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Journal of Advanced Scientific Research

J.Adv.Sci.Res, 2010, 1(2); 46-52
Research Article

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ISSN: 0976-9595

Characterization of Antioxidant activity of *Streptomyces* species VITTK3 isolated from Puducherry Coast, India

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ABSTRACT

The aim of the present study was to evaluate antioxidant activity of intracellular and extracellular metabolites of *Streptomyces* species isolated from marine soil sample collected at the Bay of Bengal coast of Puducherry, India. The sample was serially diluted and plated on Starch casein agar and the white powdery colonies formed indicated the growth of actinomycetes and selectively isolated by plated on ISP 1 medium. The broth culture of the same media was used for fermentation process and intracellular and extracellular metabolites were extracted using solvents acetone and ethyl acetate. The brown colored extract obtained was dissolved in water and screened for DPPH radical scavenging activity. The extracellular metabolite showed 96% inhibition at 5mg/ ml, however intracellular metabolites showed only 22% of inhibition at 5 mg/ ml of intracellular metabolites and the inhibition was compared with the standard antioxidant ascorbic acid which showed 97% inhibition at 5mg/ ml concentration. The potential strain was characterized by polyphasic molecular taxonomic approach. The strain was identified as *Streptomyces* species and designated as *Streptomyces* sp. VITTK3. The 16 S rDNA sequence was submitted to the Genbank under the accession number GU808333. The phylogenetic tree by neighbor joining Kimura method showed 97% similarity with *Streptomyces cebimarensis* with the bootstrap value of 20. Secondary structure and the restriction sites were predicted for the strain *Streptomyces* sp. VITTK3.

Keywords: DPPH, *Streptomyces*, Phylogenetic tree, Antioxidant activity.

INTRODUCTION

In recent years, marine actinomycetes have emerged as a rich source of novel compounds. Perhaps the first notable discovery of a biologically active marine natural product was reported over 50 years ago. The marine environment was once thought to have high salt, poor nutrition and less microbial growth. On the contrary, soil microbes are widely regarded to live in a much more crowded and competitive environment. The enormous biodiversity of marine microorganisms might have been the reason for the interest to study them. Marine environmental conditions are extremely different from the terrestrial environment, it turned out that marine microorganisms have different characteristics from those of their terrestrial counterparts therefore, might produce different types of bioactive compounds. The tremendous biochemical diversity of marine microorganisms and their biotechnological potential is becoming more and more recognized, not only by microbiologists but also by the pharmaceutical industry. Several new companies focus on the discovery of more and more effective drugs based on natural products of marine microorganisms. The focus on the physiology and the potential of bioactive substances of non-cultivable marine microorganisms is an important challenge at present and for the future. The

estimated taxonomical diversity of marine microorganisms in general indicates the powerful potential of novel bioactive substances produced in aquatic ecosystems[1].

Streptomycetes and related actinomycetes continue to be useful sources of novel secondary metabolites with a range of biological activities that may ultimately find applications as anti-infectives, anti-cancer agents or other pharmaceutically useful compounds[2]. Thus, screening, isolation and characterization of promising strains of actinomycetes producing potential antibiotics has been a major area of research by many groups worldwide for many years[3, 4].

In the course of screening for new metabolites, several studies were carried out in order to isolate new *Streptomyces* species from different habitats. Recent studies are focusing on the response of antioxidant system of bacteria, which is important in terms of biotechnology, such as *Streptomyces* growth in various oxidative stress conditions[5].

Antioxidants play an important role in inhibiting and scavenging free radicals, thus providing protection to humans against various infections and degenerative diseases[6]. Dietary antioxidants have already been shown to be protective against chronic diseases. Some degradation processes of aerobic living organisms are mediated by reactive oxygen species, such as superoxide anion radical (O_2), hydrogen peroxide radical and hydroxyl radical. Particularly O_2 has been considered as a causative species to induce inflammation[7]. Nutritional antioxidant deficiency also leads to oxidative stress, which signifies the identification of natural antioxidative agents present in the diet consumed by the human population. There are certain naturally occurring antioxidants that can give protection to liver from hepatotoxins. Modern research is now directed towards natural antioxidants from plants and microorganisms and serves as safe therapeutics[8]. The objective of this present study is to establish antioxidant potential of novel actinomycetes strain by evaluating both intracellular and extracellular metabolites.

