



Phenotypic And Genotypic Examination Of Streptomyces Tsukubaensis Produced By Double Mutagenesis

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Abstract:-

Tacrolimus is a highly valued multipurpose medication with a wide range of biological features. It is known to reduce graft rejection in humans and has the potential to cure a variety of skin problems as well as anti-inflammatory macrolactams as an immunosuppressant. Due to the structural intricacy, chemical synthesis is difficult; consequently, microbial manufacture utilizing *Streptomyces tsukubaensis* has been favored. The yield can easily be boosted by several folds using a variety of genetic manipulation and mutagenesis approaches. Understanding the biosynthesis process of Tacrolimus has relied heavily on biotechnological approaches.

Tacrolimus is a strong immunosuppressant and a polyketide macrolide generated by the *Streptomyces* species. It is commonly utilized as an anti-fibrotic drug. *Streptomyces tsukubaensis* was developed as a mutant strain utilizing a dual mutagenesis technique using mutagens (NTG +UV), and this strain demonstrated greater tacrolimus production. The genus *Streptomyces* includes high G+C Gram-positive bacteria, which present a characteristic life cycle closely connected with their ability to produce secondary metabolites. These bacteria are among the most ubiquitous soil microorganisms. After mutation these bacteria exhibit variation in their phenotype as well as in genotypic level.

Key words: -Tacrolimus, Strain improvement, Multipurpose medication, physical mutation, mutagen, phenotype, genotype

Introduction:-

The genus *Streptomyces* includes high G+C Gram-positive bacteria, which present a characteristic life cycle closely connected with their ability to produce secondary metabolites. These bacteria are among the most ubiquitous soil microorganisms. Tacrolimus is an immunosuppressive drug; inhibit the activity of patient's immune system. It is produced biosynthetically as secondary metabolites. Tacrolimus (C₄₄H₆₉NO₁₂) molecular mass 804.031g/mol. is 23-membered macrocyclic lactone. It belongs to the group of polyketide and synthesized by type I polyketide syntheses (PKSs) and non-ribosomal peptide synthetase (NRPS). Tacrolimus was first isolated from the fermentation broth of Japanese soil sample and reported as a macrolide antibiotic by Kino et al. in 1984. It is also known as FK-506 (Fermentek catalogue number 506) and fujimycin. This drug is suppresses immune system and used to prevent the rejection of transplanted organs. The immunosuppressive activities are due to its effect to reduce the activity of enzyme peptidyl-propyl isomerase and interact to the protein

immunophilin. In 1994, FDA (Food and Drug Administration) approved Tacrolimus for liver transplantation. It also has been used in patients for heart, kidney and bone marrow transplantation. It is also useful in the treatment of various autoimmune diseases. Tacrolimus has been shown to be effective in treating a number of disease such as asthma, inflammatory and hyper-proliferative skin disease and cutaneous manifestations of immunologically induced illness (European Patent No 315, 978). Members of the genus *Streptomyces* are amongst the most valuable industrial bacteria due to their ability to produce some of the most important classes of clinically active secondary metabolites. Tacrolimus is produced by a type of soil bacterium *Streptomyces tsukubaensis*. The name is derived from Tsukuba macrolide immunosuppressant. Tacrolimus was discovered by Fujisawa Pharmaceuticals Co., which merged with Astellas Pharma in 2004. *S. tsukubaensis* includes 26 genes that encode the PKS and NRPS structural proteins, proteins responsible for the biosynthesis of precursors, post-PKS tailoring of the polyketide backbone and regulation of gene expression, among others. Despite the high market value of Tacrolimus and the growing industrial interest, the laboratory fermentation process of Tacrolimus using wild type production strains often results in low yields. This has prompted several studies to improve the production of this compound, both in academia and industry. Initially, most of the efforts that were made relied on classical approaches including nutritional control, random mutagenesis and feeding strategies. To enhance the production of Tacrolimus, it is necessary to optimize the culture conditions and media ingredients. Medium optimization by the traditional method is not only time consuming but also expensive due to large number of variables, but culture optimization is easily achieved by UV irradiation and NTG treatment.

