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RESEARCH ARTICLE

MICROBIOLOGICAL AND MOLECULAR CHARACTERIZATION OF SULPHUR OXIDIZING *PSEUDOMONAS* SP.PRK786 ISOLATED FROM CATTLE MANURE COMPOST.

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Abstract

Rural based cattle manure compost inhabitant bacteria (sulphuroxidizing- *Pseudomonas* sp.PRK786**)** were characterized. The microbial physiologyaspect shows:chloramphenicolsensitive, gram-negative, non-motile, non-endospore forming, and transparent yellow color colonies were obtained. Besides the following biochemical characteristics such as:nitrate reducing,glucose dependent and non-pyocyanin pigments producing colonies were shown in thiosulphate agar medium. Moreover, it is significantly reducing the pH of the thiosulphate broth from 8.0 to 1.5.The sulfur oxidizing behavior of *Pseudomonas* sp.PRK786 was determined by sulphate ions accumulation in culture supernatant. Results, ~60% of sulphates are precipitated (without the formation of sulfur globules). Molecular aspects: It carries the ~4.5Mb size genomic DNA (A_{260}/A_{280} ratio - 1.52), 1285bp 16S rRNA gene (16S rRNA-PCR amplified product size was 1500bp), the 16S rRNA gene sequences showed 100% similarity with *Pseudomonas* sp.database, the free energy of consensus sequence of 16S rRNA was -396.80 kcal/mol and EMBL-EBI - accession number is HG931346.
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Introduction

Cattle manure compost (CMC) (mixture of dung and urine in the ratio of 3:1; approximately 88% water) is the daily dumped waste plant matters with undigested fecal residue of cow's. It is a good natural fertilizer because it contains a variety of essential nutrients for plant growth (generally, It contains crude fiber, crude protein, cellulose, hemicellulose and minerals such as N, K, S, traces of P, Fe, Co, Mg, P, Cl, Mn, etc.[28] Dark greenish brown colored often after exposure to air and sunlight. Inside of the compost very hot even we cannot touch, produce an unpleasant odor might be due to the methane, hydrogen sulphide and ammonia. It might utilize for biogas production and cow manure is used to line the walls of rustic houses caked anddried as a cheap fuel for burial ground. Olden days Indian peoples are drunk the cow urine for medicinal purpose because it is cure several diseases like cardiac, kidney problems,indigestion, stomachache, edema, and etc.Ayurveda has mentioned five important substances from cow namely Urine, Dung, Milk, Ghee, and Curd termed as "Panchagavya."[1] Now a days cow-dung based microbial fuel cell (CDFC) being innovated and got Gandhian Young Technological Innovation Awards 2013. Furthermore, five morphological and physiological distinct bacteria'swere screened from CMC[30].Sulphur oxidizing and phosphorus solubilizing *B. subtilis* was isolated from cow dung and it produced important enzymes like amylase and cellulose [28].Various bacterial strains were isolated from CMC such as *Bacillus* sp. [9, 27] and methanogenicbacteria [21].In a study,single strain of sulphur oxidizingbacteria (SOB)

Halothiobacillusneapolitanus[25]was isolated and identified in the cattle manure waste-based and sewage sludge compost. [12] Research revealed that pathogenic *Listeria monocytogenes*[3],*Escherichia coli, salmonella,* coliform bacteria and fecal coliform, were included in CMC; they were derived from the intestinal tract of animals [7]. Antimicrobial activity fresh cow urine andantifungal effect compost-inhabiting bacteria to reduce the infection in plants have been reported [24]. The pathogens like *T. harzianum, Sclerotiumrolfsii, F. oxysporum,* etc. also inhabitant in CMC [20].Until recently, few studies were carried out in the member of *pseudomonas* in cattle manure compostand well evident that Dr. C. S Nautiyal, director of Central Institute of Medicinal and Aromatic Plants and National Botanical Research Institute, briefed about the forced to work on cow dung microbes said on Oct 28, 2013. By this way, in our study to focus on microbiological and molecular characterization of sulphur oxidizing *pseudomonas* sp.PRK786 from cattle manure compost.

