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A genomic analysis of multifunctional drug rapamycin producing microbial trait Streptomyces hygroscopicus through various genetic tools

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Abstract

Streptomyces hygroscopicus produces rapamycin, a macrocyclic polyketide having immunosuppressive, antifungal, and anti-tumor activity. Rapamycin has made a substantial contribution to medicine. The wild strain's ability to produce, however, is quite limited. In this way, a computationally guided biotechnology technique was suggested to increase the production capacity of rapamycin. Based on its annotated genome and biochemical data, a metabolic model of Streptomyces hygroscopicus was built at the genome scale. Phylogenetic analysis of the 16*S* rRNA gene and genome sequences revealed that this strain unambiguously belonged to the genus *Streptomyces Hygroscopicus*, and its genomic features and functional genes were comprehensively analyzed and annotated. The core structure of rapamycin is derived from (4R, 5R)-4, 5-dihydrocyclohex-1-ene-carboxilic acid that is extended by polyketide synthase. Submerged fermentation is used by many strains of Streptomyces hygroscopicus to produce rapamycin. Due to the limited amount of medicine produced by the species, engineers confront a tremendous challenge in further developing and industrializing this essential polyketide. As a result, biotechnological manipulations are required for enhanced production of this critical antibiotic. The purpose of the current work was to describe the procedures for enhancing Streptomyces hygroscopicus species ability to synthesize rapamycin (Rap). Both UV radiation and N-methyl-N-nitro-N-nitrosoguanidine (NTG) have been employed as physical and chemical mutagens to increase rapamycin production. When rapamycin production is enhanced utilizing a sequential UV mutagenesis technique. It makes a significant difference to have access to a commercially viable amount of this important macrolide molecule. The present findings confirmed that various phylogenetically different streptomycetes strains produce rapamycin.

Keywords: Rapamycin, Strain improvement, Streptomyces hygroscopicus genome, Multipurpose medication, mutagenesis, mutagen.

Introduction

Rapamycin (RAPA $C_{51}H_{79}NO_{13}$) is an antibiotic which is commonly used as a potent immunosuppressant and antifungal drug produced by the soil born actinomycete streptomyces hygroscopics (Fang and Demain 1995). It also exhibits various pharmacological and biological actions such as anti tumor, neuroprotective and anti aging properties. It is also known as Sirolimus.

rapamycin are macrocyclicamino acid-linked polyketides isolated, respectively, from Streptomyces hygroscopicus .Streptomyces hygroscopicus are important therapeutics in immunosuppression and in combating inflammatory disease. RAPA was initially found as a lipophilic macrolide antibiotic that inhibits

the growth of filamentous fungi (Sehgal et. al., 1975). It is nitrogen containing molecule with molecular mass of 914.18 Daltons. In addition to its multifunctional activity being an antifungal, immunosuppressive, antitumor, anti-aging drug, Rapamycin is also useful as drug eluting stent to prevent restenosis of coronary arteries following angioplasty. It is inactive against many gram positive (other than Sarcina lutea and Staphylococcus aureus) as well as many gram negative bacterial strains (Cheng et al., 1995b; Sumin et al., 2013; Vezina et al., 1975; Zhu et al., 2010). This drug is highly active against Candida albicans—a yeast (Vezina et al., 1975). Harrison et al. (2009) described that it can expand the lifespan of mice which is a big research platform in medical science related to aging (Harrison et al. 2009). RAPA is getting importance day by day for its effectiveness in various medical fields (Parkinson's disease etc.). However, the low yield of RAPA production by the organism increases its production cost. Therefore, the key challenge for researchers is to scale up its production yield. In the early 1990s, a study on the biosynthesis of immunosuppressant Rapamycin was begun by Demain's research group at Massachusetts institute of technology. The purpose of this investigation is to enhance the production of the immunosuppressant drug Rapamycin by subjecting the strain to ultraviolet (UV) and N-methyl-N′-nitro-N-nitroso guanidine (NTG) mutations. Among all the mutants when tested, showed the highest activity. The effect of different factors including medium composition, pH, temperature and intensity of mixing on rapamycin production was studied. Based on the study, the optimal concentrations of soluble starch and dry yeast granules were found to be increased. The aim of the present study was to establish the taxonomic status of strains received as S. hygroscopicus.