Mutagenesis

It is well known that UV radiations have a substantial mutagenesis effect on DNA, or the genetic makeup of organisms. Although it has a deadly effect when used manually, DNA damage is not as severe at modest doses. UV light stops replication at high concentrations, but at intermediate doses, the DNA that is copied differs from the original DNA, leading to mutations.

Mutagenesis is a process by which the genetic information of an organism is changed by the production of a mutation. It may occur spontaneously in nature or as a result of exposure to mutagens. It can also be achieved experimentally using laboratory procedures. A mutagen is a mutation-causing agent, be it chemical or physical, which results in an increased rate of mutations in an organism's genetic code. In nature mutagenesis can lead to cancer and various heritable diseases, but it is also a driving force of evolution. Mutagenesis as a science was developed based on work done by Hermann Muller, Charlotte Auerbach and J. M. Robson in the first half of the 20th century. DNA may be modified, either naturally or artificially, by a number of physical, chemical and biological agents, resulting in mutations. Mutagenesis may occur endogenously (e.g. spontaneous hydrolysis), through normal cellular processes that can generate reactive oxygen species and DNA adducts, or through error in DNA replication and repair. Mutagenesis may also occur as a result of the presence of environmental mutagens that induce changes to an organism's DNA. The mechanism by which mutation occurs varies according to the mutagen, or the causative agent, involved. Most mutagens act either directly or indirectly via mutagenic metabolites, on an organism's DNA, producing lesions. Some mutagens, however, may affect the replication or chromosomal partition mechanism, and other cellular processes. Mutagenesis may also be self-induced by unicellular organisms when environmental conditions are restrictive to the organism's growth, such as bacteria growing in the presence of antibiotics, yeast growing in the presence of an antifungal agent, or other unicellular organisms growing in an environment lacking in an essential nutrient. Many chemical mutagens require biological activation to become mutagenic. While most mutagens produce effects that ultimately result in errors in replication, for example creating adducts that interfere with replication, some mutagens may directly affect the replication process or reduce its fidelity. Base analog such as 5-bromouracil may substitute for thymine in replication. Metals such as cadmium, chromium, and nickel can increase mutagenesis in a number of ways in addition to direct DNA damage, for example reducing the ability to repair errors, as well as producing epigenetic changes. Mutations often arise as a result of problems caused by DNA lesions during replication, resulting in

errors in replication. In bacteria, extensive damage to DNA due to mutagens results in single-stranded DNA gaps during replication. This induces the SOS response, an emergency repair process that is also error-prone, thereby generating mutations. In mammalian cells, stalling of replication at damaged sites induces a number of rescue mechanisms that help bypass DNA lesions, however, this may also result in errors. The Y family of DNA polymerases specializes in DNA lesion bypass in a process termed translesion synthesis (TLS) whereby these lesion-bypass polymerases replace the stalled high-fidelity replicative DNA polymerase, transit the lesion and extend the DNA until the lesion has been passed so that normal replication can resume; these processes may be error-prone or error-free.

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 RAPA is achieved via chemical mutagenesis by NTG (N-Nitrosoguanidine {CH₄N₄O}) 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

MATERIALS AND METHODS

Chemicals and Bacterial Culture Conditions

All the chemicals used were of analytical grade. NTG, lactose, glucose, yeast extract, peptone, NaCl, FeSO₄, KNO₃, acetone, aceto-nitrile, CaCl₂ and MgSO₄ and agar-type I were purchased from Hi-Media (Mumbai), SD Fine Chemicals (Mumbai), India, and accumix from Tulip diagnostics (pvt.) ltd. *S. tsukubaensis* was maintained on slants and petri plates containing medium enriched with 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.

UV Treatment

1. Lyophilized culture of *Streptomyces tsukubaensis* 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 tsukubaensis* 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.

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.