Materials and Methods

Locations and Collection of CMC

Cattle manure compost samples were collected from cattle farms in around Nagamalaipudhukottai, Madurai -19, Tamilnadu. Generally, the cow dung was dumped nearby forms approximately 1 to 2 months of period. During that time cow dung was converted into compost (ready to apply in the crop fields); Samples were withdrawn from one-month-oldcompost, the 50cm depth (~ 43^0C) ofthe pits and their transfer into sterilized polyethylene bags to store in room temperature.

Microbiological Analysis of CMC

The inhabitants of microbes in the cattle manure compost sample were enumerated by serial dilution and pour plating method, using suitable dilutions and the colonies werecounted and expressed as No.of colonies.

Isolation of SOB from CMC

The compost was composed of cattle feces, urine, and sawdust. For isolation of sulphur oxidizing bacteria, thiosulphate medium was employed by enriching with 5% of sodium thiosulphate. The thiosulphate medium contained 5.0 g $Na_2S_2O_3$, 0.1 g K_2HPO_4, 0.2 g $NaHCO_3$, 0.1 g NH_4Cl and 5.0 g of glucose in 1000 ml distilled water, with pH 8.0. Bromocresol purple used as an indicator. The colonies were maintained on thiosulphate slants. The pure cultures were labeled and used for characterization and further studies [23].

pH reduction and Conservation of SOB

The acquired isolate was inoculated in the thiosulphate broth with initial pH adjusted to 8.0. After 40 hours of incubation, the final pH of the growth media was measured using pH pen.According to Bergey`s Manual of Determinative Bacteriology, the microorganisms (0.5 ml of each pure culture was transferred into cry tubes tag along by 40% glycerol) for long term maintenance, Glycerol stocks were stored at -20^0C, whereas pure culture requires strains were incubated at 40^0C for 28 hours.

Gram Staining

A band full of overnight culture was smudged onto a microscopic slide. It was dried and heat fixed, then smear was discolored with crystal violet solution for 1 min. After rinsing the slide, flooded with Gram's iodine solution and was kept for 60 Sec. Again the slide was washed with water and decolorized using 95% ethyl alcohol for 30 Sec. And counter staining was done by flooding with safranin for 20 Sec. The slide was again washed with tap water and dried. The preparation was observedunder the light microscope.

Motility Test

A little volume of petroleum jelly was placed edges of the cover glass. Shift various loopsfuloff the 24 hours culture of the organism was placed in the center of the cover glass. Downturn slides were thumb over the cover glass, cover the culture drop and quickly inverted. The finished preparation was observed under light microscope.

Examination of Endospores

Isolated SOB has grown in Luria BertaniBroth medium for 2 days were suspended in 5µl of sterile 0.09% NaCl on a glass slide and smeared with a cover slip. Endospores were observed as glossy bodies in the cells under the contrast microscope [19].

Nitrate Reduction Test

SOB was inoculated into nitrate broth and incubated at 37^0C for 24 hours. After the incubation presence of nitrate was tested by adding 3 drops of sulphanilic acid and alpha Napthalamine reagent to the tubes. A distinct yellow color turned red which indicates the reduction of nitrate [10].

Determination of Sulphur Oxidizing ability of SOB

The sulfur oxidizing ability of the isolated strain was determined by the sulfate accumulation in a cultured medium. The thiosulphate medium with sodium thiosulphate 5 % (w/v) was added to a test tube, which was sealed with a silicone stopper and sterilized. Preincubated bacteria were inoculated into the medium. After 3 days of incubation at 37°C, the sulfate concentration produced in the medium was determined. Sulphuroxidizing ability of sulphur oxidizing bacteria were examined according to [31].

Antibiotic Susceptibility

Estimation of the susceptibility of SOB by MIC (Minimal Inhibitory Concentration), prepared nutrient agar plates were incubated after inoculation of SOB using L- rod and wells with different concentration of standard antibiotics (ampicillin, streptomycin and chloramphenicol- concentration (µg/µl) ranges -10µl, 20µl & 30µl).

Isolation, Purification and Identification Genomic DNA from SOB

The method of genomic DNA isolation and purification from sulphur oxidizing bacteria was adapted by [22, 13,&14]. The purity of DNA achieved byDNA = A_{260}/A_{280}ratio, quality was evaluated on 1.2% Agarose Gel Electrophoresis and it is used as substrate for PCR amplification.

