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# SIROLIMUS: PRODUCTION ENHANCEMENT USING MUTAGENESIS AND MEDIUM OPTIMISATION

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***Abstract:*** Sirolimus (Rapamycin) is a coveted drug substance obtained from actinobacteria named *Streptomyces hygroscopicus*, which synthesizes it as a secondary metabolite. It has been studied in regard to a variety of biological conditions such as anti-pathogenicity, immunosuppression, anti-aging, and anti-cancerous activity. Devising methodologies to obtain a sufficient amount of sirolimus is a part of eminent research prospects. For the current study, enhancement in sirolimus production has been achieved by following a sequential regime involving improvement in the fermentation media and mutation of the parent strain along with careful and continuous screening at each step. The investigation encompasses the establishment of initial production activity followed by optimization of medium components using Plackett-Burman design and OVAT methodology. The significant components obtained have been thoroughly monitored to finalize the concentration which yielded maximum production of sirolimus. Then the procured strain of *Streptomyces hygroscopicus* has been mutated using intermittent treatment with UV radiations. The stable culture colonies obtained thereby have been taken for chemical mutagenesis using NTG. The colonies offering stable and enhanced yield have been isolated to inoculate the optimized media. Finally, 2.5 to 2.8 - fold augmentation in the production of sirolimus has been observed concerning the initially recorded activity.

Keywords: fermentation, optimization, Plackett-Burman design, OVAT, *Streptomyces hygroscopicus*, UV, NTG.

## I. INTRODUCTION

Sirolimus is a 31-membered clinically important macrocyclic polyketide (Gregory et al., 2004; Risdian, Mozef, & Wink, 2019) popularly identified as a proactive antibiotic (Shah, Kute, Patel, & Trivedi, 2015). It was named as Rapamycin (Rap) owing to its isolation from the soil sample of Rapa Nui (Easter Island in Chile) (Manning, 2017; Vezina, Kudelski, & Sehgal, 1975). Sirolimus has been studied in regard of a wide range of biological activities (Dutta, Basak, Bhunia, & Dey, 2014). Initially designated as a profound drug against fungi (Bastidas, Shertz, Lee, Heitman, & Cardenas, 2012), exhibiting remarkable antimicrobial activity against *Candida albicans* (Napoli & Taylor, 2001), it is now also popular as an immunosuppressant (Dumont & Su, 1995; Law, 2005; Lamming, 2016) and an anti-cancer agent (Law, 2005) harbouring neurogenerative functions (Lamming, 2016). Its role in anti-aging, lifespan extension (Bové, Martínez-Vicente, & Vila, 2011) and related phenomenon is one gathering immense attention from the community of researchers worldwide. Sirolimus also has an established function towards prevention of coronary artery restenosis post angioplasty (Abizaid, 2007) and is thus an approved eluting stent drug (Abizaid, 2007; Ardissino et al., 2004). Furthermore, raphas has gained FDA approval as a medicine finding application in the prevention of organ-rejection during kidney transplantation (Morath et al., 2007). Considering other immunosuppressants, sirolimus has been studied to offer far more effectiveness and reduced toxicity (Cutler et al., 2014; Groth, et al., 1999). Therefore, the industrial demand of this protean drug is ever-increasing but the scaling up of its production yield is a topic of immense challenge (Dutta, et al., 2014).

The wild strain known for sirolimus production is *Streptomyces hygroscopicus* isolated by Vezina et al. (Vezina, et al., 1975). But the low yield of the drug calls for a variety of augmentation techniques ranging from induction of genetic manipulations to optimisation of the media (Yoo, Kim, Park, & Yoon, 2017; Liu, Lin, Li, Ali., & He, 2020). The first in this arena includes determination of the effects of various supplements in the fermentation media and subsequent optimization so that the best component in a precise concentration level can be included in the production regime (Dutta, Basak, Bhunia, Sinha, & Dey, 2017; Mohamed, Elkhateeb, Taha, & Daba, 2019). Following changes in the media, involvement of genetic mutations, high throughput screening (HTS) methodologies and techniques like protoplast fusion have gained momentum (Dutta et al., 2014; Patel, Patel, Goyal, & Waheed, 2019). One of the most recent and effective strain improvement technique is called as protoplast-based genome shuffling which offers a great advantage of introducing changes at various loci throughout the genome without the availability of genome sequence data (Chen, et al., 2009). In regard of mutagenesis, HTS screening post treatment with nitrosoguanidine (Dutta

et al., 2017), has also been found to be a useful method. A lot of efforts have been directed towards the study of metabolic pathways in which sirolimus participates while delivering its biological effectivity (Dutta et al., 2014; Jung et al., 2011).