MATERIALS AND METHODS

Collection of soil sample

Marine soil samples were collected from Bay of Bengal coast of Puducherry, India. About 15 grams of soil sediments were collected and stored at 4°C until further use. The soil samples were air dried for preventing bacterial contamination. Then the sample was serially diluted to till 10^{-6} dilution by adding 1 gram of soil to 10ml of distilled water[9].

Isolation of Actinomycetes

About 0.1 ml of each dilution was pour plated on Starch casein agar (SCA). After one week incubation at room temperature, the plates were analyzed for white powdery colonies[10]. These colonies were sub-cultured on International Streptomyces Project-1 (ISP-1) agar plate.

Fermentation of culture broth

The strain was inoculated into 50 ml medium containing ISP-1 broth with 25% of sea water and 75% of distilled water with the pH of 7.2 and incubated for 7 days in rotary shaker (110 rpm) at 28°C. After incubation the turbidity was checked. This culture was used as inoculums for fermentation of broth culture in bulk. These inoculums (10%) were transferred into 500 ml of ISP-1 broth in 1 L of Erlenmeyer flask. The inoculated cultures in the production medium were incubated for 10 days on a rotary shaker (110 rpm) at 28°C.

Extraction of intracellular and extracellular metabolites

After fermentation the broth culture was centrifuged at 10,000 rpm for 10 minutes. The supernatant and the pellet were separated. To the pellet, acetone was added and to the supernatant, equal volume of ethyl acetate was added. Both were kept for overnight in rotary shaker. The pellet extract was separated from the pellet and kept

for evaporation to get crude extract. The supernatant extract was separated by collecting the solvent layer and it was rotary evaporated to get a concentrated brown colored viscous extract. Both intra and extracellular metabolites were dissolved separately in sterile distilled water. These were used to be further for antioxidant activity.

Antioxidant Activity

DPPH scavenging activity

Different concentration (0.1, 0.5, 1.0 & 5.0 mg/ml) of crude extracts (extracellular and intracellular metabolites) were dissolved in water and taken in tubes separately. Ascorbic acid was also taken in different concentrations (0.1, 0.5, 1.0 & 5.0 mg/ml) and used as a reference standard. DPPH (1, 1, Diphenyl-2-Picryl hydrazyl) 0.002% was freshly prepared in methanol. DPPH (2 ml) was added to each tube containing different concentrations of extracts (2 ml) and of standard solution (2 ml). It was shaken vigorously. They were then allowed to stand for 30 minutes for room temperature in dark place. The control was prepared without any extracts. Methanol was used for base line corrections in absorbance (OD) of sample measured at 517 nm. The below formula was used to interpret the value of the sample[11].

% Radical Scavenging Activity = [(control O.D – sample O.D) / control O.D] x 100

Strain identification

The DNA was isolated by HiPurA bacterial DNA isolation and purification kit (Himedia, India) and amplified by PCR using a master mix kit, Medoxmix (Medox, India) as per user manual. Universal 16S rRNA primers were used (Forward primer FC 27 and reverse primer RC 1492). The methodology for sequencing was adapted from earlier reports[12, 13] and the 16S rRNA was sequenced bi-directionally.

The 16S rRNA partial gene sequence obtained from the isolate was compared with other bacterial sequences by using NCBI BLAST search[14, 15] for their pair wise identities. Multiple alignments of this sequence with the sequences available in the data bank were carried out by Custal W 1.83 version of DDBJ (http://clustalw.ddbj.nig.ac.jp/top-e.html) and the phylogenetic tree were constructed in MEGA 4.0 version (http://www.megasoftware.net) using the neighbor-joining (NJ) method with 100 replicates as bootstrap value and NJ belongs to the distance-matrix method[16]. The nucleotide sequences were analyzed for estimating the number of nucleotide substitutions between sequences by Kimura method. The 16S rRNA sequence was submitted to the GenBank in, EMBL (Europe), and the (DDBJ) DNA Data Bank (Japan) under the accession number GU808333 for *Streptomyces* sp.VITTK3.

