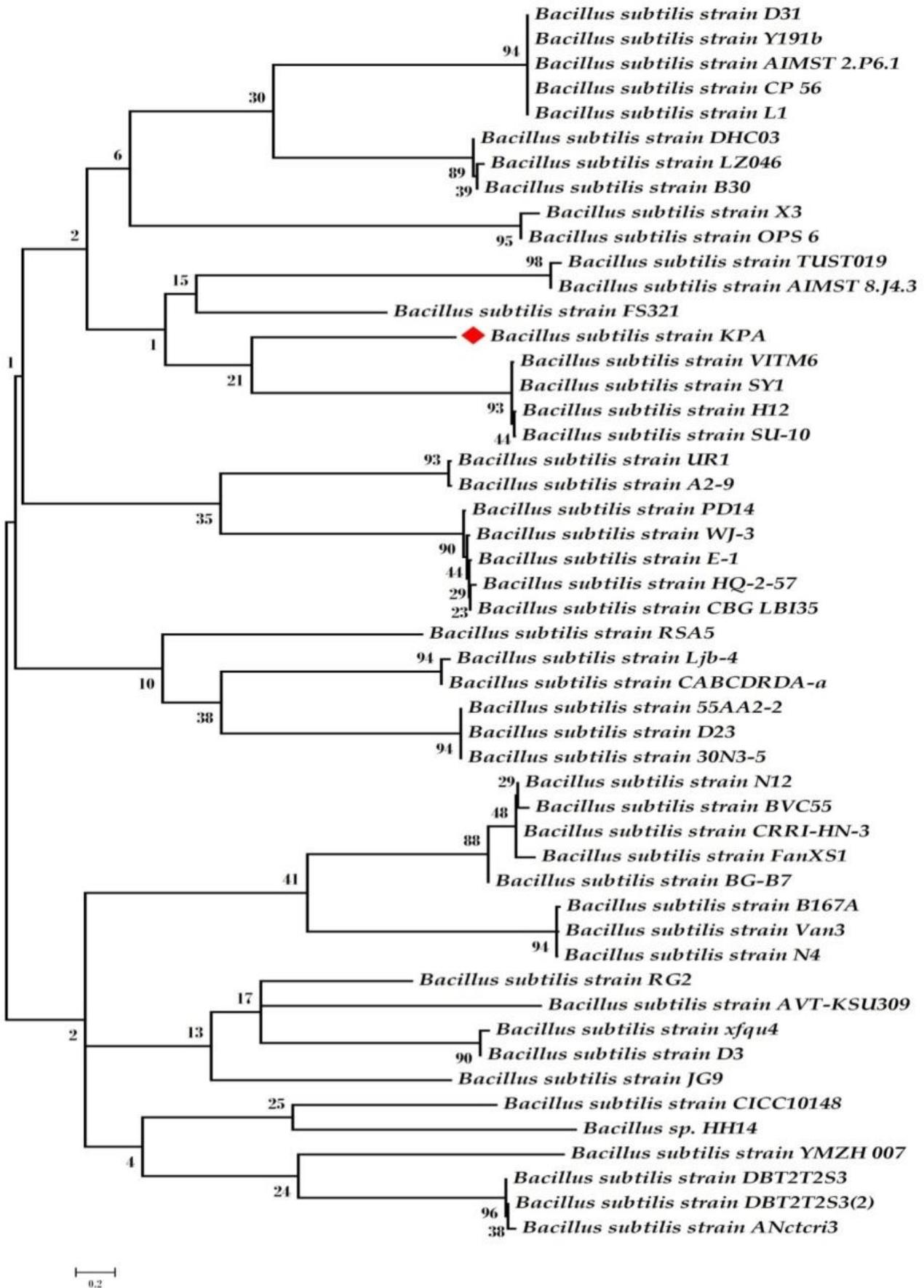


Fig- k: Shows Dendrogram depicting the phylogenetic relationship of strain KPA

Discussion

The presence of microorganisms in extreme stress conditions or contaminated environment facilitates their use in different biotechnological applications, as their enzymatic systems are encoded by genes which could be up-regulated for use in different industries¹². Bacteria are the most dominant group of enzyme producer. Bacteria belonging to *Bacillus* sp. are by far the most important source of several commercial microbial enzymes. They can be cultivated under unfavorable conditions to give rise to products that are in turn stable in a wide range of harsh environments. Previous researches had been done to identify the enzymes producing bacteria based upon the traditional methods. There are two major drawbacks of traditional methods of bacterial identification. First, they can be used only for organisms that can be cultivated *in vitro*. Second, some strains exhibit unique biochemical characteristics that do not fit into patterns that have been used as a characteristic of any known genus and species. The traditional identification of bacteria on the basis of phenotypic characteristics is generally not as accurate as identification based on genotypic methods¹³. Comparison of the bacterial 16S rRNA gene sequence has emerged as a preferred genetic technique. 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, 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 purposes¹⁴. 16S rRNA gene consists of highly conserved regions which describe the level of phylogenetic relationships. 16S ribosomal DNA (rDNA)-based molecular identification could achieve identification, for reasons including its universal distribution among bacteria and the presence of species-specific variable regions. This molecular approach has been extensively used for bacterial phylogeny, leading to the establishment of large public-domain databases and its application to bacterial identification, including that of environmental and clinical uncultured microorganisms, unique or unusual isolates and collections of phenotypically identified isolates¹⁵. The right taxonomic position of an environmental isolate could be accurately assigned to identify its novelty; this could only be done by the use of advanced molecular techniques¹⁶. In some cases, phenotypic identification alone did not verify the right taxonomic position and seemed truly necessary in case of *Bacillus* sp. and relatives¹⁷. Many studies used the natural sources as one of the medium constituents such as rice bran, wheat flour, corn bran, corn starch and orange peels to support growth of different bacteria to produce different enzymes¹⁸. *Bacillus subtilis* isolate is found to produce significant amount