Various conglomerated methodologies encompassing metabolic engineering of precursor pathways, identification, upregulation and downregulation of target genes and optimisation of the fermentation media have helped in obtaining strains and conditions which promise a higher yield of sirolimus at the industrial level (Tripathi, Singh, & luhtra, 2014). In our study, we have obtained higher yield of sirolimus identified by a sequential upgradation in production activity while performing medium optimisation and mutagenic strain improvement.

## II. MATERIALS AND METHOD

### 2.1. Microorganism

*Streptomyces hygroscopicus* was employed for the current study has been obtained from Industrial Lab, Mumbai, India. The acquired culture was maintained in Yeast extract-malt agar (YMA) medium slants. The media was prepared by mixing 4.0 g Dextrose, 4.0 g Yeast extract, 10.0 g Malt extract in 500 mL R.O water and finally making up the volume up to 1000 ml. The pH was adjusted to 7.2 and then 20.0 g agar was added. The medium was converted into molten form and then 15 mL of it was taken in 50 mL test tube which was finally sealed with cotton plugs. Following autoclaving for 40 minutes, the tubes were kept in slant position till they get solidified.

*Finally*, the slants were kept in incubator at 30°C for pre-incubation. Then 0.2 ml of the procured culture suspension was streaked on YM agar slants and incubated at 28°C till the cultured got matured.

### 2.2. Single Colony isolation

*After* 8 days of incubation, a loopful of serially diluted culture suspension, from the slants, was streaked on YM agar medium plates in order to obtain the single isolated colonies. The inoculated plates were incubated at 28°C for 7 days. The colonies were screened on the basis of their morphology.

### 2.3. Shake flask process for Sirolimus production

*Single* colonies of the microorganism were studied based on their morphological characteristics and 0.5 ml of the selected colony suspensions in 0.85% saline was inoculated in 30 mL autoclaved lab inoculum media comprising soya bean flour (18 g/L), L-lysine HCl (4.5 g/L), yeast extract (5.0 g/L) and dextrose (25 g/L).

*The* inoculated flasks were incubated at 29°C and 240 rpm. pH, PMV and microscopy were analysed. *Further*, 1 mL of matured inoculum with pH 5.4, PMV: 10.0 and age of 72 hrs was transferred to the seed media having the same composition as the lab inoculum media.

*The* flasks were incubated at 29°C and 200 rpm for 96 hours. pH, PMV and microscopy were analysed regularly.
After 96 hours of incubation, 10 % of matured inoculum having pH of 5.5, PMV: 12 was transferred to the production media.

### 2.4. Production media

The amount of sirolimus produced by the original culture was analysed using the production media which encompasses the following ingredients:

**Table 2.1: Composition of production media (30 mL in 250 mL flask)**

| Material | Quantity(g/L) |
|---|---|
| Soya flour (Defatted) | 20.0 |
| Glycerol | 40.0 |
| Dextrose | 20.0 |
| $KH2PO_4$ | 1.5 |
| $K2HPO_4$ | 1.5 |
| L-Lysine HCl | 10.0 |
| Malto Dextrin | 35.0 |
| PPG-2000 | 0.5 |
| NaCl | 2.5 |
| *Mineral solution* | 1.0 |

pH of the media at the time of inoculation was 6.5 and the flasks were incubated at 29°C at 200 rpm for 264 hours. pH, PMV and microscopy were checked at regular intervals of time.

### 2.5. Optimisation of the independent variables

#### 2.5.1. Plackett-Burman Design

After inoculation of production media with the seed culture, activity was tested. However, in the quest of obtaining increased yield, production media was optimised.

Initially, Plackett-Burman (PB) method, based on first order model, has been employed for the purpose (Luthra et al., 2021; Plackett & Burman, 1946). The method helps to recognise the essential components of the medium and to assess the collective

influence of the most significant ones on the production of secondary metabolites. All the experiments were carried out in triplicate using Equation 1 which has been stated as follows:

$$Y = \beta o + \beta i X i \ (I = 1,\ldots k)$$

For the study, the minimal and maximal levels (denoted by (0) and (1) respectively) selected for the 10 factors ($X_1$ to $X_{10}$ factors with 4 variables as dummies ($D_1$ to $D_4$) investigated using 12 run design has been shown in Table 2.

**Table 2.2: Code sheet for Plackett-Burman design**

| Independent Factors | Code | Low Value (0) (g/L) | High Value (1) (g/L) |
|---|---|---|---|
| Soya flour (Defatted) | $X_1$ | 15 | 25 |
| Glycerol | $X_2$ | 30 | 50 |
| Dextrose | $X_3$ | 15 | 25 |
| L-Lysine HCl | $X_4$ | 7.5 | 12.5 |
| Malto Dextrin | $X_5$ | 26.2 | 44 |
| PPG-2000 | $X_6$ | 0.35 | 0.65 |
| $D_1$ | $X_7$ | - | + |
| $D_2$ | $X_8$ | - | + |
| $D_3$ | $X_9$ | - | + |
| $D_4$ | $X_{10}$ | - | + |

**2.5.2. One Variable At a Time method (OVAT)**

The components screened using Plackett-Burman Design were further optimized with One Variable At a Time (OVAT) method (Luthra et al., 2021). For this procedure, the concentration of one component of the media is varied at a time while keeping the concentration of the remaining components as constant. The final concentration of the most significant ingredient of the media showing maximum enhancing effect on the activity is determined and precise composition is established.

