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Investigation On Sugar Cane Field Actinomycetes Of Erode District.

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ABSTRACT

Actinomycetes are the member of gram positive organism which has very large species diversity around the environment. The sample was collected from sugarcane field and 4 actinomycetes strains were isolated which are named as SCF-1, SCF-2, SCF-3 and SCF-4 respectively. Among the isolated strain SCF-1 showed excellent growth than other strains, so identification of the strain SCF-1 was made by biochemical characterizations such as Starch hydrolysis, casein hydrolysis, etc., and molecular level characterization. Molecular level characterization was done with the help of 16S rRNA gene sequencing and compared with online database (BLAST tool) and it was identified as *Streptomyces coelicoflavus* species and the 16S rRNA sequence of the isolated species *Streptomyces coelicoflavus* was submitted to NCBI and received the submission Id as GenBank accession no. KP780807. The phylogenetic tree of the isolated strain *Streptomyces coelicoflavus* was constructed along with the closely related organisms (as per 16S rRNA gene sequence comparison)

Keywords: Actinomycetes, Isolation, Biochemical characterisation, Starch hydrolysis, Casein hydrolysis, PCR amplification, 16S rRNA gene sequence, BLAST, Phylogenetic tree.

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INTRODUCTION

Actinomycetes is member of group of gram positive bacteria[1] that possess interesting features [2] that includes *Streptomyces* family, which is the largest family that is isolated and identified under the actinomycetes group. *Streptomyces* species are saprophytic in nature and differ from other soil bacteria (Lacey, 1971). Soil is the main source for Actinomycetes [3] and there are about 100 genera of actinomycetes existing in environment and only small fraction of the Actinobacteria family was discovered [3][4]. Although, various *Streptomyces* species have been isolated from marine water [5][6][11] and lake water[7]. Most *Streptomyces* species are capable of forming pigments [10] which depends on the media used for culturing [8] and it also produces extra cellular hydrolytic enzymes by nature, which are secondary metabolites that sometimes able to inhibit potentially the other microorganisms (anti-microbial compounds) [12][13][14] .

Actinomycetes are the prokaryotes that are most valuable economically and biotechnologically [8]. DNA of Actinomycetes is of high G+C content of about 57 - 75 %. *Streptomyces* species are generally produces number of natural metabolites which are secondary and also sometimes primary metabolites, are best known antibiotics and other bioactive components that are used worldwide as pharmaceuticals like anti-cancer agent and anti-tumor agents (Berdy 1989) and agrochemicals and more than 70% of the naturally derived antibiotics that are currently in clinical use are derived from actinomycetes [9]. These microorganisms can also be able to regulate the synthesis of inorganic materials during its metabolisms [15].

The *Streptomyces* species has been classified based on classical microbiological and chemo taxonomical methods. *Streptomyces* species are strongly variable and hard for interpretation. For references standard resources such as Bergey's Manual of Systematic Bacteriology can be used in such cases. With PCR and DNA sequencing technologies, it is agreeable get closely related taxons, and it is successful in comparison to other methods.

In this work, we identified a new strain which was named as strain SCF - 1 and it is characterized biochemically, molecularly. Pair wise sequence of 16S rRNA gene sequence was conducted to identify closely related organisms with the help of BLAST tool of NCBI webpage [http://www.nih.nov.ncbi].

MATERIALS AND METHODS

Isolation of Actinomycetes

The actinomycetes were isolated from the soil sample collected from the Sugarcane field. It was purified using Decimal dilution technique and it was cultured in starch casein nitrate agar (Starch soluble - 10g/l, Casein - 0.3 g/l, KNO_3 -2 g/l, NaCl – 2 g/l, K_2HPO_4- 2 g/l, Mg_2SO_4 – 0.05 g/l, $CaCO_3$- 0.02 g/l, Agar – 30 g/l, pH ranges at 7), and the isolated strain was named as SCF - 1. And the strain SCF - 1 culture was maintained in starch casein nitrate agar medium at -20°C.

Characterization of isolated Actinomycetes strain SCF-1

Morphological features of strain SCF-1

The isolated strain SCF-1 was cultured on starch casein nitrate agar medium and incubated for 4 days and after incubation period the strain SCF - 1 was Gram stained to know the cell wall nature and observed. The strain was also observed with naked eye to know the morphological features.