PCR and 16S rRNA gene Sequencing Analysis

PCR reaction was achieved in a thermal cycler (eppendorf). The reaction volume 50 µl contains of 20 ng of genomic DNA (A_{260} nm 0.04 OD corresponds to 20ng) , 2.5 U of Taq DNA polymerase, 5 µl of 10 X Taq buffer (100 mMTris-HCl, , 500 mMKCl pH-8.3) and 200µMdNTP. 10 pmoles each universal primers (forward -8F_S5957_003_G01 (544 bp) '5- GAGAGCG----GCATTCAA-3'and reverse-1492R_S5957_001_H01 (769 bp) '5-ACGTATTC---GAGCCCGG-3') and 1.5mM $MgCl_2$. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer.PCR ampliconwas separated on a 1.2% agarose gel in 1x TAE at 100V for one hour. The gel was stained with ethidium bromide and visualized with GELdoc system.

Construction of Phylogeny

The 16S rDNA gene sequence was used to carry out BLAST with the NCBI gene bank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 4. [26, 6, 15&29]

Modeling of RNA secondary structure

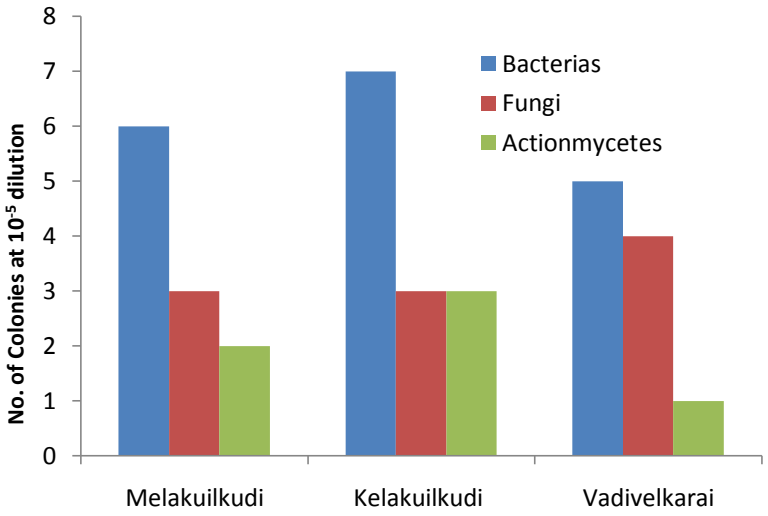
The NUPACK 3.0 tool (http://www.nupack.org)was also used for designing of secondary structure of 16S rRNA gene. The program to predict the minimum free energy (MFE) secondary structureRNA or DNA sequences. Algorithms are assigned in terms of ordered complexes,each corresponding to the structuralband, $\Omega(\pi)$, of all linked polymer graphs with no crossinglines for a specific commanding, π, of a set of strands [2].

NCBI accession number

The 16S rRNA gene sequences of *Pseudomonas* sp. PRK786has been submitted to EMBL-EBIunder accession number: HG931346.

Results and Discussion

The study was governed at three locations in around Nagamalaipudhukottai, Madurai -19, Tamilnadu. In every spot of the sample area, we are followed uniform collection procedure and the collected cow dung samples were brought to the laboratory for further investigations. The brought samples were enumerated the total population of microbes and the results are presented in Graph. 1. The secret information that the total microbial communities ranged from 6 to 18 colonies in the CMC (Cattle manure Compost) and the towering microbial communities (13 colonies) were observed in kelakuilkudi dumbed CMC.The total fungal and actinomycetes inhabitants were fluctuated form 3 to 4 and 1 to 3 colonies were observed all samples respectively at (10^{-5} dilution).



Graph.1 Enumerated the Total Population of Microbes in Different Locations

Pick out the individual colonies (diaphanous, wrinkled and with unclear edges) and streak into thiosulphate agar medium with bromocresol purple for isolated the SOB, the colonies were show in Fig.1, The yellow color zone around the colonies indicates acidic pH through bromocresol purple turned into yellow by oxidization thiosulphate into sulphuric acid. Besides, the observed yellow color is not a pyocyanin pigments, because we are not supplied the magnesium chloride, potassium sulfate, and glycerol in the thiosulphate agar medium. The pathogenic *Pseudomonas aeruginosa*onlyproduces a water soluble, blue color pigment called pyocyanin.[11] Additionally, only growth was observed in the thiosulphate medium contains glucose (as a carbon source), so it's might belongs to chemolithoheterotorbic oxidizer.