The newly designed production media post optimisation was utilised and the activity was recorded using HPLC.

**2.6. Quantification using HPLC**

Analysis of sirolimus in broth samples was performed using HPLC. For this, 5g of the sample was taken in 25.0 mL volumetric flask. 15 ml of the diluent (methanol) was added to it and the mixture was sonicated for 50 minutes. The volume of the diluent was made up following final shaking for 10 minutes. The contents were then filtered through the Whatman filter paper. The filtrate obtained was re-filtered using 0.45 µ nylon membrane filter and finally injected into the HPLC comprising C-18 column (Hypersil BDS, 5µ C18 (250 mm X 4.6 mm). Concentration of sirolimus was calculated by comparison of peak areas with standard sirolimus and subsequently the calculated activity was recorded.

**2.7. Mutagenesis**

In order to experiment for enhanced activity, the actinomycete was exposed to physical and chemical mutagens.

**2.7.1. Treatment with UV radiation**

Intermittent exposure to UV rays as opposed to conventional UV irradiations, was experimented in the current study. The mature culture of *S. hygroscopicus* was mixed with 5 mL normal saline and serially diluted up to $10^{-6}$ concentration. 5 mL of the latter dilutions ($10^{-5}$ and $10^{-6}$) were distributed into sterile petriplates. The culture-containing plates were exposed to 254 nm UV radiations at a distance of 15 cm from the source in an intermittent fashion for different time intervals between 30 to 120 seconds in UV chamber, with a brief period of no UV after every 30 seconds. The process was performed in a dark room to avoid any photoreaction.

0.1 ml of these exposed spores were spread on the basal media containing 4.0 g Dextrose, 4.0 g Yeast extract, 10.0 g Malt extract and 20.0 g/L agar. Plates were incubated at 28°C for 12 days. Later, the percentage survival was calculated and the samples with a death rate of 95.0% and above were subjected to subsequent isolation.

**2.7.2. Treatment with chemical mutagen**

Following several rounds of screening and isolation post UV exposure, the stable colony suspension was dispersed in 2 mL Eppendorf tubes, each comprising 1.5 mL culture suspension. After centrifugation, the pellet obtained was washed with 0.01 M phosphate buffer, the culture was resuspended in the same buffer. Then the contents were split into an array of test tubes to contain 1 mL culture suspensions. 0.5 mL of the same was used as the control. The test tubes containing 1.0 mL of the culture suspensions were sequentially treated with NTG at variable dilutions keeping constant the amount of culture (1: 1, 1: 2, and 1: 3). NTG stock concentration was 10 mg/mL in 0.2 M phosphate buffer at pH 6. Post mutagenic treatment, the tubes were kept in incubator for 180 minutes at 28 °C and 200 rpm for proper interaction.

All the chemically treated samples were processed further separately along with control by adding 1 ml of 5% sodium thiosulphate. Following centrifugation, the pellets obtained were washed twice with sterile water and then the culture suspensions in the respective Eppendorf tubes were serially diluted to $10^{-5}$. 1 ml of the latter dilutions ($10^{-3}$, $10^{-4}$ and $10^{-5}$) were inoculated on basal media to calculate the percentage survival. Samples with 95% and more as the death rate were subjected to subsequent

isolation. Further screening involved the spreading of 0.1 mL of the treated culture suspensions on basal media, following incubation for 12 days at 28ºC.

Thereafter the stable mutants obtained following mutagenesis were detected by natural selection based on the consistent expression of the phenotypic character up to 5 generations and were then maintained on basal slants for experimental purposes. Production activity testing was eventually done using the optimised production media.

### 2.8. Crude extraction

The broth obtained from the production flask was filtered and the cell mass extraction was performed using toluene. The rich solvent was further concentrated and mixed with acetonitrile. The components were then allowed to undergo carbon treatment. The product obtained was crystallized and eventually quantified using HPLC.

## III. RESULTS AND DISCUSSION

### *3.1. Streptomyces hygroscopicus* colony characteristics during single colony isolation

For the isolation of single colonies of *Streptomyces hygroscopicus*, the characteristic features which were considered are as follows:

**Table 3.1: Visual and microscopic characterization**

| Colony Characteristics | Description |
|---|---|
| Diameter | About 5 mm |
| Shape | Round |
| Sporulation | Absent |
| Pigmentation | Absent |
| Elevation | Flat, slightly elevated in the centre, cream in colour |

### 3.2. Initial production activity

Prior to any changes towards enhancement in the production of sirolimus from the microorganism, the initial production activity was recorded.