of keratinase under optimized conditions using horn meal as a substrate in the presence of peptone as nitrogen and dextrose as carbon source¹⁹. Members of the genus *Bacillus* were found to be predominant and a prolific source of alkaline protease²⁰. As it is clear from the previous reports that *Bacillus subtilis* is an important source of industrially important enzymes so in view of this the first part of this study was dedicated to find the position of new strain of bacteria isolated from poultry farm via morphological and biochemical identification. Then the potential of 16S rRNA sequencing was evaluated to rapidly identify *B. subtilis* strain KPA. Sreedevi and Reddy²¹ isolated new strain of Phytase producing *Bacillus* sp. through 16S rRNA gene sequencing which was 98% similar to *Bacillus subtilis*. In the present investigation the new strain of *Bacillus subtilis* was isolated from poultry farm with similarity percentage of 99% from other strains of the same genus and species. Reports of several workers showed that *Bacillus* sp. was considered as prime producer of keratinase²². Jahan *et al*²³ characterized keratinolytic bacteria isolated from poultry waste. According to the study, *Bacillus* Z4 is a potent producer of keratinase, which can be used for production of the enzymes at large scale. Mehta *et al*²⁴ isolated new strain of *Bacillus* species with keratinolytic activity using Microscopic, Biochemical and 16S rRNA gene sequencing. New strain of *Bacillus subtilis* was identified and characterized from poultry farm using 16S rRNA gene sequencing²⁵. The 16S rRNA gene is now used as a framework for the modern classification of bacteria including *Bacillus* sp. However, 16S rRNA gene sequences sometimes show limited variation for members of closely related taxa (Fox *et al*²⁶) due to the conserved nature of the gene. In such cases, DNA sequencing of certain housekeeping genes can provide more sensitive DNA sequencing subtyping than 16S rRNA sequencing for a number of bacterial species²⁷. Minimum free energy for the prediction of optimal secondary structure is the method for searching the structure with stable energies. First a dot matrix analysis is carried out to highlight complementary regions (diagonal indicates succession of complementary nucleotides). The energy is then calculated for each predicted structure by summing negative base stacking energies. Using one sequence can determine structure of complementary regions that are energetically stable. Minimum free energy value determines it as a stable model. As a DNA sequence based identification scheme for *Bacillus*²⁸, we consider 16S rRNA sequences appropriate for the identification of poultry farm *Bacillus* sp. The isolate was examined as *Bacillus subtilis* strain KPA depending on their