Biochemical Characterization

Amylase was detected using Starch hydrolysis medium, production of Caseinase enzyme by casein hydrolysis test, MacConkey agar growth test, Indole production test, Methyl red test, Voges-Prauskauer test, Citrate Utilization tests were done.

Starch Hydrolysis Test

The Starch hydrolysis test is for determining the ability of microbes to produce extra cellular hydrolytic enzymes (Amylase) which degrades the polymeric carbohydrate starch into Glucose. Starch Agar Medium (starch - 2g/L, peptone - 5g/L, beef extract - 3g/L, agar -30g/L) was prepared and sterilized. The continuous streak inoculation technique was adopted; the sample organism was streaked in the centre of the plate and incubated for 24 hrs at 37ºC. After incubation the plate was flooded with Iodine solution for the observation of clear zone.

IMViC Test

On the basis of differentiation of the gram negative enteric bacteria from the family of enterobacteriaceae, the IMViC is performed. It includes four types of test such as (i) Indole production from tryptophan, (ii) Methyl Red, (iii) Voges Proskauer test, (iv) Citrate utilization test.

Indole Production Test

The Tryptone Broth (tryptone - 10g/L) was pre-sterilized and inoculated with the sample culture. The test tubes were incubated at 37ºC for 48 hrs, then Kovac's reagent is added and shaken vigorously for confirmation.

Methyl Red Test

The MR-VP broth (peptone - 7g/L, glucose - 5g/L, potassium phosphate - 5g/L) was prepared and sterilised perfectly, then inoculated with sample and incubated at 37ºC for 48hrs. And Control was maintained which fresh uninoculated medium. After incubation five drops of methyl red indicator were added and observed for color change.

Voges-Proskauer test

The MR-VP broth (peptone - 7g/L, glucose - 5g/L, potassium phosphate - 5g/L) was prepared and sterilised perfectly, then inoculated with sample and incubated at 37ºC for 48hrs. After incubation, few drops of Barrit's reagent were added and the tubes were gently shaked for 2 mins and incubated for 15mins for the complete reaction. And Control was maintained which fresh uninoculated medium. Further the color changes were observed.

Citrate Test

Citrate agar medium was prepared form readymade mix Simmon's Citrate Agar Medium, and sterilised and mad Slants in Test tubes and by stabbing and streaking method the samples were inoculated in the respective slants and incubated at 37ºC for 48 hrs. And Control was maintained which fresh uninoculated medium. The results were examined.

Casein Hydrolysis Test

Casein is the name for a family of related phosphoproteins which is a complex protein. This test is to determine the ability of microorganisms to produce proteolytic enzymes. The milk agar medium (skim milk powder-100g/L, peptone -5g/L, agar -15g/L) was prepared and sterilised and poured into the petri dishes. Inoculation of the sample as a single streak was done and incubated at 37ºC for 48hrs. The results were noted.

MacConkey Agar Test

MacConkey agar test is a differential test which supports only the gram negative bacteria. MacConkey Agar medium (peptone-17g/L, protease peptone-3g/L, lactose- 10g/L, bile slats- 1.5g/L, sodium chloride- 5g/L, neutral red- 0.03g/L, crystal violet- 0.001g/L, agar-13.5g/L) was prepared, sterilised and inoculated with organism. The results were observed after four days of incubation at room temperature.

DNA Isolation and Manipulation

The Isolated actinomycetes strain SCF-1 was grown for 5 days on starch casein agar plate. Then the spore was inoculated into the starch casein nitrate broth and incubated at room temperature for 4 days. After incubation culture broth was centrifuged at 10000 rpm for 10 minutes. The pellet of the centrifuged culture was used to extract Genomic DNA of isolated actinomycetes (Vijay kumar *et al*., 2010)[16]. And the isolated DNA was analysed by 1% agarose gel electrophoresis.