**Table 3.2: Production flask observation (initial)**

| Age Hrs | pH | PMV% | Activity mg/L |
|---|---|---|---|
| 120 | 5.2 | 40 | - |
| 144 | 5.2 | 50 | 105 |
| 168 | 5.2 | 50 | 108 |
| 192 | 5.2 | 50 | 110 |
| 216 | 5.2 | 50 | 111 |
| 240 | 5.2 | 45 | 120 |
| 264 | 5.2 | 45 | 128 |

### 3.3. Medium Optimisation

The chief medium components ($X_1$ to $X_8$) were screened through 10 variable 12 run design of Plackett Burman (PB). The design along with the response have been configured in Table 5.

**Table 3.3: Plackett Burman design and its response**

| Run | $X_1$ | $X_2$ | $X_3$ | $X_4$ | $X_5$ | $X_6$ | $X_7$ | $X_8$ | $X_9$ | $X_{10}$ | Response (mg/L) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 124 |
| 2 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 236 |
| 3 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 159 |
| 4 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 251 |
| 5 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 274 |
| 6 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 242 |
| 7 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 223 |
| 8 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 198 |
| 9 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 209 |
| 10 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 185 |
| 11 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 167 |
| 12 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 179 |

According to the experiments based on the PB design, we observed that the runs involving higher coded values for soya flour ($X_1$), L-lysine HCl ($X_4$) and Maltodextrin ($X_5$) showed comparatively more yield as compared to the others. Also, lower coded value for glycerol ($X_2$) was found significant towards production. The maximum activity obtained using this model was during the $5^{th}$ run. We experimented with various concentration levels of the discussed factors.

Further, OVAT helped to finalise the most optimum concentration of the significant variables obtained through PB model. The effect of varying concentrations of the most eminent factors pertaining to the yield of sirolimus, as part of the OVAT methodology, has been included in the tables that follow:

**Table 3.4: Effect of glycerol concentration on activity**

| Concentration (g/L) | Activity (mg/L) |
|---|---|
| 28 | 176 |
| 30 | 250 |
| 32 | 272 |
| 34 | 263 |
| 36 | 252 |

**Table 3.5: Effect of L-lysine HCl concentration on activity**

| Concentration (g/L) | Activity (mg/L) |
|---|---|
| 08 | 244 |
| 10 | 252 |
| 12 | 281 |
| 14 | 256 |
| 16 | 232 |

**Table 3.6: Effect of Malto dextrin concentration on activity**

| Concentration (g/L) | Activity (mg/L) |
|---|---|
| 43 | 254 |
| 46 | 278 |
| 49 | 265 |
| 51 | 261 |
| 54 | 252 |

Through OVAT analysis, we obtained the highest yielding concentration of the most significant variables assessed through the entire optimisation strategy. These values helped in designing the final production media comprising of the following composition:

**Table 3.7: Final production media**

| Material | Quantity(g/L) |
|---|---|
| Soya flour (Defatted) | 25.0 |
| Glycerol | 36.0 |
| Dextrose | 30.0 |
| KH2PO4 | 1.0 |
| K2HPO4 | 1.0 |
| L-Lysine HCl | 22.0 |
| Malto Dextrin | 46.0 |
| PPG-2000 | 0.5 |
| NaCl | 2.5 |
| *Mineral solution* | 1.0 |

**3.4. Mutagenesis and subsequent screening for enhanced activity**

In an attempt to obtain high-producing strains of *Streptomyces hygroscopicus*, physical and chemical mutagenesis was sequentially performed and the potent colonies were screened by natural selection. In the current study, UV irradiation was used for inducing physical mutagenesis and followed by selection of stable mutants, treatment with NTG was carried out simultaneously with different UV treated colony suspensions.

After each mutagenic treatment, production activity of the selected mutants was analysed using shake flask method and the most promising strain was taken for the next mutagenic treatment.

In case of UV irradiation, intermittent exposure to light was done. It was found that the colonies exposed for 90 seconds with a period of 2 seconds of darkness after each 30 seconds showed the survival rate of 10 % only, which was also the lowest in comparison to the control. These colonies were stabilised and isolated so that chemical mutagenesis can be performed on them. For the second experiment involving treatment with NTG, the stock concentration of the mutagen was 10 mg/ml 1 mL of the UV mutated colonies were treated with NTG diluted to $10^{-1}$, $10^{-2}$and $10^{-3}$. The suspensions treated with $10^{-2}$ dilution offered colonies showing an approximate increase of 1.5 - 2 times in activity compared to the initial.