Mutagenesis

UV rays have been shown to have a severe mutagenic effect on DNA, the genetic makeup of animals. Although it has a highly fatal effect, DNA damage is not as severe with a modest dose. Strong UV light prevents replication, while intermediate amounts cause mutations because the DNA that is duplicated differs from the original in some ways. Mutagenesis is the process through which an organism's genetic makeup is altered through the occurrence of a mutation. It could happen naturally on its own or as a result of being exposed to mutagens. Additionally, it can be accomplished experimentally in a lab setting. A mutagen is a chemical or physical factor that causes mutations by increasing the frequency of genetic changes in an organism. In the natural world, mutagenesis can cause cancer and other inheritable illnesses, but it also promotes evolution. In the early half of the 20th century, research by Hermann Muller, Charlotte Auerbach, and J. M. Robson served as the foundation for the science of mutagenesis. Numerous factors can alter DNA either naturally or intentionally Endogenous mutagenesis, such as spontaneous hydrolysis, common biological activities that can result in reactive oxygen species and DNA adducts, and mistakes in DNA replication and repair are other ways that mutagenesis can take place. The presence of environmental mutagens, which cause alterations to an organism's DNA, can also lead to mutagenesis. According to the mutagen, or the causative agent, involved, the method by which mutation arises varies. Most mutagens cause lesions in an organism's DNA either directly or indirectly through mutagenic byproducts. However, some mutagens may interfere with biological functions such as chromosomal partitioning or replication. If the environment is unfavorable to an organism's growth, such as when it is home to bacteria that can only grow in the presence of antibiotics, yeast that can only grow in the presence of an antifungal agent, or other unicellular organisms that can only grow in an environment devoid of a crucial nutrient, mutagenesis may also be self-induced by those organisms. In order to cause mutations, many chemical mutagens need to be biologically activated. While the majority of mutagens have side effects that ultimately cause replication problems, such as forming adducts that obstruct replication, some may have direct impacts on replication or lower its fidelity. In replication, thymine could be replaced by a base analogue like 5-bromouracil. In addition to causing direct DNA damage, metals like cadmium, chromium, and nickel can also alter the epigenome and reduce the body's capacity to repair errors, both of which can increase mutagenesis. When DNA lesions during replication cause issues and replication mistakes, mutations frequently result. When bacteria replicate, single-stranded DNA gaps occur as a result of mutagens' significant DNA damage. This triggers the SOS reaction, a quick but error-prone repair method, leading to mutations. A multitude of rescue mechanisms are triggered in mammalian cells when replication stalls at damaged locations. These mechanisms help mammalian cells avoid DNA lesions, but they may also lead to mistakes.

UV irradiation

Ultraviolet (UV) light induces specific mutations on the cellular as well as genetic levels. UV induces specific types of mutation: base substitutions of cytosine (C) → thymine (T) at dipyrimidine sites and CC → TT tandem base substitutions, although the latter rarely occur. These two types of mutation are also called UV signature and their detection suggests past exposure to UV. UV light also affects other organisms. UV light can cause mutations in bacteria that lead to a visible change in their appearance as well as their activity of production of metabolites.

NTG (Nitrosoguanidine) treatment

Enhanced production of Rapamycin is achieved via chemical mutagenesis by NTG (N-Nitrosoguanidine {CH_4N_4O} has been widely used to induce mutations in bacteria. It has proved highly effective, so that it has been suggested to be the most potent chemical mutagen. NTG has also proved a very effective mutagen for actinomycete.

1. Lyophilized culture of *Streptomyces hygroscopicus* was dissolved in 1.5 ml of sterile water in centrifuge tube and spread on the agar plate (supplemented with growth media)
2. Plates were kept in incubator at 28°C up to 08 days.
3. After 08 days colonies were obtained and harvested with sterile water and spread on different agar plates and again kept at 28°c.
4. Wild type culture is irradiated sequentially in 30 seconds for 1.30 minutes with UV radiation and each time inoculated on agar plate and then incubated at 28°c.
5. After 08 days final colonies count carried out with digital colony counter.
6. After counting colonial characteristics were matched and record the results.