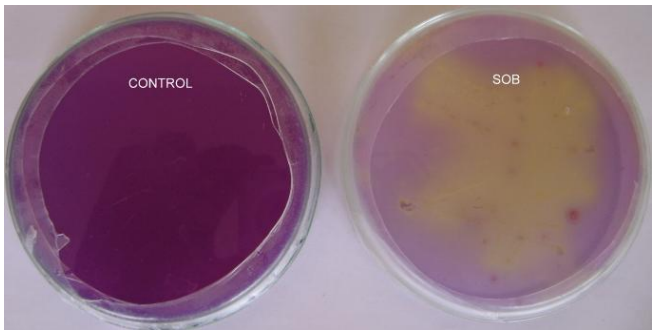


Fig.1 Isolated SOB form Cattle Manure Compost using Thiosulphate agar medium

The purple color turned to yellow color followed by orange color builded on the pH reduction, from the initial pH of 8.0, could reduce to pH 1.5 shown Fig.2 that was in accommodation to [31].

Table.1 Effects of pH reduction by SOB on the Thiosulpate medium

S.No	Time (Hrs)	pH	Color of medium
1	0	8.0	Purple
2	20	4.9	Orange
3	30	3.2	Yellow
4	40	1.5	Pal yellow

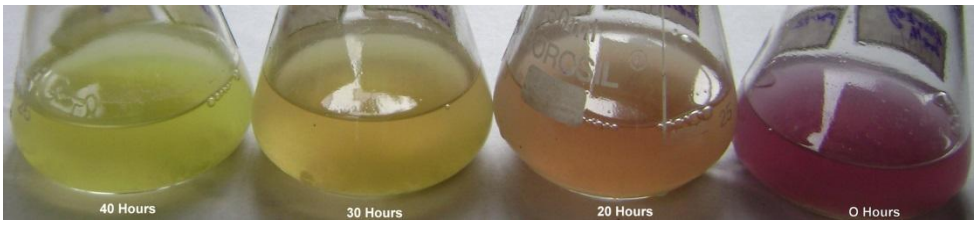


Fig.2pH reduction of Thiosulphate Broth by SulphurOxidizing Bacteria

Under observation of SOB was gram-negative rods, non-motile and non-endospore forming bacteria. These above characteristics were also coined in[17].However, most of them have one or more polar flagella are providing motility. The non-motile behavior was except here and it's have ability to reduce the nitrate into nitrite by the action of nitrite reductase enzyme the result shown in Fig.3, the results showed closest similarity to [8,16] based on that activity it may be used for nitrogen-fixing bacterium in future. The sulfur oxidizing behavior of SOB was determined by sulphate accumulation in culture supernatant, after addition $Bacl_2$ powder into the culture supernatant, a minute ago ~60% of sulphates are precipitated (without the formation of sulfur globules) in the form of $BaSO_4^-$ against the 5% of K_2SO_4 standard. The sulphate accumulation might be due to the basis of their potential to oxidation inorganic sulfur compounds, generally the production sulphate from thiosulphate by the SOB requires periplasmicthiosulphate – oxidizing multienzyme complex (SOX Complex). Many number of sulfur-oxidizing bacteria (SOB) that convert thiosulfate to sulfate with and without the formation of sulfur globules as intermediate[18].Especially, *Pseudomonas stutzeri* group of obligately heterotrophic bacteria's could oxidized the thiosulfate to tetrathionate under anaerobic condition [5]

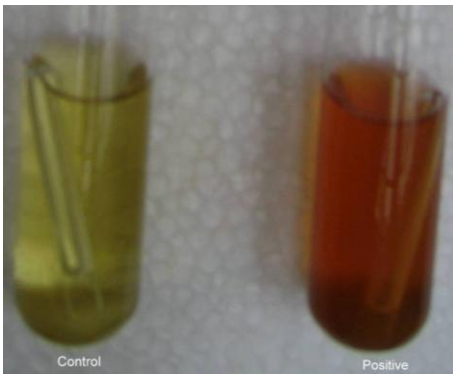


Fig.3 Nitrate Reduction test

Fig.4Screening of Antibiotic Susceptibility of SOB by MIC

Predominantly *pseudomonas* sp. is unaffected by majority of the beta-lactam antibiotics such as penicillin, cephalosporin etc. Here, screenings of antibiotics susceptibility of SOB were tested against ampicillin, streptomycin and chloramphenicol and finding were denoted as MIC (Minimal Inhibitory Concentration). From the results chloramphenicol (µg/µl) (10µl- 4.5mm, 20µl- 5mm & 30µl-8mm) was significantly inhibit the growth of manure born SOB than the other antibiotics. In addition chloramphenicol to inhibits both gram positive and negative bacteria's. Eligible zone of inhibition was not observed against ampicillin, because it is closely related to penicillin