Secondary structure prediction and restriction site analysis

The RNA secondary structure of the isolate VITTK3 was predicted using Genebee online software by greedy method and the restriction sites of the DNA of the strain was analyzed by NEB cutter Version 2.0.

RESULTS AND DISCUSSION

Screening of actinomycetes isolated from marine sediment samples collected at the Bay of Bengal coast, Pudhucherry, India resulted in isolation of potential strain having antioxidant (DPPH scavenging) activity. The isolate produced white powdery colonies over Starch casein agar at the dilution of 10^{-4}. The isolate was subcultured and maintained on ISP 1 agar medium. Intracellular (acetone) and extracellular (ethyl acetate) metabolites extracted were tested for DPPH scavenging activity.

DPPH scavenging activity

The extracellular metabolite showed concentration dependent DPPH scavenging activity and it was increased linearly with gradual increase in concentration and exhibited 96% inhibition at 5 mg/ ml. Similar trend was observed with ascorbic acid used as positive control (97% inhibition at 5 mg/ ml) (Fig 1). It is clear from the results that the extracellular metabolite extrated form the isolate possesses significant DPPH scavenging activity comparable to standard ascorbic acid.

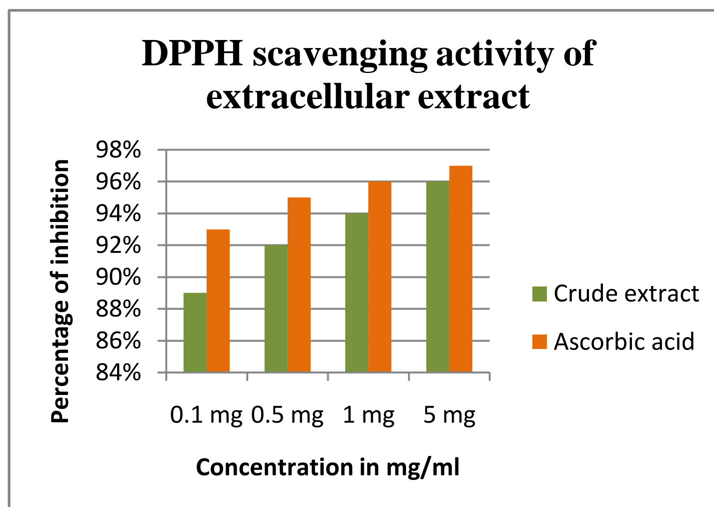
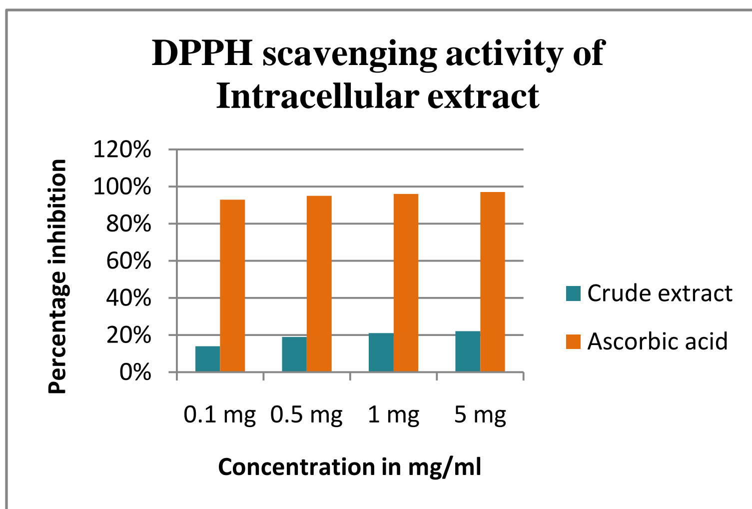


Fig.1: DPPH scavenging activity of extracellular extract of *Streptomyces* sp.VITTK3



The intracellular extract scavenged DPPH only to 22% at 5 mg/ ml and compared with that of positive control. At the same concentration ascorbic acid (5mg/ml) showed 97% DPPH scavenging activity. The results indicated the mild DPPH scavenging activity of intracellular extract when compared to the positive control (Fig 2).