NTG Treatment

1. Lyophilized culture of *Streptomyces hygroscopicus* was dissolved in 1.5 ml of sterile water in centrifuge tube and spread on the agar plate (supplemented with growth media).
2. Plates were kept in incubator at 28°C up to 08 days.
3. After 08 days colonies were obtained and harvested with sterile water and spread on different agar plates and again kept at 28°c.
4. After reculturing microbial cells were inoculated on to NTG containing agar plate with the help of inoculating loop.
5. After inoculation plate were kept at 28°c for 08 days in BOD incubator.
6. After 08 days final colonies count carried out with digital colony counter.
7. After counting colonial characteristics were matched and record the results.

MATERIALSANDMETHODS

Chemicals and Bactrial Culture Condition

All the chemicals used were of analytical grade. NTG, lactose, glucose, yeast extract, peptone, NaCl, $FeSO_4$, KNO_3, acetone, aceto-nitrile, $CaCl_2$ and $MgSO_4$ and agar-type I were purchased from HiMedia (Mumbai) Sigma, SD Fine Chemicals (Mumbai), India and accumix from Tulip diagnostics(pvt.) ltd. The bacterial culture was maintained on slants and petri plates containing 4 g/l glucose, 10 g/l, malt extract, 2 g/l peptone and 17 g/l agar having pH 7.3, and finally incubated at 28°C.

Molecular reagents

All the reagents and chemicals were of Analytical Grade Standard Streptomyces hygroscopicus was procured from MTCC. Microbiological media used for the growth and maintenance of rapamycin producing Actinomycetes & electrophoresis reagents, molecular grade chemicals used for DNA isolation were purchased from Hi-Media & SD fine Chemicals.

Microorganism

Streptomyces hygroscopicus were procured in a lyophilized state. One loopful of culture was inoculated aseptically to sterile media and kept at 28 °C for 5–6 days in a BOD incubator.

UV Treatment

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Genomic analysis method

STEPS FOLLOWED:

1. Genomic DNA was isolated from the sample.
2. The ~1.5 kbp, 16s-rDNA fragment was amplified using high–fidelity PCR polymerase.
3. The PCR product was sequenced Bi-directionally.
4. The sequence data was aligned and analyzed to identify the Bacteria and its closest neighbors.

DNA Extraction:

A strain was used as the PCR template. The suspension was made as follows:

1. The sample was picked up and placed in a mortar and homogenized with 1 ml of extraction buffer and the homogenate was transferred to a 2 ml-microfuge tube.
2. An equal volume of Phenol: Chloroform: Isoamlyalcohol (25:24:1) was added to the tubes and mixed well by gently Shaking the tubes.
3. The tubes were centrifuged at room temperature for 15 min at 14,000 rpm.
4. The upper aqueous phase was collected in a new tube and an equal volume of Chloroform: Isoamly alcohol (24:1) was added and mixed.
5. The upper aqueous phase obtained after centrifuging at room temperature for 10 min at 14,000 rpm was transferred to a new tube.
6. The DNA was precipitated from the solution by adding 0.1 volume of 3 M Sodium acetate pH 7.0 and 0.7 volume of Isopropanol.
7. After 15 min of incubation at room temperature the tubes were centrifuged at 4ºC for 15 min at 14,000 rpm.
8. The DNA pellet was washed twice with 70% ethanol and then very briefly with 100% ethanol and air dried.
9. The DNA was dissolved in TE (Tris-Cl 10 mM pH 8.0, EDTA 1 mM).
10. To remove RNA 5 µl of DNAse free RNAse A (10 mg/ml) was added to the DNA.

PCR was performed with Gene Amp PCR System (Biokart Genomic Lab #5M-612, 5th Main Road, OMBR Layout, Bengaluru, Karnataka 560043, Bengaluru - 560043, Karnataka, India.)