Protocol:

DNA Extraction:

- 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.
- An equal volume of Phenol: Chloroform: Isoamylalcohol (25:24:1) was added to the tubes and mixed well by gently Shaking the tubes.
- The tubes were centrifuged at room temperature for 15 min at 14,000 rpm.
- The upper aqueous phase was collected in a new tube and an equal volume of Chloroform: Isoamyl alcohol (24:1) was added and mixed.
- The upper aqueous phase obtained after centrifuging at room temperature for 10 min at 14,000 rpm was transferred to a new tube.
- 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.
- After 15 min of incubation at room temperature the tubes were centrifuged at 4°C for 15 min at 14,000 rpm.
- The DNA pellet was washed twice with 70% ethanol and then very briefly with 100% ethanol and air dried.
- The DNA was dissolved in TE (Tris-Cl 10 mM pH 8.0, EDTA 1 mM).
- To remove RNA 5 µl of DNase free RNase A (10 mg/ml) was added to the DNA.

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.

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 AND DISCUSSION

Isolation and Screening of Mutants

The use of mutagenesis to increase the production of metabolites is supported by enough scientific evidence. *Streptomyces clavuligenus* has been shown to produce more clavulanic acid when exposed to UV mutagenesis. Chemical mutagens, in particular NTG, have been employed successfully for a number of purposes to improve the strains of bacteria and fungus. NTG has been shown to be the most effective chemical mutagen for *Streptomyces* species. for improved macrolide synthesis. Similarly, in this investigation, *S. tsukubaensis* received treatments with different mutagens (NTG and UV) for differing lengths of time. In order to maximise the amount of mutagens, the survival rate was evaluated. It was discovered that for 50% survival, the UV treatment period needed to be 3 minutes at a concentration of 100 M NTG (data not shown). When several mutants were screened based on tacrolimus yield, it was discovered that control *S. tsukubaensis* was able to create little tacrolimus, while other mutants, such as those that were exposed to UV light for three minutes or those that were exposed to NTG produced more tacrolimus. Tacrolimus synthesis significantly increased when chemical and physical mutagens were applied together (dual mutation) compared to the previously isolated wild type strain. Maximum amounts of tacrolimus were produced by the dual mutant treated with physical and chemical mutagens.

Phenotypical Comparison of Control and Mutant Cells

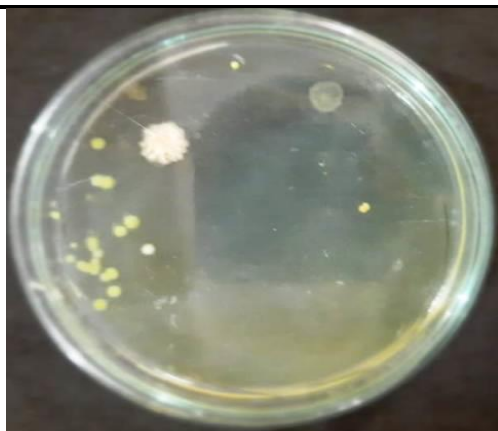
The developing mutant was compared to see whether there were any potential morphological character changes. Diagram depicts the morphological alterations in control *S. tsukubaensis* cells after exposure to UV, NTG.

At Macroscopic Level

A diagram revealed that in control cells, the bacterial colonies were spherical, smooth, and milky white. The bacterial colonies subjected to several mutagens showed noticeable morphological changes. Control *S. tsukubaensis* cells had white colonies with smooth borders, but colonies of UV-treated *Streptomyces* mutants were off-white in colour with thick margins. Likewise, the mutant colonies treated with NTG had irregularly shaped borders and were off-gray in colour with fuzz in the centre. The NTG exposed colonies were off gray with blackish fuzz in the centre, and it was observed that these colonies were larger than the colonies of control *S. tsukubaensis*. The colonies that had been exposed to UV light were identified as having wavy borders and a blackish colour. At the centre of each colony, there was noticeable dark fuzz.