Fig 2: DPPH scavenging activity of Intracellular extract of *Streptomyces* sp.VITTK3

It was reported that the crude butanol extract of two *Streptomyces* sp. isolated from soil samples collected in Western Ghats of Agumbe, Karnataka showed 58.71% and 59.97% DPPH scavenging activity at 0.5 mg/ml concentration [17]. Comparison of DPPH radicals scavenging activity of both extracellular and intracellular extracts revealed the effectiveness of extra cellular metabolite prepared from cell free supernatant of isolate using ethyl acetate solvent on scavenging DPPH radicals (Fig 3).

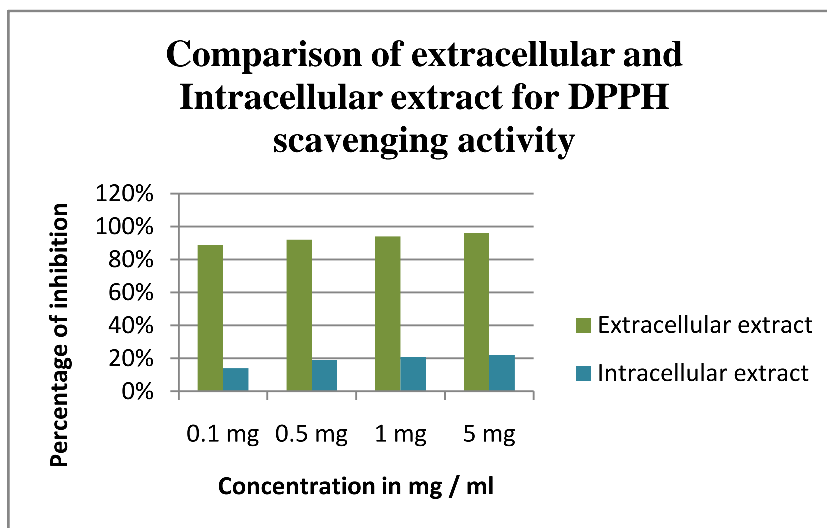


Fig 3: Comparison of extracellular and intracellular extract for DPPH scavenging activity of *Streptomyces sp.* VITTK3