(β-Lactams are a group of antibiotics binds to inhibits the cell wall synthesizing enzymes like carboxypeptidases and transpeptidases) and streptomycin are slightly inhibits the growth of SOB[4].

Isolation of genomic DNA from sulphur oxidizing bacteria wassuccessfully by modified alkaline lysis method. 0.7M $CaCl_2$was used to remove the RNA residues without disturb the genomic DNA. A_{260}/A_{280} ratio of SOB genomic DNA was 1.52 designates the purity of DNA. Generally, *Pseudomonas* genomes typically have sizes between 6 and 7 Mbp. Our results coincident to 75% homology. Control and SOB Genomic DNA are shown in figure.5, as revealed from the figure, the ~4.5Mb size genomic DNA from SOB of showed definite bands, while the marker bands showed uncertain due to the concentration of DNA.

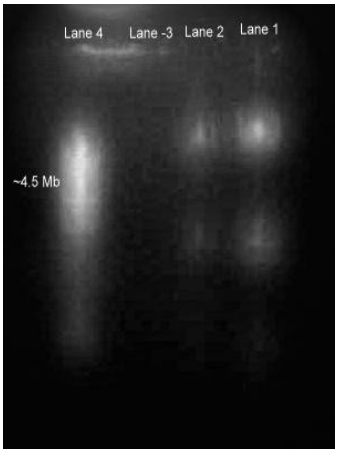


Fig. 5 Agarose gel electrophoretic pattern (1.2%) of genomic DNA of sulphur oxidizing bacteria. Lane-1 & Lane-2 are markers (E.coliDBH10 Genome ~4.6 Mb (isolated) and lambda DNA- 0.465 Mb), Lane-3 Negative Control and Lane-4 SOB Genomic DNA.

Generally, portions of the rDNA sequence from distantly homologous organisms are wonderfully similar. rRNA have been used extensively to determine taxonomy, phylogeny and contains small ribosomal subunit plays an important role in the protein synthesis. Hence, molecular level characterization of SOB based on the 16S rRNA. In the present study, consensus sequence of 1285bp 16S rRNA gene was generated from forward and reverse sequence data using aligner software.The 16S rRNA ribosomal PCR amplified product size was 1500bp shown in Fig 6.

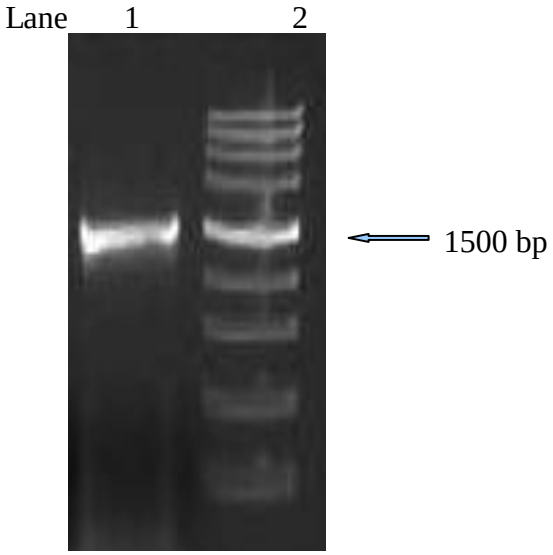


Fig.6Agarose gel image of 16SrDNA amplicon
(Lane 1: 16S rDNAamplicon band Lane 2: DNA marker)

The 16S rRNA gene sequences showed 100% similarity with *pseudomonas* sp. the existing NCBI database. Fig. 7. The evolutionary history was inferred using the Neighbor-Joining method [26]. The bootstrap consensus tree inferred from 500 replicates [6] is taken to represent the evolutionary history of the taxa analyzed [10]. 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 (500 replicates) is shown next to the branches [10]. The evolutionary distances were computed using the Kimura 2-parameter method [15] and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were 1285 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 [29].