Amplification and sequencing of the 16S rRNA gene

PCR amplification of the 16S rRNA of the isolated actinomycetes strain SCF-1was performed using two universal primers, forward primer 27F-5'AGAGTTTGATCMTGGCTCAG 3' and reverse primer 1492R-5' TACGGYTACCTTGTTACGACTT 3'. The PCR reaction mixture contains 100 ng of template chromosomal DNA, 30 pmol of each primer, 200 µM of each dNTPs , 25 µl TE buffer, 2.5 units of Taq polymerase , 50 µl polymerase buffer, 1.5 mM $Mgcl_{2.}$ Amplification was done with initial denaturation step at 94°C for 2 min followed by 30cycles of amplification for 1 min at 94°C,1 min of annealing at 55°C, and 2 min of extension at 72°C and final extension step of 72°C for 2 min. This amplification was done with Eppendorf Thermo-cycler 96. The PCR product was analysed by agarose gel electrophoresis and visualised by ultraviolet (UV) fluorescence after ethidium bromide staining, the remaining amplified product was purified using a HiPurA™ PCR product purification spin kit. The 16S rRNA gene was sequenced on both strands (Sanger *et al*.1977).

Sequence identities and phylogenetic analysis

The BLAST (Basic Local Alignment Search Tool) program [http://www.nih.nov.ncbi]was employed in order to assess the degree of DNA similarities and identities. The partial 16S rRNA sequence of srainSCF-1was aligned manually with available *Streptomyces* (16S rRNA) sequences retrieved from GenBank using Clustal X version. Phylogenetic trees were inferred by using tree making algorithms, the neighbour joining and maximum likelihood algorithms from the Phylip package.

RESULTS AND DISCUSSION

Origin of strain SCF-1

The collection of sample from sugarcane field soil yielded colonies of isolated strain in starch casein nitrate medium in dilution of $10^{-2}$of soil sample. And it was named as strain SCF-1.

Fig. 1 - The isolated actinomycetes strain (after 1 day of incubation)

Characteristics of isolated strain SCF-1

Morphological features of strain SCF-1

The isolated strain SCF-1has white aerial mycelium with red pigment formation in the starch casein nitrate medium. It is also observed as Gram positive (G+) organism by Gram staining method.

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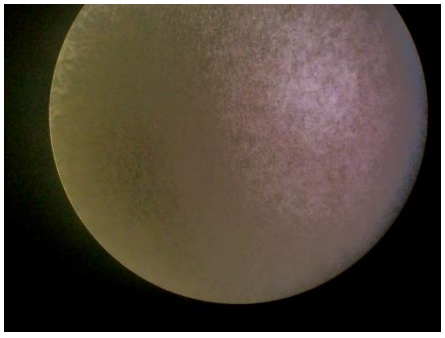

Fig. 2a Gram staining of the isolated strain SCF- 1 shows that the organism is gram positive.

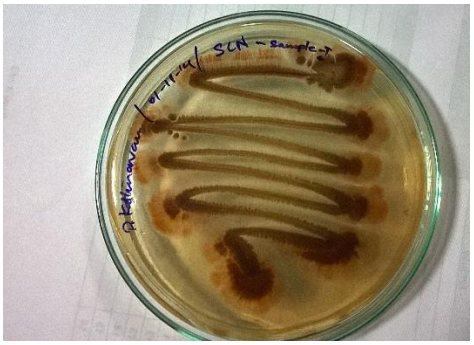

Fig. 2b shows the red pigment produced by the isolated strain SCF – 1 on starch casein nitrate medium.

Biochemical characteristics

Fig. 3a & 3b – starch hydrolysis test of isolated strain SCF –1

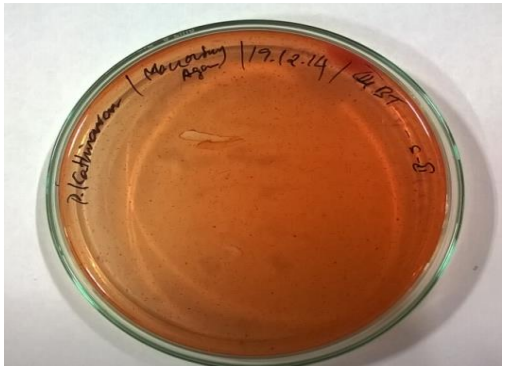

Fig . 4– growth ability of isolated strain SCF – 1 on MacConkey agar media

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The isolated strain SCF - 1 showed amylase activity (Fig. 3a & 3b), caseinase activity (Fig .4), which was confirmed by starch hydrolysis test, and casein hydrolysis test. MacConkey agar media growth test (Fig. 4), Indole production (Fig. 5), citrate utilization test (Fig. 6), Voges-Prauskouer test (Fig. 7), Methyl red test (Fig. 8), starch hydrolysis, and casein hydrolysis results were shown in the table 1.