Molecular taxonomy of the isolate

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   1 agagtttgat cctggctcag gacgaacgct ggcggcgtgc ttaacacatg caagtcgaac
  61 gatgaagccg cttcggtggt ggattagtgg cgaacgggtg agtaacacgt gggcaatctg
 121 ccctgcactc tgggacaagc cctggaaacg gggtctaata ccggatacga cacaggaagg
 181 catcttctct gtgtggaaag ctccggcggt gcaggatgag cccgcggcct atcagcttgt
 241 tggtggggtg atggcctacc aaggcgacga cgggtagccg gcctgagagg gcgaccggcc
 301 acactgggac tgagacacgg cccagactcc tacgggaggc agcagtgggg aatattgcac
 361 aatgggcgca agcctgatgc agcgacgccg cgtgagggat gacggccttc gggttgtaaa
 421 cctctttcag cagggaagaa gcgcaagtga cggtacctgc agaagaagca ccggctaact
 481 acgtgccagc agccgcggta atacgtaggg tgcgagcgtt gtccggaatt attgggcgta
 541 aagagctcgt aggcggcttg tcacgtcgga tgtgaaagcc cggggcttaa ccccgggtct
 601 gcattcgata cgggcaggct agagttcggt aggggagatc ggaattcctg gtgtagcggt
 661 gaaatgcgca gatatcagga ggaacaccgg tggcgaaggc ggatctctgg gccgatactg
 721 acgctgagga gcgaaagcgt ggggagcgaa caggattaga taccctggta gtccacgccg
 781 taaacgttgg gaactaggtg tgggcgacat tccacgtcgt ccgtgccgca gctaacgcat
 841 taagttcccc gcctggggag tacggccgca aggctaaaac tcaaaggaat tgacgggggc
 901 ccgcacaagc ggcggagcat gtggcttaat tcgacgcaac gcgaagaacc ttaccaaggc
 961 ttgacataca tcggaaagcc gtagagatac ggcccccctt gtggtcggtg tacaggtggt
1021 gcatggctgt cgtcagctcg tgtcgtgaga tgttgggtta agtcccgcaa cgagcgcaac
1081 ccttgttctg tgttgccagc atgcctttcg gggtgatggg gactcacagg agactgccgg
1141 ggtcaactcg gaggaaggtg gggacgacgt caagtcatca tgccccttat gtcttgggct
1201 gcacacgtgc tacaatggcc ggtacaatga gttgcgatgc cgtgaggtgg agcgaatctc
1261 aaaaagccgg tctcagttcg gattggggtc tgcaactcga ccccatgaag tcggagtcgc
1321 tagtaatcgc agatcagcat tgctgcggtg aatacgttcc cgggccttgt acacaccgcc
1381 cgtcacgtca cgaaagtcgg taacacccga agccggtggc ctaacccccct tgtggggagg
1441 gaatcgtcga aggtgggact ggcgattggg gacgaagtcg taacaaggta gccgtaccgg
1501 aagggtgcgg ctggatcacc ct
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The DNA was extracted from the isolate and it was amplified with universal forward and reverse primers. Then it was sequenced bi-directionally and yielded 1522 base pairs. Sequenced base pairs are as follows (Fig 4)

Fig 4: 16S rDNA sequence of *Streptomyces* sp. VITTK3 (1522 bp)

Construction of phylogenetic tree

NCBI BLAST search analysis showed that the sequence was 97% similar with the sequence of *Streptomyces cebimarensis* with the bootstrap value of 20. The 16S rDNA sequence was submitted to the GenBank, EMBL (Europe), and the DNA Data Bank (Japan) under the accession number GU808333. A neighbour-joining tree based on 16 S rDNA sequences showed that the isolate occupies a distinct phylogenetic position within the radiation including representatives of the *Streptomyces* family. A phylogenetic tree based on Kimura method also showed distinct position of the isolate (Fig 5).