RNA secondary structure (1285 bases of 16S rRNA of *Pseudomonas* sp. PRK786) was drawn using online NUPACK 3.0 tool. The Free energy of consensus sequence of 16S rRNA (-396.80 kcal/mol) showing fig.8. We are not comparing with other genetically related *pseudomonas* mentioned on phylogenetic tree. Generally, the free energy variation occurs due to the mutation in the 16S rRNA sequences.

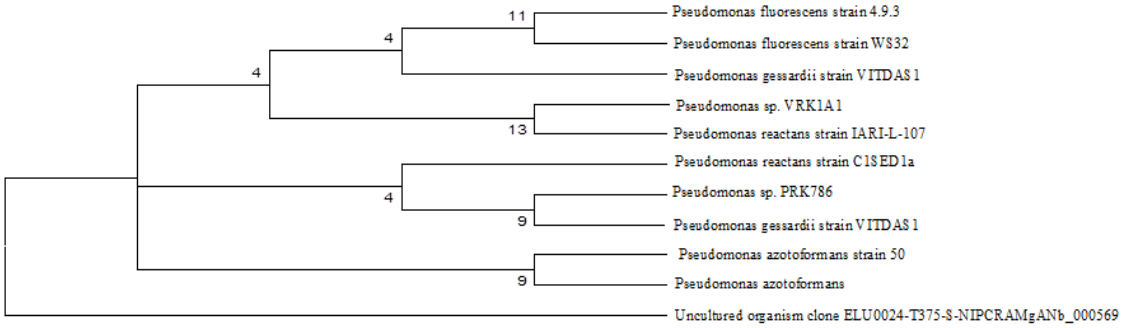


Fig.7 Phylogenetic Position between Strains and its closest relatives in the Genera *Pseudomonas* based on the 16S rDNA.

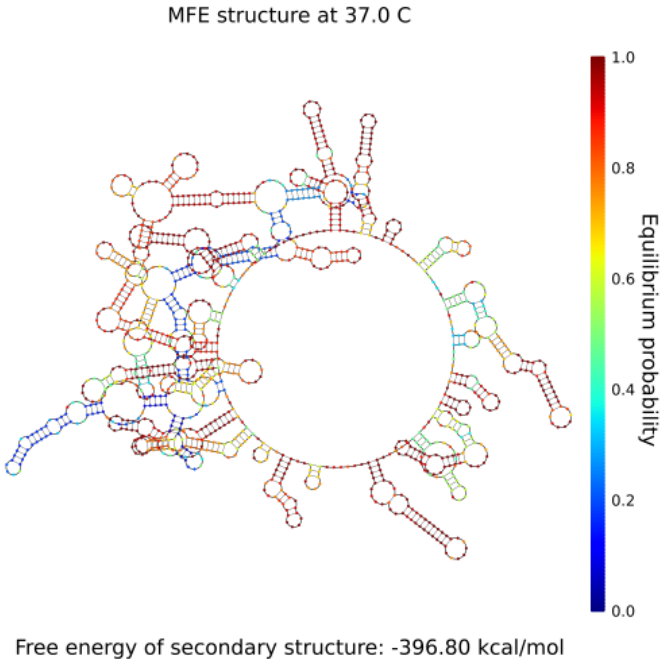


Fig.8 Modeling of RNA Secondary Structure of 16rRNA of *Pseudomonas* sp. PRK786 (-396.80 kcal/mol)

Conclusion

The collected cow dung samples have been involved to microbiological and molecular characterizations, revealed that cow dunghave highly inhabitants of bacteria, fungi, and actinomycetes.The transparent, glucose dependent and non-pyocyanin pigments producing colonies (SOB) were show inthiosulphate agar medium, significantly reducing the pHfrom 8.0 to pH 1.5 and nitrate into nitrite. In addition, chloramphenicolsensitive manure born SOBwas capable to oxidize the thiosulphate into sulphate and the size genomic DNA is ~4.5Mb. In view of taxonomy, phylogeny1285bps 16S rRNA gene (PCR amplified product size was 1500bps) was provided it belong to *Pseudomonas* sp.andconsensus sequence of 1285bps were submitted to EMBL-EBI and assigned *Pseudomonas* sp. PRK786(accession number: HG931346).

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