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Introduction of Successive Mutagenesis and Medium Optimisation for Enhanced Production of Ascomycin from Bacteria

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Abstract: ***Ascomycin is an important pharmacological product whose industrial yield is primarily credited to a bacteria named Streptomyces hygroscopicus var. ascomyceticus. The current investigation conglomerates a sequential approach adopted to genetically modify the parent strain via physical and chemical mutagenesis. The agents which we have used for inducing mutation include UV radiations, EMS and NTG. We have evaluated the effect of different individual mutagens on the parent strain. Post several rounds of screening and establishment of stable strain, we have used it to inoculate the optimised production media. For optimization of medium components, Plackett-Burman (PB) design and one-variable-at-a-time method (OVAT) have been included. At each step of the different methodologies adopted, shake flask activity has been fruitful in understanding the effect of strain modification as well as medium optimization. We have quantified crude ascomycin using HPLC. Finally, we have been able to enhance the productivity of ascomycin from the maximum of 205 mg/L with the initial conditions to 755 mg/L as a result of successive mutations and optimized production media.***

Keywords: ascomycin, *Streptomyces hygroscopicus*, mutagens, optimization, HPLC

1.Introduction

Ascomycin (FK520) is recognized as a 23-membered macrocyclic antibiotic belonging to the group of polyketides [1, 2]. It has been identified as an eminent pharmaceutical compound [3, 4] exhibiting a multitude of biological activities such as immunomodulation [5, 6, 7], antimalarial [7], anti-tumor [9, 10], anti-inflammatory [11, 12] and anti-fungal [13, 14, 23]. Ascomycin and its derivatives have found importance in the pharmacological sector [14, 15] with respect to disease conditions such as autoimmune disorders [16], atopic dermatitis [7, 17], organ transplant rejections [18, 19], psoriasis [20, 21]. Being known to aid in multifarious medical conditions, ascomycin has gained colossal significance as an eminent molecule to be considered for industrial production on a large scale. For this purpose, the highly coveted organism is a bacteria named *Streptomyces hygroscopicus* var. *ascomyceticus*. This microorganism produces ascomycin as a secondary metabolite. Various types of fermentation methodologies in combination with strategies to aid in the production and extraction of ascomycin have been adopted by pharmaceutical companies across the world [18, 25, 30]. But, with a wide array of clinical significance, the yield of ascomycin in sufficient amounts is still a topic of detailed discussion and non-exhaustive industrial research [18, 22, 26].

In the current investigation, we have tried to devise a sequential approach using which we have obtained highly promising results in the context of enhanced production of ascomycin which can be implied to solve issues pertaining to low industrial availability of the secondary metabolite.

2.Literature Survey

Ascomycin is a compound characterized by high structural complexity [3], therefore it is difficult to synthesize it chemically from scratch. Thus, its production with the help of microbes gains an immense amount of significance [23, 24]. For the industrial production of ascomycin, as stated before, the highly preferred microorganism is *Streptomyces hygroscopicus* var. *ascomyceticus* [22, 25] which synthesizes it as part of its secondary metabolic pathways [25, 27]. Fermentation technology and associated downstream processing further help in obtaining marketable amount of ascomycin [18, 22, 28]. But, the low industrial yield of this compound necessitates the strategic upgradation of the methodologies followed. For enhancement in the production of ascomycin, different kinds of genetic modifications have been employed in regard to strain improvement and various optimization approaches have been used to design the best medium conditions [27, 29]. In an approach, Femtosecond laser irradiation technology was used to study the improvement in the ascomycin production from *Streptomyces hygroscopicus* var. *ascomyceticus* NT2-11 which helped in obtaining 45 % more ascomycin in comparison to the parental strain [8, 18]. One of the recent studies involved the conglomeration of traditional mutagenesis along with computational models to design experimental media which helped in obtaining three times more the initial yield [31]. Various other efforts in this respect have made use of more sophisticated technologies like pathway engineering and precise genome modifications [18, 31, 32, 33]. Several transcriptional factors or reaction intermediates have been studied in respect of their role in

the augmented yield of ascomycin [34]. In an interesting in-silico approach, an updated network model was redesigned using ^{13}C-metabolic flux analysis which was further utilized to predict gene targets having a role in increased ascomycin production [35].

Using specific mutagens on the parental strain to study various alterations in regard to secondary metabolite production has been a very common practice [32, 36, 38]. For our study, we have tried to analyze the effects of mutagens and their specific concentrations while checking the productivity at each level. Successive mutagenesis using UV radiations, followed by EMS and NTG for different spore suspensions has been done. We have made efforts to analyse the effect of both the alkylating agents on our strain, post UV exposure. Optimisation has been employed for designing the production media in order to investigate the most significant factors with respect to augmented productivity. We have followed a simple methodological approach to present a recognizable increase in ascomycin production.