Table 1- Biochemical characteristics of strain SCF-1

Sl. no	Name of the test	Result	Inference
1.	Starch Hydrolysis	+	Shows amylase production
2.	Growth on MacConkey agar	_	Confirmed as Gram +ve microorganism.
3.	Indole Production	_	It does not hydrolyses the Tryptophan in the medium by producing Indole.
4.	Methyl red test	+	Produces organic acids
5.	Voges-Prauskouer test	_	Does not undergoes glucose metabolism
6.	Citrate Utilization	_	Citrate is not utilized

('+' positive, '–' negative)

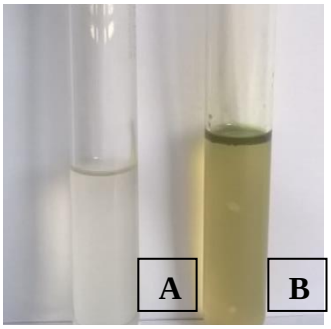

Fig.5 Indole test showing Control (A) and negative result in test (B),

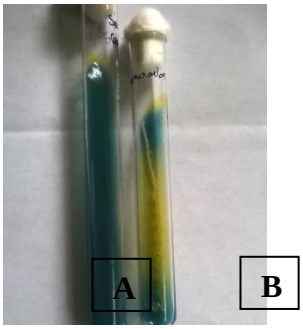

Fig.6 Citrate Utilisation Test showing Control (A) and negative result in Test (B)

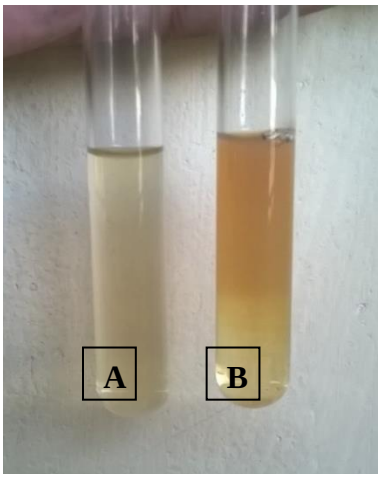

Fig. 7 Voges-Prauskouer test showing Control (A) and negative result for the test (B),

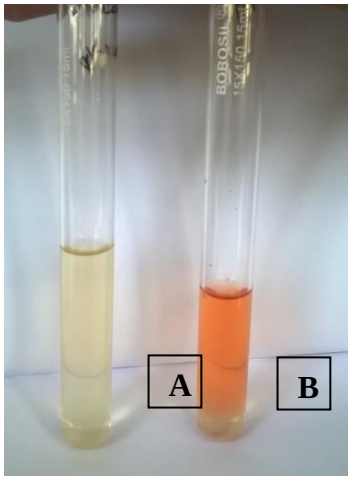

Fig.8 Methyl Red test showing Control (A) and Positive result for test (B).

Sequence Analysis

The molecular differences in species, (i.e.) to the nucleotide level was studied via multiple sequence global alignment. 16S rRNA sequence of strain SCF-1 compared with gene sequence (query sequence Icl 42679) of *Streptomyces* databases through BLAST tool. The results indicate that the pair wise aligned query sequence Icl 42679, with 16S rRNA gene sequence of the type strains *Streptomyces diastaticus* (NR 043486), *Streptomyces graminearus* (EF371437) and *Streptomyces fradiae* (AB184068), which shows 99% Sequence identity. And it was found to be *Streptomyces coelicoflavus* and the 16S rRNA gene sequence was submitted to

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NCBI and received the Genbank accession Id as KP 780807. The phylogenetic tree was constructed for isolated *Streptomyces coelicoflavus* and with closely related species, by online phylogenetic tree producing algorithm.