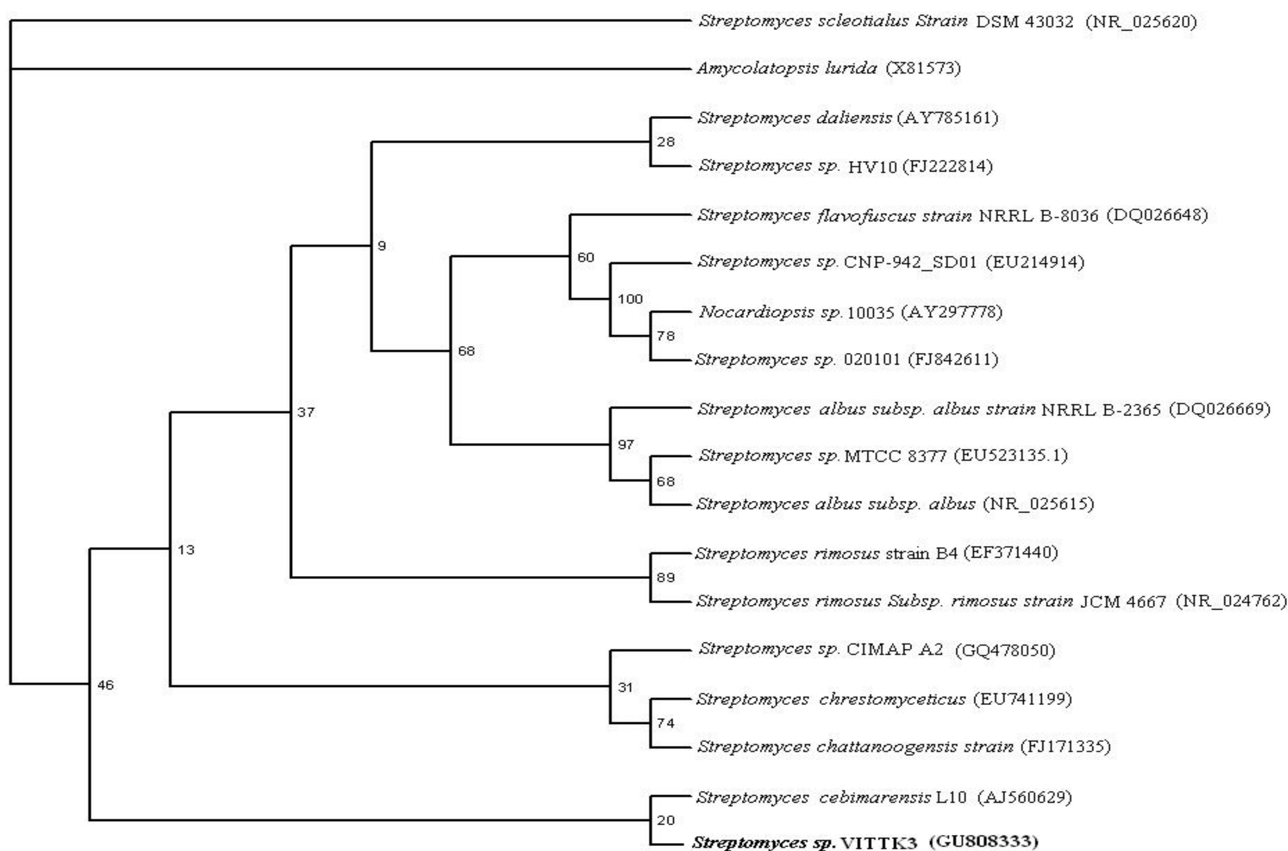


Fig 5: Phylogenetic tree view of *Streptomyces* sp.VITTK3

Based on the molecular taxonomy and phylogeny the strain was identified as *Streptomyces* and designated as *Streptomyces* sp. VITTK3.

Secondary structure prediction and Restriction site analysis

The RNA secondary structure was predicted for the16s r DNA sequence of *Streptomyces* sp. VITTK3 and shown in Fig. 6. It showed that the free energy of structure is -338.8 kkal / mol, threshold energy is -4.0 with cluster factor, conserved factor 2 and compensated factor 4 and conservativity is 0.8.