Polymerase chain reaction

The sequence mix composition and PCR condition are as follows:

10µl sequencing Reaction

Big Dye Terminator Ready Reaction mix: 4 µl

Template (100ng/ µl): 1µl

Primer(10pmol/λ) : 2 µl

Milli Q Water : 3 µl

PCR Conditions: (25 cycles)

Initial Denaturation : 96°C for 5 min.

Denaturation : 96°C for 30 sec.

Hybridization : 50°C for 30 sec.

Elongation : 60°C for 1.30 min.

Instrument and chemistry details

Sequencing Machine: ABI 3130 Genetic Analyzer Chemistry Cycle sequencing kit: Big Dye Terminator version 3.1” Polymer & Capillary Array: POP_7 pol Capillary Array. Analysis protocol: BDTv3-KB-Denovo_v 5.2 Data Analysis: Seq Scape_ v 5.2 Software Reaction Plate: Applied Biosystem Micro Amp Optical 96-Well Reaction plate.

Nucleotide sequencing and phylogenetic analysis.

The initial sequencing of Streptomyces sp. was conducted by the Biokart Genomic Lab using a GS FLX platform. Eight primers were set in approximately regular inter-vals on the forward and reverse sides. PurifiedPCR products were directly sequenced using the BigDyeTerminatorCycleSequencingReadyReactionKit(AppliedBiosystems Japan Ltd.) according to the manufacturer’sprotocol. Sequence analysis and comparison using the Basic Local Alignment Search Tool (BLAST) multiple sequence alignment and phylogenetic tree analysis were done using Clustal X 2.0 and Molecular Evolutionary Genetics Analysis (MEGA) version 6.0 (www.megasoftware.net) software respectively, based on the internal transcribed spacer sequences of similar fungal species.

Phylogenesis Identification software details:

Phylogentic Tree Builder uses sequences aligned with System Software aligner. A distance matrix is generated using the Jukes-Cantor corrected distance model. When generating the distance matrix, only alignment model positions are used, alignment inserts are ignored and the minimum comparable position is 200. The tree is created using Weighbor with alphabet size 4 and length size 1000.

Weighbor Tree: Weighbor is a weighted version of Neighbor Joining that gives significantly less weight to the longer distances in the distance matrix. The weights are based on variances and covariances expected in a simple Jukes-Cantor model.

Jukes-Cantor Correction: The Jukes-Cantor distance correction is a model which considers that as two sequences diverge, the probability of a second substitution at any nucleotide site increases. For distance-based trees such as Weighbor, the difference in nucleotides is considered for the distance, therefore, second substitutions will not be counted and the distance will be underestimated. Jukes and Cantor createad a formula that calculates the distance taking into account more than just the individual differences (1969; Evol.of Protein Molecules, Academic Press)

Bootstrap: Bootstrapping is a statistical method for estimating the sampling distribution by resampling with replacement from the original sample. In making phylogenetic trees, the approach is to create a pseudoalignment by taking random positions of the original alignment. Some columns of the alignment could be selected more than once or not selected at all. The pseudoalignment will be as long as the original alignment and will be used to create a distance matrix and a tree. The process is repeated 100 times and a majority consensus tree is displayed SH-48owing the number (or percentage) of times a particular group was on each side of a branch without concerning the subgrouping