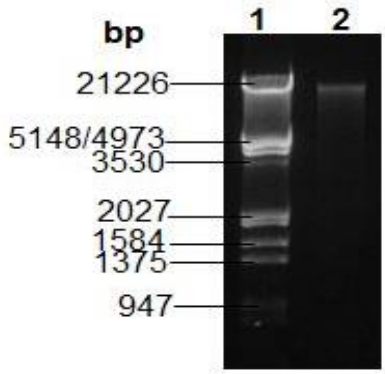

Fig. 9 Agarose Gel (1%) Photo of Genomic DNA of SCF- 1
Lane 1 – Lambda DNA/EcoRI + Hind III Digest Marker
Lane 2- Genomic DNA of SCF-1

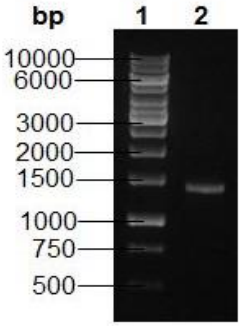

Fig. 10 Agarose Gel (1%) photo of PCR product of SCF-1
Lane 1 – 1Kb DNA Ladder
Lane 2 – PCR Product of SCF-1

GGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAACCACCTTCGGGTGGGGATTAGTG
GCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGAAACGGGGTCTAAT
ACCGGATACTGACCTGCCAAGGCATCTTGGCGGGTCGAAAGCTCCGGCGGTGCAGGATGAGCCCGCGGCC
TATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGC
CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGC
AAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGA
AGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG
GCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCGCGTCGGTTGTGAAAGC
CCGGGGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCT
GGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACT
GACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTG
GGCACTAGGTGTGGGCAACATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGA
GTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAA
TTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAGCATCAGAGATGGTGCCCCCCT
TGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCA
ACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAACTCTTCGGAGGTTGGGGACTCACGGGAGACCGCCGGG
GTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCT
ACAATGGCCGGTACAATGAGCTGCGATACCGCAAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGG
ATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCCTA

Fig.11 – represents the gene sequence of 16S rRNA gene of isolated strain SCF – 1 which found to be *Streptomyces coelicoflavus* (GenBank submission accession no. KP780807)

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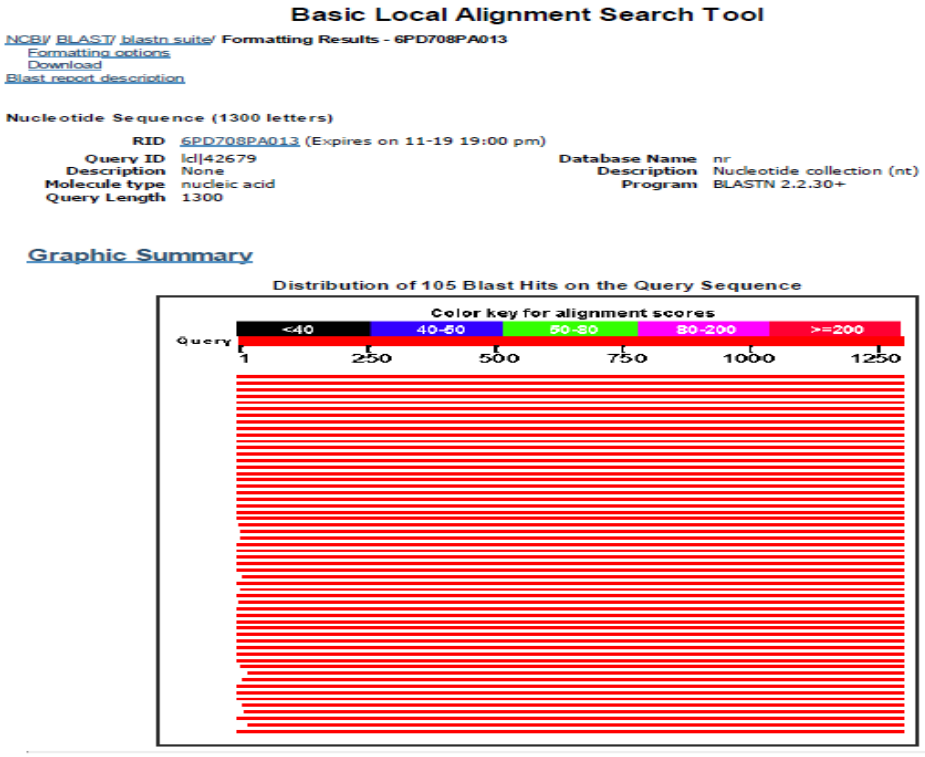