Observation table:

Strain type	Size	Shape	Margin	Elevation	Texture	Opacity	Pigmentation
Wild strain	Large	Circular	Entire	Convex	Smooth	Translucent	White
Mutant strain UV/NTG	Small	Circular	Thick/ Irregular	Convex with fuzz/ blackish fuzz	Smooth	Translucent	off-white /off- gray

**Wild type colony****Mutant colony****At Genomic level****DNA Quantification:**

S.No	Sample ID	DNA (ng/μl)
1.	STMT07	177

PCR Conditions**PCR Amplification of 16S Gene:**

177 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 – STMT07 (1528 bp)

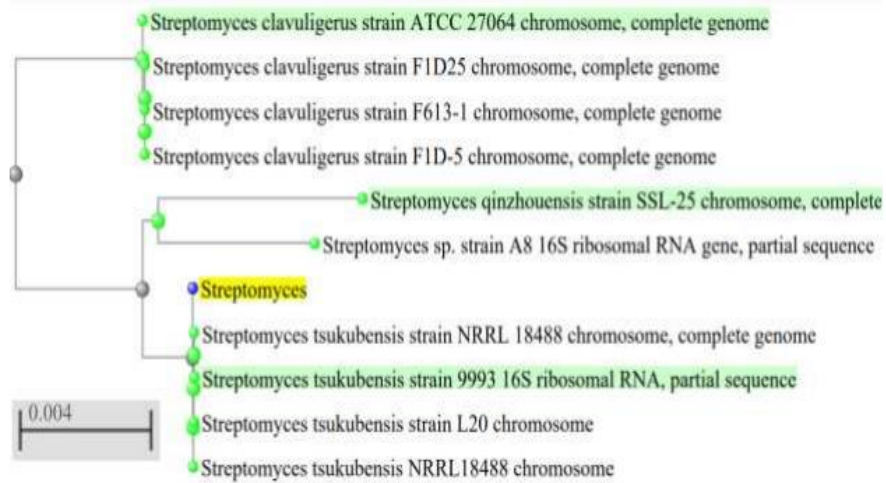
>Streptomyces

ACGGAGAGTTTGATCTGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAAGCCGCTTCGGTGGTGGAT
TAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATAATAC
CTTCGGGGGCATCCTTGAAGGTTGAAAGCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTGGTGGGGTGATGGCCTACC
AAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGGCCAGACTCCTACGGGAGGCAGCAGT
GGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAACCTCTTCAGCAG
GGAAGAAGCGAGAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGTAATACGTAGGGCGCAAGCGTT
GTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTACAGTCGGGTGTGAAAGCCCGGGCTTAACCCCGGGTCTGCATTGCA
TACGGGCAGGCTAGAGTGTGGTAGGGGAGATCGGAATCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCG
AAGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTA
AACGTTGGAACTAGGTGTTGGCGACATTCCACGTCGTCGGTGCCGAGCTAACGCATTAAGTTCCCCGCCCTGGGGAGTACGGCCGCA
AGGCTAAAACCTCAAAGGAATTGACGGGGGCCCGCACAAAGCAGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAA
GGCTTGACATACACCGGAAAGCATTAGAGATAGTGCCCCCTTGTGGTTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTG
TGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTGTCAGCAAGCCCCCTTGTGGGGTGTGGGGACTCACGGG
AGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCTTATGTCTTGGGCTGCACACGTGCTACAATGG
CCGGTACAAAGAGCTGCGATGCCGTGAGGCGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCAT
GAAGTCGGAGTTGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCCGGCCTTGTACACACCGCCCGTCACGTCACGAA
AGTCGGTAAACCCCGAAGCCGGTGGCCCAACCCCTTGTGGGAGGGAGCTGTGCAAGGTGGGACTGGCGATTGGGACGAAGTCGTAAC
AAGGTAGCCGTACCGGAAGGTGCGGCTGGATCACCTCCTTT