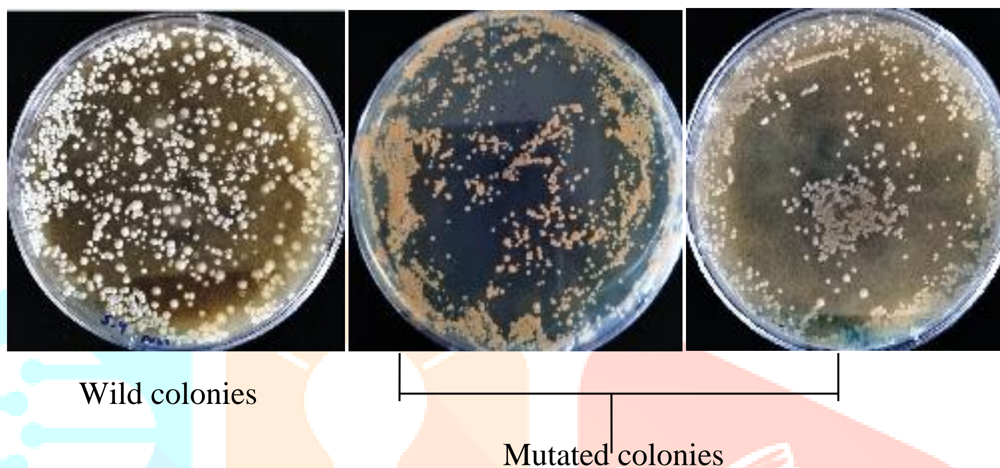
Results

Effect of UV mutagenesis

Each plate was given a maximum UV exposure duration of 150 s. When was previously documented, bright-field micrographs showed that as the UV exposure increased, the number of colonies reduced, as shown in the diagram. As a result, following the final digital colony count, a killing ratio of about 85% was reached. After initial mutation screening, several colonies were obtained.

Effect of NTG mutagenesis

At the end of the NTG treatment, the results showed that more than 90% of the cells had been destroyed, and rapamycin production was assessed in live colonies. The nature of the colony counts' exponential decline with NTG treatment time was noticed. NTG 30 through NTG 180, as well as NTG 60, 90, 120, 150, and 180 (30, 60, 90 etc. correspond to the treatment time of NTG on cell suspension).



Wild colonies

Mutated colonies

At Genomic level

DNA Quantification:

S.No	Sample ID	DNA (ng/µl)
1.	Streptomyces hygroscopicus	155

PCR Conditions

PCR Amplification of 16S Gene:

155 ng of Extracted DNA is used for amplification along with 10pM of each primer

Composition of TAQ Master MIX:

1) High-Fidelity DNA Polymerase

2) 0.5mM dNTPs

3) 3.2mM MgCl2

4) PCR Enzyme Buffer

Cycling Conditions		
Initial Denaturation	3 minutes at 94°C	
Denaturation	1 minutes at 94°C	30 Cycles
Annealing	1 minutes 50°C	
Extension	2 minutes at 72°C	
Final Extension	7 minutes at 72°C	

PCR Amplification conditions	Volume
DNA	1 ul
16s Forward Primer	2 ul
16s Reverse Primer	2 ul
dNTPs (2.5mM each)	4 ul
10X Taq DNA polymerase Assay Buffer	10 ul
Taq DNA Polymerase Enzyme (3U/ ml)	1 ul
Water	30 ul
Total reaction volume	50 ul

Primer Details - The PCR product size ~1.5 kb

No.	Oligo Name	Sequence (5`à 3`)	Tm (°C)	GC- Content
1	16s Forward	GGATGAGCCCGCGGCCTA	57	72.22%
2	16s Reverse	CGGTGTGTACAAGGCCCGG	58	68.42%

Aligned Sequence Data of Sample – Streptomyces hygroscopicus (1524 bp)

>Streptomyces hygroscopicus
ACGGAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAACCGGTTT
CGGCCGGGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGAAAC
GGGGTCTAATACCGGATATGACTGCCGACCGCATGGTCTGGTGGTGGAAAGCTCCGGCGGTGCAGGATGAGCCCGCG
GCCTATCAGCTTGTTGGTGGGGTGATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCAC
ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGAT
GCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGCAAGTGACGGT
ACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATT
ATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCGCGTCGGATGTGAAAGCCCGGGGCTTAACTCCGGGTCTGCATTCG
ATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGA
ACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAG
ATACCCTGGTAGTCCACGCCGTAAACGTTGGGAACTAGGTGTGGGCGACATTCCACGTTGTCCGTGCCGCAGCTAAC
GCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGG
CGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACATCGGAAACCTCTGGAGACA
GGGGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCG
CAACGAGCGCAACCCTTGTCCTGTGTTGCCAGCATGCCCTTTGGGGTGATGGGGACTCACAGGAGACTGCCGGGGTC
AACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCG
GTACAATGAGCTGCGAAGCCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTC
GACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACA
CCGCCCGTCACGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGCCCAACCCTTGTGGGGGGAGCCGTCGAAGGTG
GGACTGGCGATTGGGACGAAGTCGTAACAAGGTAGCCGTACCGGAAGGTGCGGCTGGATCACCTCCTT