taxonomic positions. The phylogenetic analysis and similarity analysis of the new strain undoubtedly reflects diversity within the targeted DNA regions.

Conclusion

Though several studies have been performed from various sources and environment, only relatively very few studies have been carried out on isolation of *Bacillus* species from poultry farm. It is clear from the present and previous reports that 16S rRNA gene sequence information has an expanding role in the identification of bacteria. Economically valuable enzymes can be commercially produced on large scale from novel isolated strains of *Bacillus* species. In future, 16S rRNA gene sequencing will continue to be the standard and accurate method for identification of most bacteria which will produce a new era for the production of industrially important enzymes at large scale. Further research is necessary to determine the potential of valuable enzymes produced by strain KPA in the field of Biotechnology and Pharmacy. Another research should be continued to find out the improved methods of molecular characterization of this strain other than 16S rRNA gene sequencing.

Acknowledgement

The authors wish to acknowledge Department of Plant Biology and Biotechnology, Loyola College for fully supporting this research activity.

References

1. Priest FG, Goodfellow M, Todd C. A numerical classification of the genus *Bacillus*. *J Gen Microbiol* 1988, 143, 1847-1882.
2. Popham DL, Sengupta S, Setlow P. Heat, hydrogen peroxide and UV resistance of *Bacillus subtilis* spores with increased core water content and with or without major DNA-binding proteins. *Appl Environ Microbiol* 1995, 61, 3633-3638.
3. Nicholson WL, Munakata N, Horneck G, Melosh HJ, Setlow P. Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. *Microbiol Mol Biol Rev* 2000, 64(3), 548-572.
4. Riesenman PJ and Nicholson WL. Role of the spore coat layers in *Bacillus subtilis* pore resistance to hydrogen peroxide, artificial UV-C, UV-B and solar UV radiation. *Appl Environ Microbiol* 2000, 66, 620-626.
5. Sussman JI, Baron EJ, Tenenbaum MJ, Kaplan MH, Greenspan J *et al.* Viridans streptococcal endocarditis: clinical, microbiological, and echocardiographic correlations. *J Infect Dis* 1986, 154, 597-603.
6. Bottger EC. Rapid determination of bacterial ribosomal RNA sequences by direct sequencing of enzymatically amplified DNA. *FEMS Microbiol Lett* 1989,65, 171-176
7. Kolbert CP and Persing DH. Ribosomal DNA sequencing as a tool for identification of bacterial pathogens. *Curr Opin Microbiol* 1999, 2, 299-305.
8. Woese CR, Stackebrandt E, Macke TJ, Fox GE. A phylogenetic definition of the major Eubacterial taxa. *Syst Appl Microbiol* 1985, 6,143-151.
9. Shah KP, Chandok HK, Rathore P, Sharma MV, Yadav M, Nayariseri SA. Screening, isolation and identification of Polygalacturonase producing *Bacillus tequilensis* strain EMBS083 using 16S rRNA gene sequencing. *Europ J Biol Sci* 2013, 5(1), 09-13
10. Thompson JD, Higgins DG, Gibson TJ. CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Res* 1994, 22, 4673-4680.
11. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* 2007, 24, 1596-1599.
12. GomaaOM and Momtaz OA. 16S rRNA characterization of a *Bacillus* isolate and its tolerance profile after subsequent subculturing. *Arab J Biotech* 2007, 10 (1), 107-116.
13. Krieg NR and Garrity GM. On using the manual, In: Bergey's manual of systematic bacteriology, Second edition, 2001, pp.15-19 (Garrity G. M., ed.), Bergey's Manual Trust, Springer.