Fig. 12 shows the distribution of blast hits of the submitted gene sequence of the isolated strain Streptomyces coelicoflavus

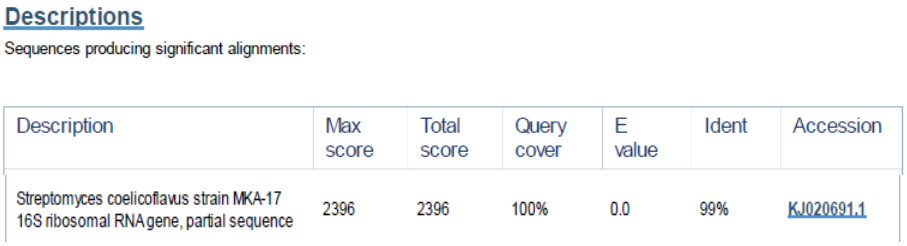

Fig. 13 shows the closely related *Streptomyces* sp. by the comparison of 16S rRNA gene sequence of the *Streptomyces coelicoflavus.*

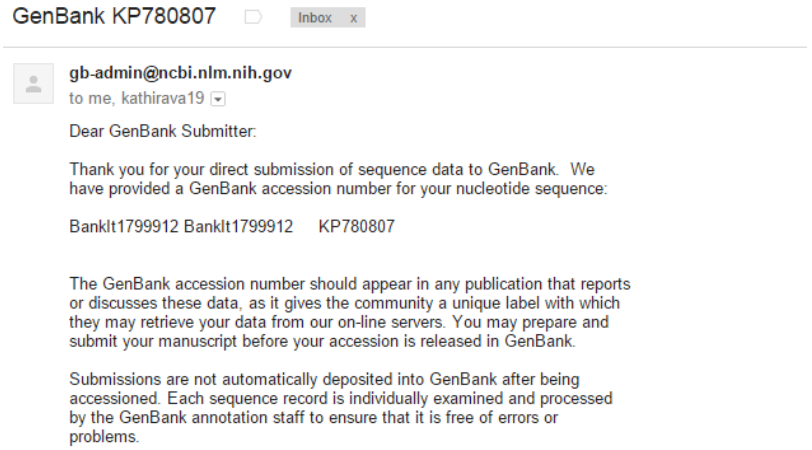

Fig. 14 screen shot page of gene sequence submitted at NCBI.

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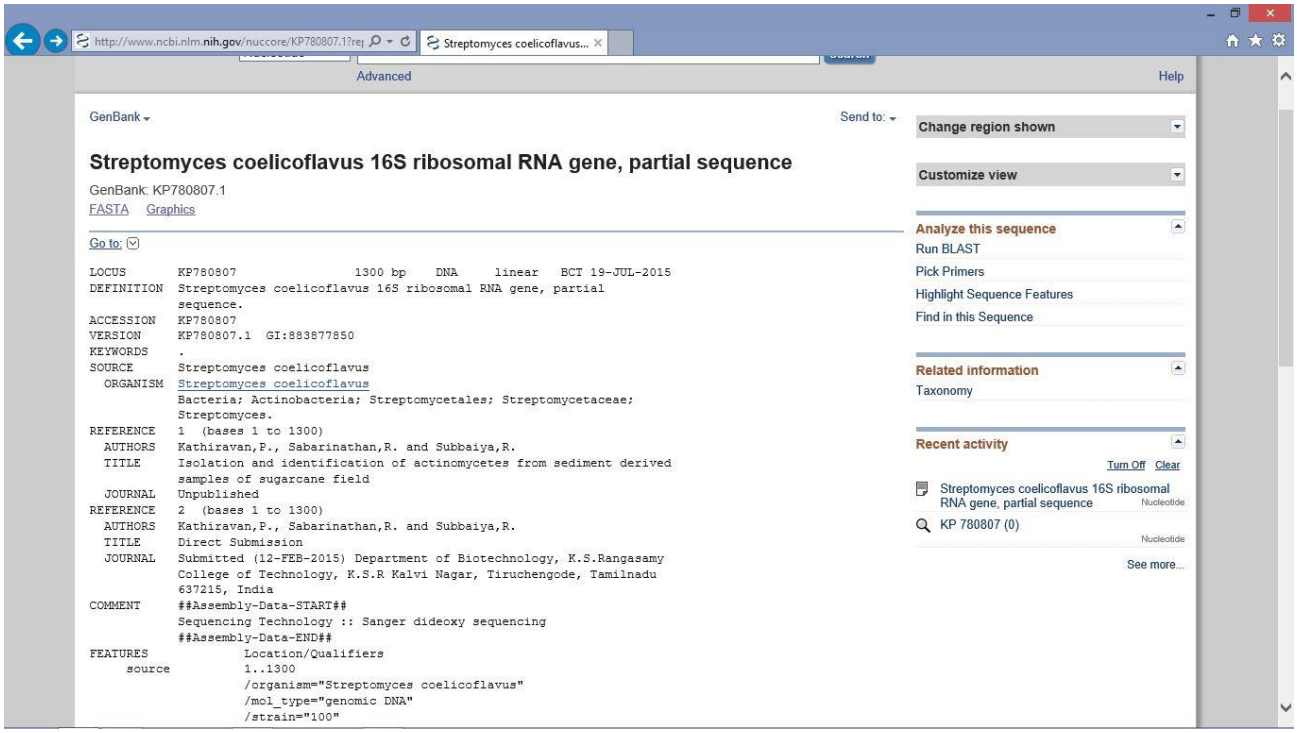