Sample: Streptomyces

- The Microbe was found to be Streptomyces tsukubensis strain NRRL 18488 chromosome
- Sequence ID: CP020700.1
- The next closest homologue was found to be Streptomyces tsukubensis strain L20
- chromosome Sequence ID: CP070379.1

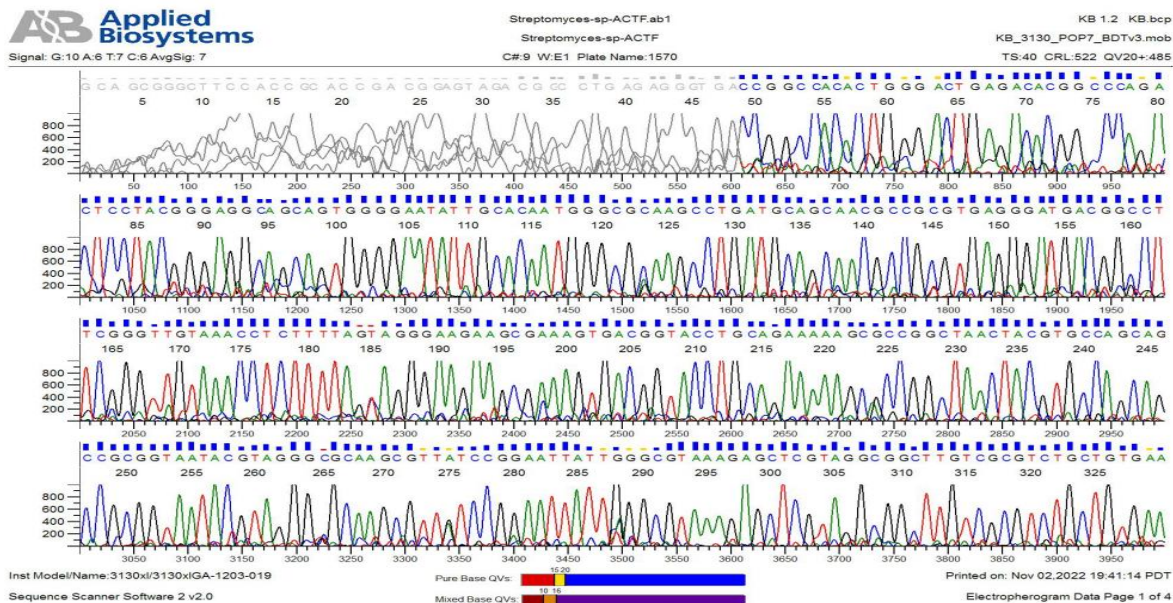
Phylogenetic Tree



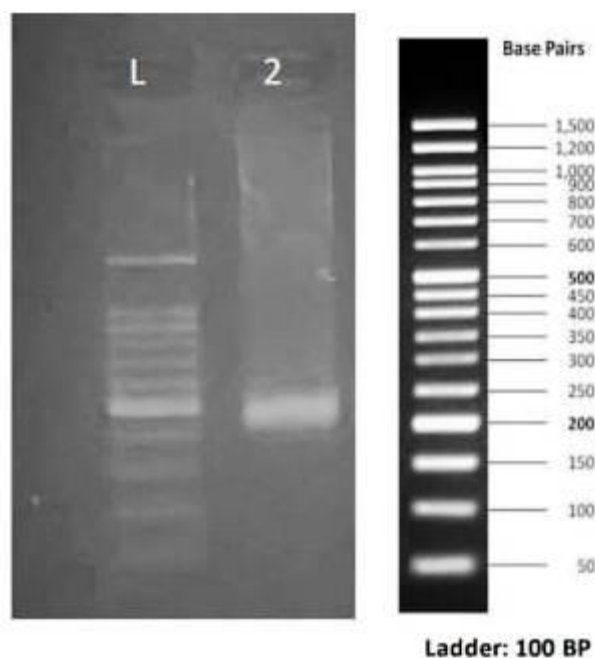
BLAST DATA

Sl. No	Organism Name	Accession No.	% Match
1	Streptomyces tsukubensis strain NRRL 18488 chromosome	CP020700.1	100.00%
2	Streptomyces tsukubensis strain L20 chromosome	CP070379.1	100.00%
3	Streptomyces tsukubensis NRRL18488 chromosome	CP029159.1	100.00%
4	Streptomyces tsukubensis strain 9993 16S ribosomal RNA	NR_134824.1	100.00%
5	Streptomyces qinzhouensis strain SSL-25 chromosome	CP042266.1	99.35%
6	Streptomyces sp. strain A8 16S ribosomal RNA gene	MH688754.1	99.24%
7	Streptomyces sp. strain A8 16S ribosomal RNA gene	CP065236.1	98.56%
8	Streptomyces clavuligerus strain ATCC 27064 chromosome	CP027858.1	98.50%
9	Streptomyces clavuligerus strain ATCC 27064 chromosome	CP032052.1	98.50%
10	Streptomyces clavuligerus strain F613-1 chromosome	CP016559.1	98.50%

Sanger's report



PCR Amplified Image



CONCLUSIONS

Researchers have reported the enhanced yield of metabolites in *Streptomyces* spp. and other bacterial species using optimization of process conditions and strain improvement methods. In this study results showed that UV and NTG treated *Streptomyces tsukubaensis* mutants produced higher amount of tacrolimus in comparison to wild type strain. This study also proposes an impressive model of metabolic regulation for enhanced tacrolimus production through newly developed dual mutant of *Streptomyces tsukubaensis* and this model might be helpful to obtain higher production of tacrolimus.

The amplification products of almost full length (ca. 1528 bp) 16S rRNA were obtained from all strains used by direct PCR. 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 BLAST search results, the strain most similar to the *Streptomyces tsukubaensis* or tacrolimus producers in this study was *Streptomyces*, and showing more than 98.0% similarity. All strains fell into the *Streptomyces* cluster in the 16S rRNA Phylogenetic tree. From these results, all were considered to belong to the genus *Streptomyces*. Were independently isolated, but their 16S rRNA 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 tsukubaensis* NRRL18488 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.