14. Patel JB. 16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory. *Mol Diagn* 2001, 6, 313–321
15. Drancourt M, Bollet C, Carlouz A, Martelin R, Gayral JP, Raoult D. 16S Ribosomal DNA Sequence Analysis of a Large Collection of Environmental and Clinical Unidentifiable Bacterial Isolates. *J Clin Microbiol* 2000, 38(10), 3623-3630.
16. Yumoto I, Kusano T, Shingyo T, Nodasaka Y, Matsuyama H, Okuyama H. Assignment of *Pseudomonas* sp. strain E-3 to *Pseudomonas psychrophilia* sp. nov., a new facultatively psychrophilic bacterium. *Extremophiles* 2001, 5, 343-349.
17. Guinebretiere MH, Berge O, Normand P, Morris C, Carlin F, Nguyen C. Identification of bacteria in pasteurized zucchini purees stored at different temperatures and comparison with those found in other pasteurized vegetable purees. *Appl Environ Microbiol* 2001, 67(10), 4520-4530
18. Joo HS and Chang CS. Production of protease from a new alkalophilic *Bacillus* sp. I-312 grown on soybean meal. Optimization and some properties. *Process Biochem* 2005, 40, 1263-1270.
19. Kumar R, Balaji S, Uma TS, Mandal AB, Sehgal PK. Optimization of influential parameters for extracellular keratinase production by *Bacillus subtilis* (MTCC9102) in solid state fermentation using horn meal—a biowaste management. *Appl Biochem Biotechnol* 2010, 160, 30–39.
20. Deng A, Wu J, Zhang Y, Zhang GQ, Wen TY. Purification and characterization of a surfactant-stable high alkaline protease from *Bacillus* sp. B001. *Bioresour Technol* 2010, 101, 7100-7106
21. Sreedevi Sand Reddy BN. Identification of Phytase producing Bacteria C43 isolated from cattle shed soil samples of Hyderabad, A.P. *Helix* 2013, 1, 238-242
22. Lin X, Kelemen DW, Miller ES, Shih JCH. Nucleotide sequence and expression of kerA, the gene encoding a keratinolytic protease of *Bacillus licheniformis* PWD-1. *Appl Environ Microbiol* 1995, 61, 1469-1474.
23. Jahan Z, Khan SN, Hoq MM. Screening of keratinolytic bacteria from poultry wastes. *Bangladesh J Sci Ind Res* 2010, 45(3), 261-266,
24. Mehta RS, Jholapara RJ, Sawant CS. Isolation of a novel feather-degrading bacterium and optimization of its cultural conditions for enzyme production. *Int J Pharm Pharm Sci* 2014, 6 (1), 194-201
25. Khusro A, Aarti C, Preetamraj JP, Panicker SG. Antibacterial activity of different solvent extracts of garlic against new strains of pathogenic bacteria: An *In vitro* study. *Int J Appl Biol Pharm Technol* 2013, 4(4), 316-321
26. Fox GE, Wisotzkey JD, Jurtshink JP. How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. *Int J Syst Bacteriol* 1992, 42, 166–170.
27. Mollet C, Drancourt M, Raoult D. rpoB sequence analysis as a novel basis for bacterial identification. *Mol Microbiol* 1997, 26, 1005–1011.
28. Goto K, Omura T, Hara Y, Sadaie Y. Application of the partial 16S rDNA sequence as an index for rapid identification of species in the genus *Bacillus*. *J Gen Appl Microbiol* 2000, 46, 1–8.

www.medipharmsai.com