The structure of the colonies obtained post mutation was found to be roughly round with slightly wrinkled edges, having an elevated centre. Figure 1 highlights the morphology of a colony screened after physical and chemical mutagenesis.

**Figure 3.1: Sirolimus colony post mutagenesis**

### 3.5. Final Production flask Observation

The production media was optimised so that simultaneous checking of activity of the stable strains can be performed. Now post mutagenesis, we have used the mutated colonies to be checked for sirolimus production in the final optimised media. The result has been included in the table below:

**Table 3.8: Production flask observation (final)**

| Age Hrs | pH | PCV% | Activity mg/L | Remarks |
|---|---|---|---|---|
| 144 | 6.5 | 30 | - | Dense Mycelial growth |
| 168 | 6.5 | 40 | 268 | Dense Mycelial growth |
| 192 | 6.5 | 40 | 284 | Dense Mycelial growth |
| 216 | 6.5 | 50 | 306 | Dense Mycelial growth |
| 240 | 6.5 | 50 | 322 | Dense Mycelial growth |
| 264 | 6.5 | 60 | 357 | Dense Mycelial growth |

HPLC analysis for sirolimus production against its standard chromatogram has been shown in the following figures:

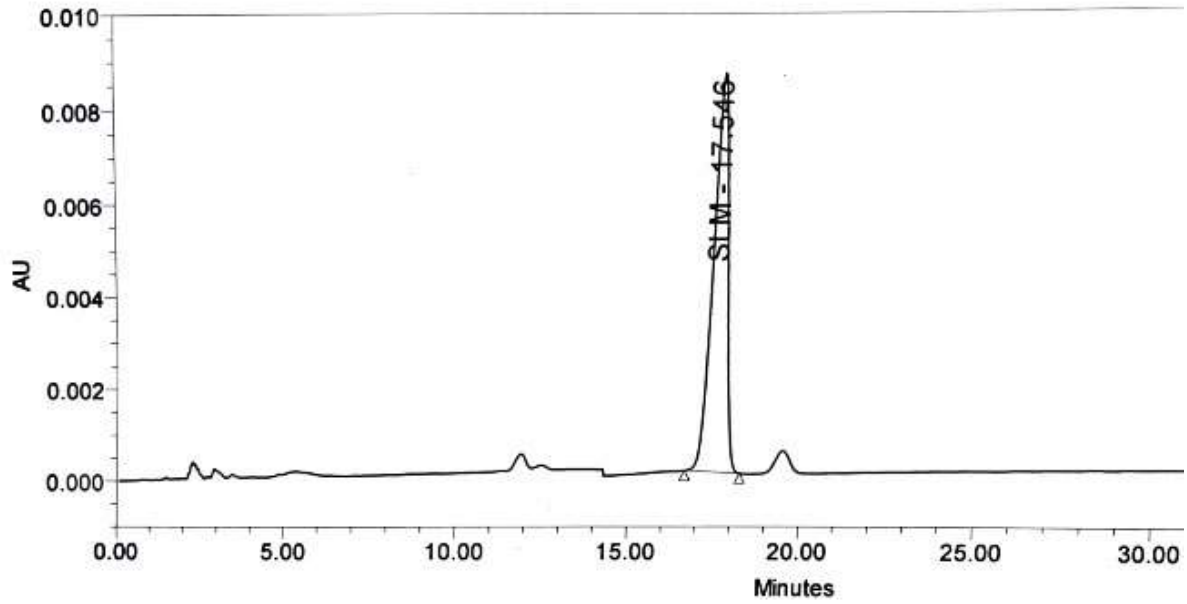


**Figure 3.2: Sirolimus (Standard)**

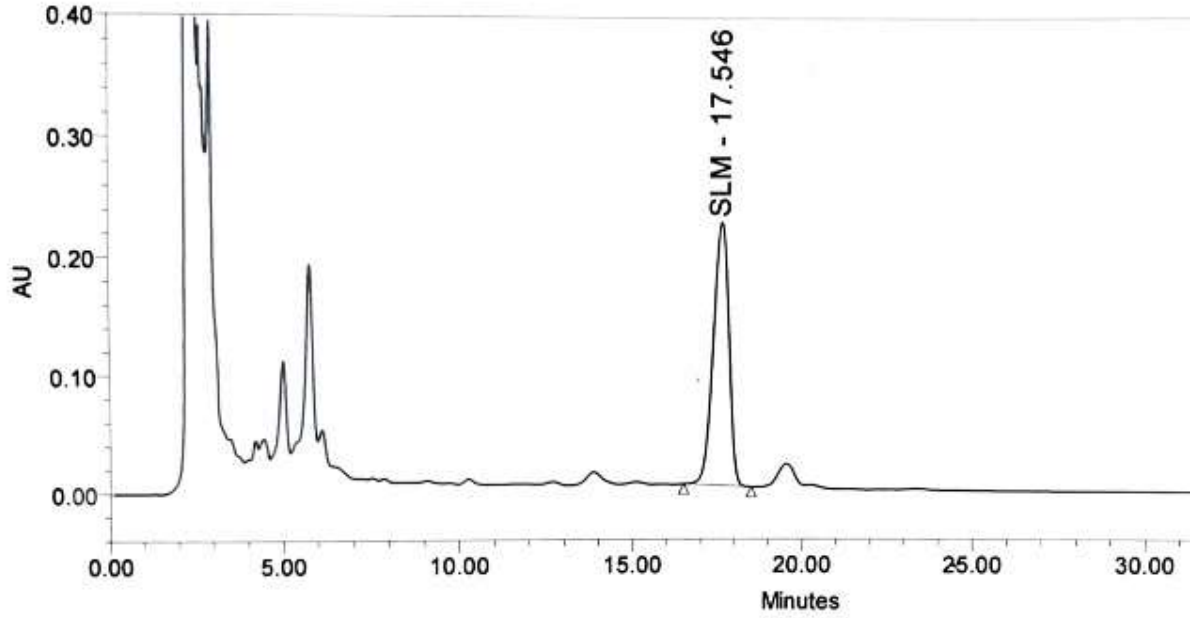


**Figure 3.3: Sirolimus (Sample broth)**

The change in the sirolimus production following analysis at each step of the sequential methodology adopted in the present investigation has been clearly indicated through the graphical representation below:

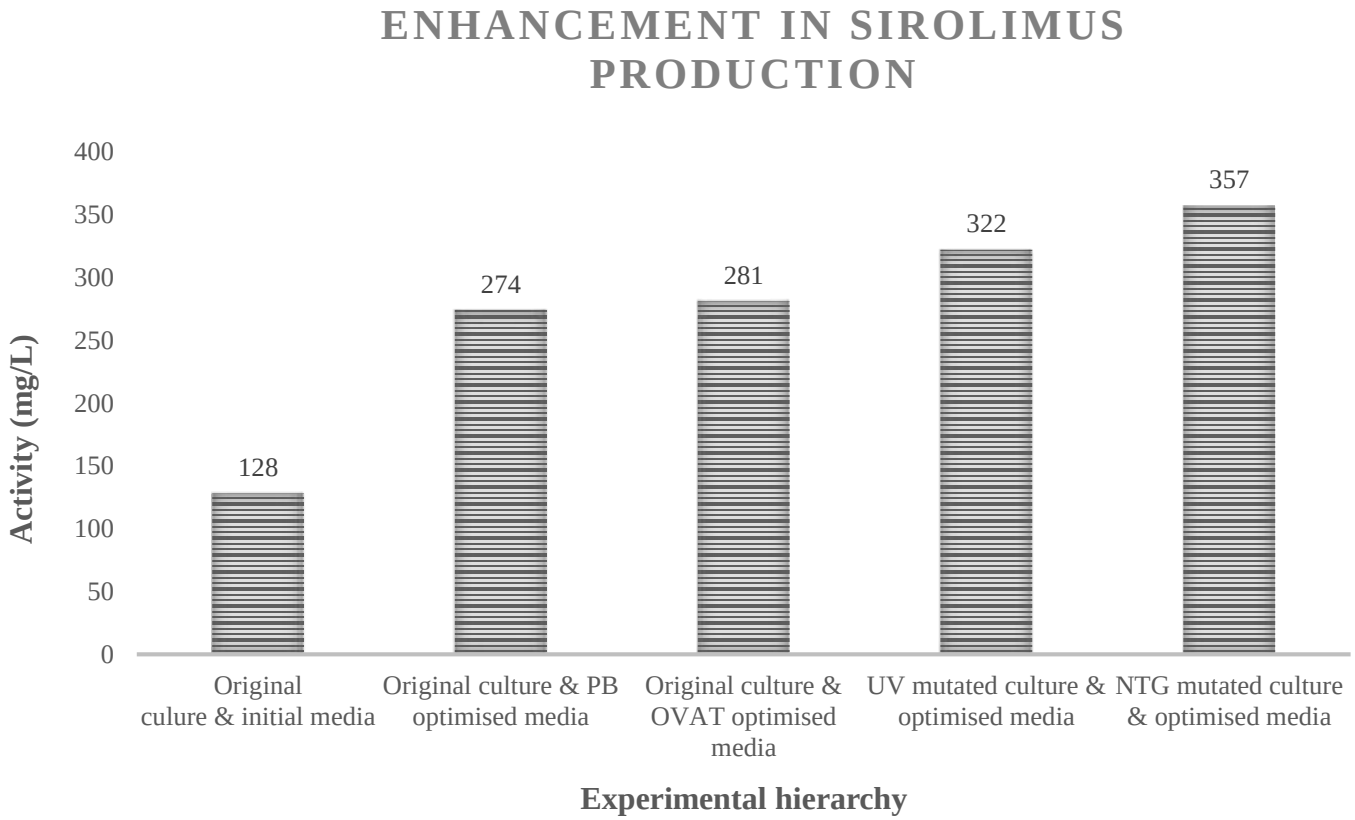


**Figure 3.4: Graphical representation of the experiments and results**

## IV. CONCLUSION

The present study encompasses a sequential approach beginning with establishment of appropriate concentration of the medium components. This was carried out using Plackett-Burman design modelling, which was made precise using OVAT approach. Then we have performed mutagenesis using UV radiations in an intermittent fashion. This was done in the hope of obtaining better and stable colonies faster than the traditional method. The colonies obtained thereby were treated with varying dilutions of NTG. We concluded that mutagenesis was not too much effective in enhancing the production of sirolimus but the collective result of the approach followed during the current study has been found useful in increasing sirolimus production by 2.5 to 2.8-fold. The initial production activity checked with the originally procured strain was 128 mg/L and finally, post optimisation and mutagenesis, the activity was recorded to be 357 mg/L.

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**REFERENCES**

[1] Abizaid, A. 2007. Sirolimus-eluting coronary stents: a review. *Vascular health and risk management*, *3*(2): 191. doi:10.2147/vhrm.2007.3.2.191

[2] Ardissino, D, Cavallini, C, Bramucci, E, Indolfi, C, Marzocchi, A, Manari, A, ... & SES-SMART Investigators. 2004. Sirolimus-eluting vs uncoated stents for prevention of restenosis in small coronary arteries: a randomized trial. *Jama*, *292*(22): 2727-2734. doi:10.1001/jama.292.22.2727

[3] Bastidas, RJ, Shertz, CA, Lee, SC, Heitman, J, & Cardenas, ME. 2012. Rapamycin exerts antifungal activity in vitro and in vivo against Mucor circinelloides via FKBP12-dependent inhibition of Tor. *Eukaryotic cell*, *11*(3): 270-281. doi:10.1128/ec.05284-11