REFERENCE:-

1. Alexandra S.B.GoebelReinhard H.H.NeubertJohannesWohlrab Dermal targeting of tacrolimus using colloidal carrier systems.International Journal of PharmaceuticsVolume 404, Issues 1–2, 14 February 2011,
2. Anderson, S. & Wellington, E. M. H. (2001). The taxonomy of *Streptomyces* and related genera. *Int J Syst Evol Microbiol* 51, 797–814.
3. Atalan, E., Manfio, G. P., Ward, A. C., Kroppenstedt, R. M. & Goodfellow, M. (2000). Biosystematic studies on novel streptomycetes from soil.
4. Antonie Van Leeuwenhoek 77, 337–353. Cerco's, A. P. (1954). *Streptomyces rutgersensis* var. *castelarensis* n. var. Nuevas propiedades de la canfomicina. *Rev Invest Agric* 8, 263–283 (in Spanish).
5. Chamberlain, K. & Crawford, D. L. (1999). In vitro and in vivo antagonism of pathogenic turfgrass fungi by *Streptomyces hygroscopicus* strains YCED9 and WYE 53. *J Ind Microbiol Biotechnol* 23, 641–646. DSMZ (1998).
6. Catalogue of Strains. Braunschweig: Deutsche Sammlung von Mikroorganismen und Zellkulturen.
7. Duangmal, K., Ward, A. C. & Goodfellow, M. (2005). Selective isolation of members of the *Streptomyces violaceoruber* clade from soil. *FEMS Microbiol Lett* 245, 321–327.
8. Ettliger, L., Corbaz, R. & Hu"tter, R. (1958). Zur Systematik der Actinomyceten.
9. Eine Arzteilung der Gattung *Streptomyces* Waksman & Henrici. *Arch Mikrobiol* 31, 326–358.
10. Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *J Mol Evol* 17, 368–376.
11. Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39, 783–791.
12. Felsenstein, J. (1993). PHYLIP (phylogeny inference package) version 3.5c. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle, USA.
13. Ferguson, E. V., Ward, A. C., Sanglier, J. J. & Goodfellow, M. (1997). Evaluation of *Streptomyces* species-groups by pyrolysis mass spectrometry. *Zentralbl Bakteriol* 285, 169–181.
14. Fitch, W. M. & Margoliash, E. (1967). Construction of phylogenetic trees: a method based on mutation distances as estimated from cytochrome c sequences is of general applicability. *Science* 155, 279–284.
15. Garrity, G. M.; B. K. Heimbuch, H. Motamedi & A. Shafiee:Genetic relationships among actinomycetes that produce the immunosuppressant macrolides FK506, FK520/FK523 and rapamycin. *J.Ind.Microbiol.*12,42–47,1993
16. Gonzalez, J. M. & Saiz-Jimenez, C. (2005). A simple fluorimetric method for the estimation of DNA-DNA relatedness between closely related microorganisms by thermal denaturation temperatures. *Extremophiles* 9, 75–79.
17. Goodfellow, M., Ferguson, E. V. & Sanglier, J. J. (1992). Numerical classification and identification of *Streptomyces* species: a review. *Gene* 115, 225–233.
18. Goodfellow, M., Manfio, G. P. & Chun, J. (1997). Towards a practical species concept for cultivable bacteria. In *Species, The Units of Biodiversity*, pp. 25–59. Edited by M. F. Claridge, H. A. Dawah & M. R. Wilson. London: Chapman & Hall.
19. Goodfellow, M., Kumar, Y., Labeda, D. P. & Sembring, L. (2007). The *Streptomyces violaceusniger* clade: a home for streptomycetes with rugose ornamented spores. *Antonie Van Leeuwenhoek* 92, 173–199.
19. HideyukiMuramatsu,SeriIntanMokhtar,MasaakiKatsuokaandMasamiEzaki(2005) Phylogenetic Analysis of Immunosuppressant FK506-ProducingStreptomyceteStrains *ACTINOMYCETOLOGICA*33-39
20. Johnson, L. E. & Dietz, A. (1971). Scopafungin, a crystalline antibiotic produced by *Streptomyces hygroscopicus* var. *enhygrus* var. *nova*. *Appl Microbiol* 22, 303–308.
21. Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, vol. 3, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.
22. Jurado, V., Laiz, L., Gonzalez, J. M., Hernandez-Marine, M., Valens, M. & Saiz-Jimenez, C. (2005). *Phyllobacterium catacumbae* sp. nov., a member of the order 'Rhizobiales' isolated from Roman catacombs. *Int J Syst Evol Microbiol* 55, 1487–1490.
23. Ka"mpfer, P., Kroppenstedt, R. M. & Dott, W. (1991). A numerical classification of the genera