3.Material and Methods

Slant preparation and single colony isolation

Culture of *Streptomyces hygroscopicus* var. *ascomyceticus* was obtained from Industrial Lab, Mumbai, India and it was maintained on slant medium composed of 5.0 g/L starch, 5.0 g/L dextrose monohydrate, 2.0 g/L bacteriological peptone, 3.0 g/L yeast extract, and 25.0 g/L agar with pH adjusted at 7.0. Post-medium sterilization for 30 minutes at 121°C, the slant culture was prepared by taking 0.2 mL of the procured spore suspension and spread over the medium, following which the slants were incubated at 28°C in the incubator for 12 days.

After maturation, the slants were wrapped in parafilm & aluminum foil and stored in the refrigerator at 5^{o}C.

For the purpose of single colony isolation, a loopful of serially diluted spore suspension from slant culture was streaked on plate medium and incubated at 28°C for 12 days.

The colonies were screened on the basis of morphology under the stereo-microscope.

Shake flask Method

Post 15 days of incubation for colony isolation, the spores were collected with 0.85% normal saline and then homogenized. Finally, 2mL of colony spore suspension was transferred into 150 mL in 1000 EMF seed/inoculum medium. The components of the media have been described below:

Table 1: Lab seed/ inoculum medium

Material	Quantity (g/L)
Dextrose monohydrate	7.5
Corn Starch	15.0
Soya bean meal	7.5
Inactive Dried yeast	1.0
Sodium Chloride	1.0
Yeast extract powder	1.0
Initial pH – 6.90, pH Adjusted – 7.00 Sterilization Time – 30 mins at 121°C, pH after Sterilization – 6.80	

The seed, post-inoculation was incubated on a shaking incubator at 28°C; and 240 rpm for 72 hrs.
The mature culture had pH 7.0 with 14% PMV. Various other physical parameters were analyzed at regular intervals of time.

Determination of physical parameters:

The physical process parameters like pH, %PMV, and microscopic characteristics were estimated at different intervals (from day 1 to day 11 at an interval of every 24 hours).

For measuring the pH of culture broth, the pH meter was calibrated with neutral (7.0), acidic (4.01) and alkaline (10.01) buffer. pH electrode was rinsed with demineralized water and was then wiped carefully with a tissue paper. The electrode was immersed into the sample and the stable pH readings were noted down.

Packed mycelial volume (PMV) of the sample was determined by putting 10 mL broth sample in 15 mL graduated tube and centrifuged at 5000 rpm for 5 min. The PMV was the pellet volume expressed as a percentage (%) of measured total volume of the tube. Equation 1 mentioned below highlights the same:

$$PMV\% = \frac{\text{Precipitate volume(mL)}}{\text{Total Volume(mL)}} X\ 100$$

For analysing the microscopy, a smear was prepared by taking loopful of culture suspension that is to be stained. The smear was heat fixed so that the culture gets firmly mounted onto the slide. Gram staining was done and observed under the microscope.

Initial production activity

After 72 hours of incubation, 3 mL of the matured lab seed culture with pH 7.0 and 14% PMV, was transferred to the production flask containing media having the following composition:

Table 2: Lab production medium

Material	Quantity (g/L)
Corn starch	38.0
Malto Dextrin	32.0
Maltose	25.0
Glycerol	24.0
Soya bean flour	40.0
Calcium Carbonate	2.6
Dried yeast	5.0
Initial pH – 7.80	

During cultivation, the culture conditions were maintained with pH of 7.4, the temperature being 28 ^{o}C and 240 rpm and the activity was recorded continuously for definite intervals of time.

Quantification methodology

Analysis of ascomycin in broth samples was performed using HPLC. Precisely, the concentration of ascomycin produced in the culture broth was determined by HPLC (Water, pump-alliance (2695), auto sample-alliance (2695); detector –UV (2489) with empower software) method. Acetonitrile was used to extract the product from the fermentation broth. For this, 5 gm of the sample was weighed and dissolved in acetonitrile and filtered. A mobile phase of 0.01% trifluoroacetic acid and acetonitrile in 1: 1 ratio was used in isocratic mode. Ascentis Express C18, 100×4.6 mm, 2.6 µ column was used to estimate ascomycin concentration. The flow rate was set at 1.0 mL/min. The resulting solution (20 µl) was injected into the HPLC for the estimation of ascomycin.

Concentration of ascomycin present in the fermentation broth was calculated by comparison of peak areas with that of the ascomycin standard.

Preparation of solutions:

A. Standard Solution: 10 mg ascomycin standard was weighed in 50 mL volumetric flask and made up to volume with acetonitrile.
B. Sample Solution: 5 gm of broth sample was weighed in 25 mL volumetric flask, then 10 mL of acetonitrile was added and sonication was done. Finally, the volume was made with acetonitrile.