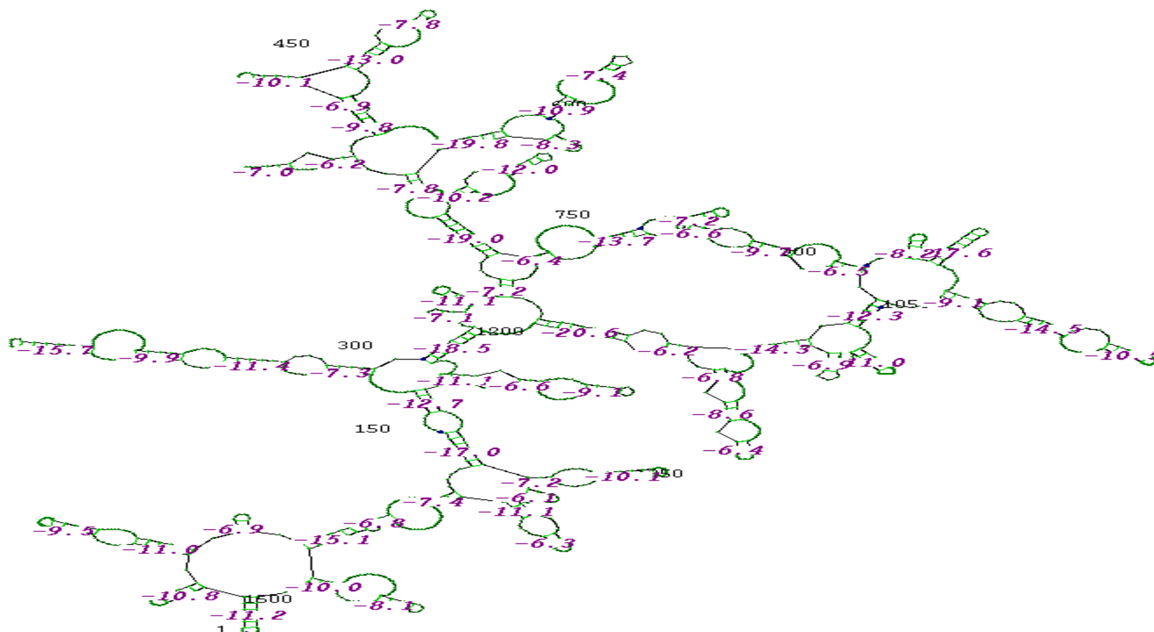
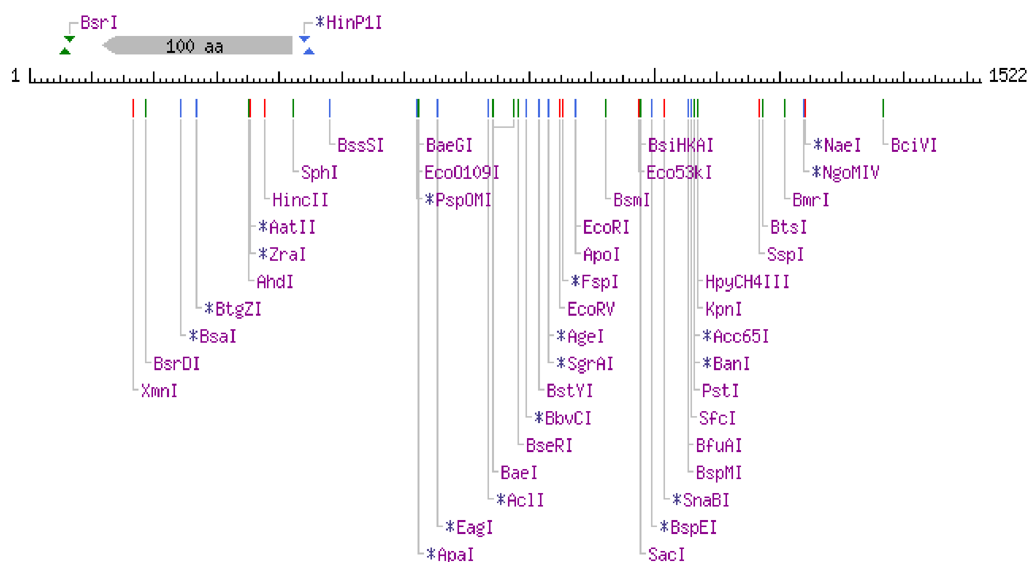


Fig 6: Secondary structure prediction of 16S rRNA sequence of *Streptomyces* sp.VITTK3



The prediction for 16S rDNA sequence of the strain VITTK3 showed the restriction sites for various enzymes such as Xmn I, BsrD I, Bsa I, BgZ I etc. (Fig 7). It showed GC and AT content of 59% and 41% respectively.

Fig 7: Restriction sites of the 16S rRNA sequence of *Streptomyces* sp.VITTK3

CONCLUSION

The results of this study indicated that *Streptomyces* sp. VITTK3 possess significant DPPH free radical scavenging activity. Based on the results it can be concluded that the isolate produces extracellular secondary metabolite capable of scavenging DPPH free radicals. However further studies are needed to identify the chemical nature of extracellular secondary metabolite produced by *Streptomyces* sp.VITTK3.

ACKNOWLEDGEMENT

The researchers thank the management of VIT University for providing the facilities to carry out this study.

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