[4] Bové, J, Martínez-Vicente, M, & Vila, M. 2011. Fighting neurodegeneration with rapamycin: mechanistic insights. *Nature Reviews Neuroscience*, *12*(8): 437-452. doi:10.1038/nrn3068

[5] Chen, X, Wei, P, Fan, L, Yang, D, Zhu, X, Shen, W, ... & Cen, P. 2009. Generation of high-yield rapamycin-producing strains through protoplasts-related techniques. *Applied microbiology and biotechnology*, *83*(3): 507-512. doi:10.1007/s00253-009-1918-7

[6] Cutler, C, Logan, B, Nakamura, R, Johnston, L, Choi, S, Porter, D, ... & Antin, JH. 2014. Tacrolimus/sirolimus vs tacrolimus/methotrexate as GVHD prophylaxis after matched, related donor allogeneic HCT. *Blood, The Journal of the American Society of Hematology*, *124*(8): 1372-1377. doi:10.1182/blood-2014-04-567164

[7] Dumont, FJ, & Su, Q. 1995. Mechanism of action of the immunosuppressant rapamycin. *Life sciences*, *58*(5): 373-395. doi:10.1016/0024-3205(95)02233-3

[8] Dutta, S, Basak, B, Bhunia, B, & Dey, A. 2014. Bioprocess engineering aspects of rapamycin (sirolimus) production: a review on past achievements and recent perspectives. *Journal of Bioprocess Engineering and Biorefinery*, *3*(4): 243-256. doi:10.1166/jbeb.2014.1105

[9] Dutta, S, Basak, B, Bhunia, B, Sinha, A, & Dey, A. 2017. Approaches towards the enhanced production of Rapamycin by Streptomyces hygroscopicus MTCC 4003 through mutagenesis and optimization of process parameters by Taguchi orthogonal array methodology. *World Journal of Microbiology and Biotechnology*, *33*(5): 90. doi:10.1007/s11274-017-2260-3

[10] Gregory, MA, Gaisser, S, Lill, RE, Hong, H, Sheridan, RM, Wilkinson, B, ... Leadlay, PF. 2004. Isolation and Characterization of Pre-rapamycin, the First Macrocyclic Intermediate in the Biosynthesis of the Immunosuppressant Rapamycin by S. hygroscopicus. *Angewandte Chemie International Edition*, *43*(19): 2551-2553. doi:10.1002/ange.200453764