Mutagenesis

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

Treatment with UV radiation:

The mature spores of *S. hygroscopicus* var. *ascomyceticus* were harvested with 5 mL normal strain and serially diluted up to 10^{-6}.5 mL of the latter dilutions (10^{-5} and 10^{-6}) were distributed into sterile petriplates. The plates were exposed to 254 nm UV radiations at a distance of 15 cm from the source, for variable time intervals between 30 to 120 seconds in UV chamber. 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 5.0 g/L starch, 5.0 g/L dextrose monohydrate, 2.0 g/L bacteriological peptone, 3.0 g/L yeast extract and 25.0 g/L agar. Plates were incubated at 28^{o}C for 12 days. Later the percentage survival was calculated and the samples with a death rate of 99.0% were subjected to subsequent isolation.

Treatment with chemical mutagen:

Post several rounds of screening and isolation, the concentration of 10^{7} spores/mL of the UV mutated slant spore suspension was dispersed in four, 2 mL Eppendorf tubes, each containing 1.5 mL suspension. The tubes were centrifuged and the pellet was washed twice with 0.01 M phosphate buffer. The spores were again resuspended in the same buffer.

Each of the suspensions was then split in test tubes to contain 1mL and 0.5 mL of the suspension. While using 0.5 mL aliquot as control and treating 1 mL aliquot with 20 µg/mL of EMS, both of them were left for incubation at 28^{o}C for different time intervals between 30 to 120 minutes at 100 rpm. The chosen EMS concentration was finalized based on the lowest survival rate.

Different UV mutated spore suspensions were employed for the treatment with the next mutagen. For NTG mutation, I mg/mL of the mutagen was added into the other two spore suspensions at 28 °C and 100 rpm for variable time intervals between 30 to 120 minutes.

All the chemically treated samples were processed further separately along with control by adding 1 mL of 5% sodium thiosulphate. Then, centrifugation was done and the pellets obtained were washed twice with sterile water and finally, the spore 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 90% as the death rate were subjected to subsequent isolation. Further screening involved the spreading of 0.1 mL of the treated spore suspensions on basal media, following incubation for 12 days at 28^{o}C.

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 done at regular intervals of time.

Medium Optimisation

To study further enhancement in the production activity, optimization of the medium components was performed using Plackett-Burman (PB) method and One Variable At a Time (OVAT) model.

PB was based on first-order model and it helps to identify the essential components of the medium and to understand their influence on the production rates of secondary

metabolites [37]. All the experiments were carried out in triplicate using the following equation:

Equation 2:

$$Y = \beta o + \beta iXi \ (I = 1,\ldots.k)$$

For the current study, the importance of 6 factors was investigated using 2 dummies, 8 variables, 12 runs design. The code sheet for Plackett Burman design has been shown in Table 3.

Table 3: Code sheet for Plackett Burman design

Independent Factors	Code	Low Value (0) (g/L)	High Value (1) (g/L)
Corn Starch	X_1	36.0	60.0
Malto Dextrin	X_2	24.0	40.0
Maltose	X_3	18.75	31.25
Glycerol	X_4	18.0	30.0
Soya bean flour	X_5	30.0	50.0
Dried yeast	X_6	3.75	6.25
D_1	X_7	-	+
D_2	X_8	-	+

After PB, the results were analyzed appropriately and OVAT [39] was performed further in which the concentration of one component is changed at a time while keeping constant the remaining components. The final concentration of the most significant component which shows maximum activity is determined. Finally, the values pertaining to highly optimised medium variables are included in designing the new production media.

Final production activity

Shake flask and production activity were then carried out using the stable mutant strains and optimised medium components. The activity was recorded at regular intervals of time.

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.

4.Results and Discussion

Initial Production Activity

The originally procured strains were tested for initial activity and the results were recorded at regular intervals of time for the time duration of 24 to 192 hours.

Table 4: Initial production activity

Age (Hrs)	pH	PMV (%)	Activity (mg/L)
24	7.0	20	-
48	8.0	25	-
72	7.5	35	-
96	7.0	35	.
120	5.7	35	108
144	5.3	38	156
168	5.4	40	189
192	5.5	30	205

According to the above table, the maximum activity obtained prior to any changes in the medium components and strain was found to be 205 mg/L.

HPLC analysis with respect to the standard has been represented through the chromatograms shown in figure 1 and figure 2.