Sample: Streptomyces hygroscopicus

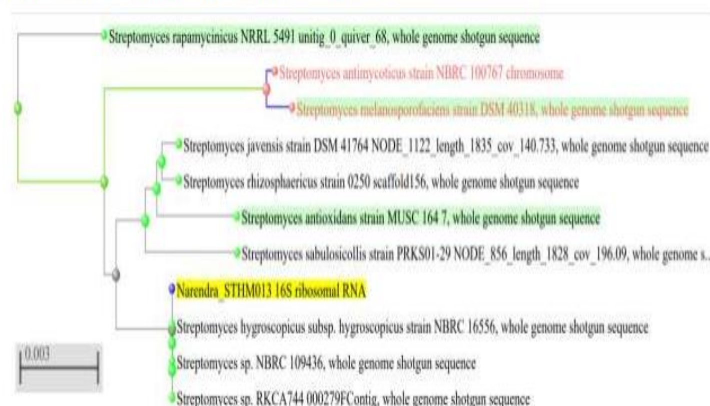
- The Microbe was found to be Streptomyces sp. strain GKU220 16S ribosomal RNA gene
- Sequence ID: KY923054.1
- The next closest homologue was found to Streptomyces hygroscopicus subsp. hygroscopicus strain NRRL 2339 16S ribosomal RNA
- Sequence ID: NR_043379.1

Phylogenetic tree

Phylogenetic Tree

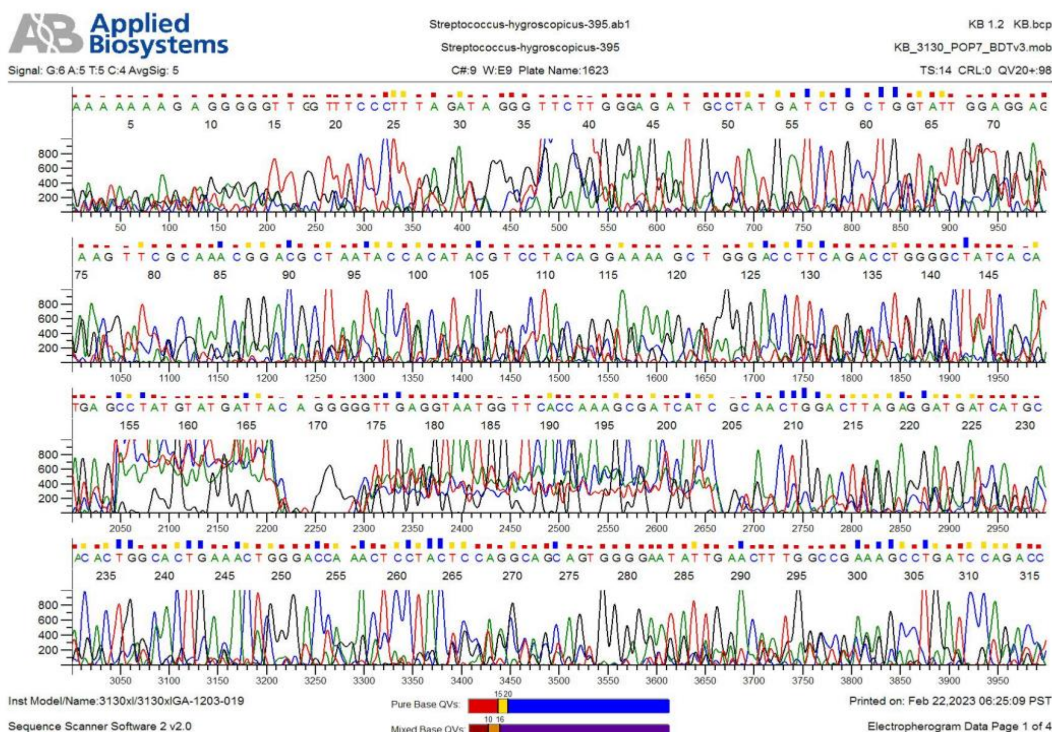
The phylogenetic tree was constructed with BLAST neighbour joining distance tree method with the maximum sequence difference of 0.75.