- Streptomyces and Streptovorticillium using miniaturized physiological tests. *J Gen Microbiol* 137, 1831–1891.
24. Kino, T.; H. Hatanaka, M. Hashimoto, M. Nishiyama, T.Goto, M., Okuhara, M. Kohsaka, H. Aoki&H.Imanaka:FK-506, anovel immunosuppressant isolatedfroma *Streptomyces*. Fermentation, isolation, and physico chemical and biological characteristics. *J. Antibiot.* 40:1249–1255, 1987
25. Labeda, D. P. (1998). DNA relatedness among the *Streptomyces fulvissimus* and *Streptomyces griseoviridis* phenotypic cluster groups. *Int J Syst Bacteriol* 48, 829–832.
26. Labeda, D. P. & Lyons, A. J. (1991). The *Streptomyces violaceusniger* cluster is heterogeneous in DNA relatedness among strains: emendation of the descriptions of *Streptomyces violaceusniger* and *Streptomyces hygroscopicus*. *Int J Syst Bacteriol* 41, 398–401.
27. Monaghan, R.L.; N.H. Sigal, L. Kaplan, K.M. Byrne, R. P. Borris, F. J. Dumont, G. M. Garrity & D. L. Zink: Novel immunosuppressant agent. EP0323865, 1989
28. Motamedi, H. & A. Shafiee: The biosynthetic gene cluster for the macrolactone ring of the immunosuppressant FK506. *Eur. J. Biochem.* 256:528–534, 1998
29. Okuhara, M.; H. Tanaka, T. Goto, T. Kino & H. Hatanaka: Method of immunosuppression. US Patent 6,028,097, 2000
30. Pernodet, J.L.; F. Boccard, M. T. Alegre, J. Gagnat & M. Guérineau: Organization and nucleotide sequence analysis of ribosomal RNA gene cluster from *Streptomyces ambofaciens*. *Gene* 79:33–46, 1989
31. Rossello-Mora, R. & Amann, R. (2001). The species concept for prokaryotes. *FEMS Microbiol Rev* 25, 39–67.
32. Rossi Doria, T. (1891). Su di alcune specie di “Streptothrix” trovate nell’aria studate in rapporto a quelle già note a specialmente all’ “Actinomyces”. *Ann Int Igiene Sper Univ Roma* 1, 399–438 (in Italian).
33. Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4, 406–428.
34. Sembiring, L., Ward, A. C. & Goodfellow, M. (2000). Selective isolation and characterisation of members of the *Streptomyces violaceusniger* clade associated with the roots of *Paraserianthes falcataria*. *Antonie van Leeuwenhoek* 78, 353–366.
35. Shirling, E. B. & Gottlieb, D. (1966). Methods for characterization of *Streptomyces* species. *Int J Syst Bacteriol* 16, 313–340.
36. Shirling, E. B. & Gottlieb, D. (1968). Cooperative description of type cultures of *Streptomyces*. II. Species description from first study. *Int J Syst Bacteriol* 18, 69–189.
37. Thompson, J.D.; T.J. Gibson, F. Plewniak, F. Jeanmougin & D.G. Higgins: The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 25:4876–4882, 1997
38. William J. Bruno, Nicholas D. Socci, and Aaron L. Halpern (2000). Weighted Neighbor Joining: A Likelihood-Based Approach to Distance-Based Phylogeny Reconstruction, *Mol. Biol. Evol.* DDSKVG13(1): 189-197. 2E. O. Wiley, D. R. Brooks, D. Siegel-Causey, V. A. Funk (1991). *The Compleat Cladist: A Primer of Phylogenetic Procedures*. Freely available at <http://taxonomy.zoology.gla.ac.uk/teaching/CompleatCladist.pdf>
39. Xu, C., Wang, L., Cui, Q., Huang, Y., Liu, Z., Zhang, G. & Goodfellow, M. (2006). Novel neutrotolerant acidophilic *Streptomyces* species isolated from a soil in China: *Streptomyces guandensis* sp. nov., *Streptomyces paucisporeus* sp. nov., *Streptomyces rubidus* sp. nov. and *Streptomyces yanglinensis* sp. nov. *Int J Syst Evol Microbiol* 56, 1109–1115.
40. Yashawant Kumar and Michael Goodfellow Five new members of the *Streptomyces violaceusniger* 16S rRNA gene clade: *Streptomyces castelarensis* sp. nov., comb. nov., *Streptomyces himastatinicus* sp. nov., *Streptomyces mordarskii* sp. nov., *Streptomyces rapamycinicus* sp. nov. and *Streptomyces ruanii* sp. nov. *International Journal of Systematic and Evolutionary Microbiology* (2008), 58, 1369–1378
41. Zbinovsky, V., Hausmann, W. K., Wetzell, E. R., Borders, D. B. & Patterson, E. L. (1968). Isolation and characterisation of antibiotic LL-AC 541. *Appl Microbiol* 16, 614–616.