Fig. 15 Screenshot of GenBank webpage of submitted DNA sequence of *Streptomyces coelicoflavus*

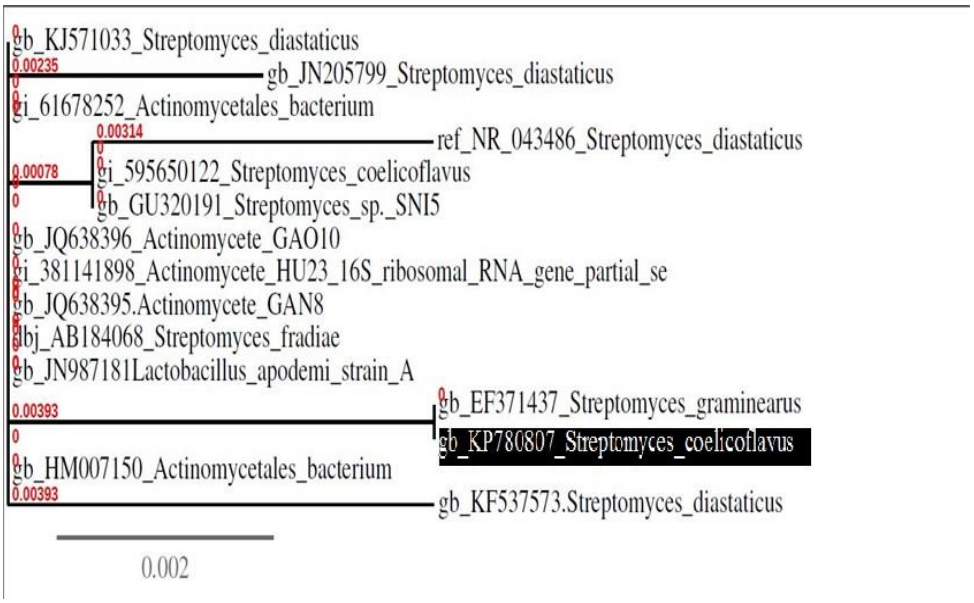

Fig. 16 The unrooted phylogenetic tree of isolated strain *Streptomyces coelicofavus* was constructed along with closely related *Streptomyces* sp., showing the evolutionary relation between *Streptomyces coelicofavus* with other *Streptomyces* species on the basis of 16S rRNA sequence evolutionary distances.

CONCLUSION

In the present study, Actinomycetes were isolated from sugarcane field with the help of preliminary level identification such as biochemical characterisation and other pigment production characteristics. It determines that cultural characteristics of present study reveal that excellent growth when compared to the soil sample collected from the dry land. With the help of the current research work, the results indicates that the isolated strain *Streptomyces coelicoflavus* can be used for many applications such as cytotoxicity study against the different cancer cell lines, bioactive compound productions such as antibiotics, proteins,etc.,

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