Phylogenetic Tree: (Streptomyces hygroscopicus)



BLAST DATA

Sl. No.	Organism Name	Accession No.	% Match
1	Streptomyces sp. strain GKU220 16S ribosomal RNA gene	KY923054.1	100.00%
2	Streptomyces hygroscopicus subsp. hygroscopicus strain NRRL 2339 16S ribosomal RNA	NR_043379.1	100.00%
3	Streptomyces sp. strain 5-4 16S ribosomal RNA gene	MK356356.1	100.00%
4	Streptomyces hygroscopicus subsp. hygroscopicus strain NBRC 100766 16S ribosomal RNA	NR_041412.1	100.00%
5	Streptomyces hygroscopicus partial 16S rRNA gene, strain NRRL B-1477	AJ391819.1	100.00%
6	Streptomyces sporocinereus 16S rRNA gene, type strain LMG 20311	AJ781368.1	100.00%
7	Streptomyces hygroscopicus strain NBRC 13472 16S ribosomal RNA gene	NR_041145.1	100.00%
8	Streptomyces hygroscopicus strain KUBOT5 16S ribosomal RNA gene	MK680216.1	100.00%
9	Streptomyces endus gene for 16S rRNA	AB564290.1	100.00%
10	Streptomyces hygroscopicus strain JY-22 16S ribosomal RNA gene	HM481473.1	100.00%



Discussion & conclusion

When compared to direct irradiation methodology, sequential UV mutagenesis was found to be an innovative and efficient way to increase Rapamycin yield with the least amount of harm to cell morphology and genotypic activity. At first, it improved rapamycin yield in comparison to wild type cultivation. Mutant survival rates were found to decline with increasing mutagen (both physical and chemical) exposure. However, the current study confirms that, in terms of strain improvement, chemical mutagens (NTG) were shown to be more efficient than physical mutagens (UV ray) (Naveena et al. 2012). According to research, NTG produces mutants more frequently than UV (Baltz 2014). Actinomycete, Streptomyces hygroscopicus has a genome with a high percentage of GC (GC), around 72%. (Baltz 1998, 2014). As a result, NTG therapy made the GC→AT transition mutation even more active, strong, and consistent. A progressive UV mutagenesis (physical mutagen) approach and chemical mutagenesis (NTG) is used to increase the production of Rapamycin. While chemical mutagenesis yielded powerful mutants for rapamycin synthesis, sequential UV mutagenesis proved to be a viable approach for increased productivity. After exposure to mutagens, strains with higher survival rates produce more. One hypothesis is that phenotypic expression of mutants is influenced by genotypic alteration during the vegetative stage. Theoretical studies showed that an optimum setting can result in about 72% of increased productivity.

The amplification products of almost full length(ca.1524 bp)16SrRNA were obtained from all strains used by direct PCR with a washed mycerial suspension. The sequences were determined by direct sequencing of these products. There are some actinomycetes strains from which the extraction of DNA is difficult; however, the employed PCR method seemed to be simple and effective.

According to the BLASTA search results, the strain most similar to the Streptomyces Hygroscopicus or rapamycin producers in this study was *Streptomyces*, and showing more than 98.0%similarity. All strains fell into the *Streptomyces* cluster in the16SrRNA Phylogenetic tree. From these results,all were considered to belong to the genus *Streptomyces.* Were independently isolated, but their16SrRNA sequences were identical. They also had similar phenotypic characteristics). From these results, they were considered to belong to the same species. Although the nearest neighbor species of these strains was *Streptomyces hygroscopicus NBRC100766* the similarity value was relatively low(100%)and in addition,the morphology of their spore chains was different.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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