[11] Groth, CG, Bäckman, L, Morales, JM, Calne, R, Kreis, H, Lang, P, ... & Sirolimus European Renal Transplant Study Group. 1999. SIROLIMUS (RAPAMYCIN)-BASED THERAPY IN HUMAN RENAL TRANSPLANTATION: Similar Efficacy and Different Toxicity Compared with Cyclosporine: 1, 2. *Transplantation*, *67*(7): 1036-1042. doi:10.1097/00007890-199904150-00017

[12] Jung, WS, Yoo, YJ, Park, JW, Park, SR, Han, AR, Ban, YH, ... & Yoon, Y. J. 2011. A combined approach of classical mutagenesis and rational metabolic engineering improves rapamycin biosynthesis and provides insights into methylmalonyl-CoA precursor supply pathway in Streptomyces hygroscopicus ATCC 29253. *Applied microbiology and biotechnology*, *91*(5): 1389-1397. doi:10.1007/s00253-011-3348-6

[13] Lamming, DW (2016). Inhibition of the mechanistic target of rapamycin (mTOR)–rapamycin and beyond. *Cold Spring Harbor perspectives in medicine*, *6*(5), a025924. doi:10.1101/cshperspect.a025924

[14] Law, BK. 2005. Rapamycin: an anti-cancer immunosuppressant?. *Critical reviews in oncology/hematology*, *56*(1): 47-60. doi:10.1016/j.critrevonc.2004.09.009

[15] Liu, Q, Lin, Q, Li, X, Ali, M, & He, J. 2020. Construction and application of a “superplasmid” for enhanced production of antibiotics. *Applied microbiology and biotechnology*, *104*(4): 1647-1660. doi:10.1007/s00253-019-10283-6

[16] Luthra, U, Babu, P, Remya, RR, Julius, A, Patel, Y, Veera, RJ, & Majeed, I. 2021. Medium optimization and downstream process design for the augmented yield of β-Carotene using fungi Blakeslea trispora. *Pigment & Resin Technology*. doi:10.1108/prt-05-2021-0055

[17] Manning, BD. 2017. Game of TOR—the target of rapamycin rules four kingdoms. *New England Journal of Medicine*, *377*(13): 1297-1299. doi:10.1056/nejmcibr1709384

[18] Mohamed, MA, Elkhateeb, WA, Taha, MA, & Daba, GM. 2019. New Strategies in Optimization of Rapamycin Production by Streptomyces hygroscopicus ATCC 29253. *Research Journal of Pharmacy and Technology*, *12*(9): 4197-4204. doi:10.5958/0974-360x.2019.00722.4

[19] Morath, C, Arns, W, Schwenger, V, Mehrabi, A, Fonouni, H, Schmidt, J, & Zeier, M. 2007. Sirolimus in renal transplantation. *Nephrology Dialysis Transplantation*, *22*(suppl_8): viii61-viii65. doi:10.1093/ndt/gfm652

[20] Napoli, KL, & Taylor, PJ. 2001. From beach to bedside: history of the development of sirolimus. *Therapeutic drug monitoring*, *23*(5): 559-586. doi:10.1097/00007691-200110000-0001

[21] Patel, GK, Patel, GK, Goyal, R, & Waheed, SM. 2019. Current Update on Rapamycin Production and its Potential Clinical Implications. *High Value Fermentation Products: Human Health*, *1*, 145-163. doi:10.1002/9781119460053.ch6

[22] Plackett, RL, & Burman, JP. 1946. The design of optimum multifactorial experiments. *Biometrika*, *33*(4): 305-325. doi:10.1093/biomet/33.4.305

[23] Risdian, C, Mozef, T, & Wink, J. 2019. Biosynthesis of polyketides in Streptomyces. *Microorganisms*, *7*(5), 124. doi:10.3390/microorganisms7050124

[24] R.Tripathi, A, Singh, NK, & luhtra, U. 2014. Optimization of Nutrient Components for Tacrolimus Production by Streptomyces tsukubaensis using Plackett-Burman followed by Response Surface Methodology. IOSR Journal of Pharmacy (IOSRPHR), 4(7): 66–75. doi:10.9790/3013-0407066075

[25] Shah, PR, Kute, VB, Patel, HV, & Trivedi, HL. 2015. Therapeutic drug monitoring of sirolimus. *Clinical Queries: Nephrology*, *4*(3-4), 44-49. doi: 10.1016/j.cqn.2015.11.005
[26] Vezina, C, Kudelski, A, & Sehgal, SN. 1975. Rapamycin (AY-22, 989), a new antifungal antibiotic I. Taxonomy of the producing streptomycete and isolation of the active principle. *The Journal of antibiotics*, *28*(10): 721-726. doi:10.7164/antibiotics.28.721
[27] Yoo, YJ, Kim, H, Park, SR, & Yoon, YJ. 2017. An overview of rapamycin: from discovery to future perspectives. *Journal of Industrial Microbiology and Biotechnology*, *44*(4-5): 537-553. doi:10.1007/s10295-016-1834-7