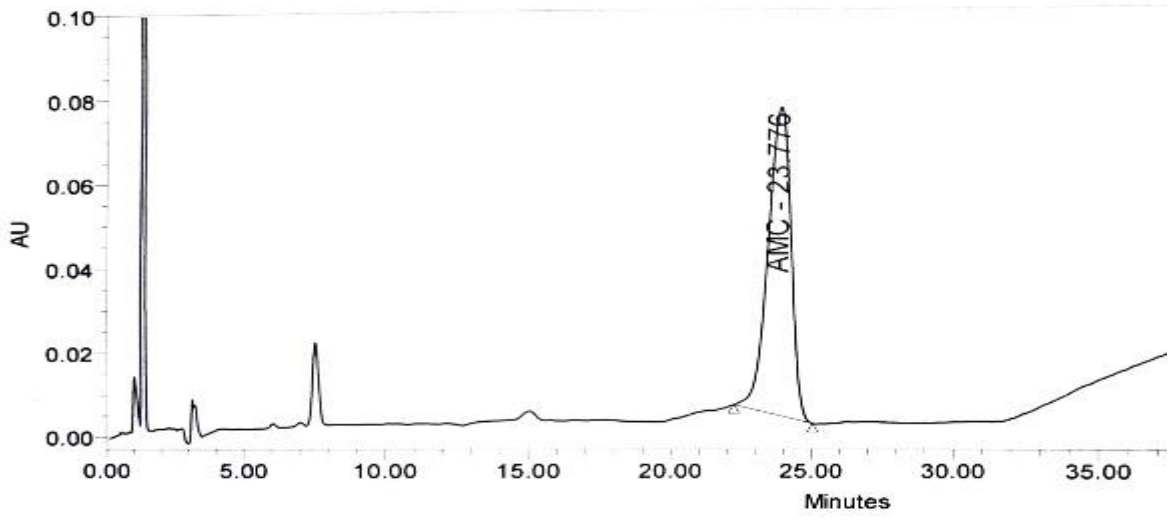


Figure 1: Ascomycin standard chromatogram

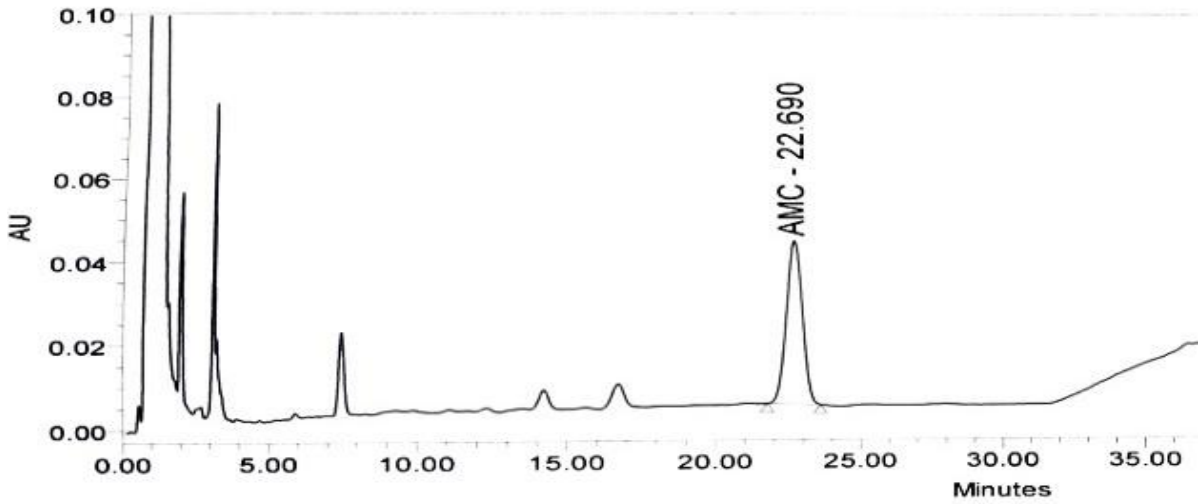


Figure 2: Sample broth chromatogram for ascomycin

Mutagenesis and subsequent screening for enhanced activity

In an attempt to obtain high-producing strains of *Streptomyces hygroscopicus* var. *ascomyceticus*, 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 EMS and NTG was performed simultaneously with different UV treated spore suspensions.

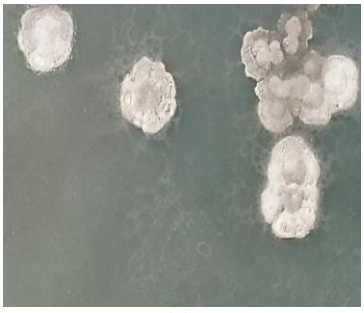
After each mutagenic treatment, the production activity of the selected mutants was analysed using shake flask method and the most promising strain was taken for the next mutagenic treatment.

In case of UV irradiation, out of all the different radiation doses given, the colonies resulting after exposure for 90 s showed the survival rate of 10 % which was the lowest in comparison to the control. For the second experiment involving the successive treatment of UV mutated spore suspension with EMS and NTG, an approximate increase of 1.5-2 times in the activity was obtained. The table below shows comparative results for EMS and NTG treated spore populations with respect to the activity obtained prior to mutagenesis, for different intervals of time:

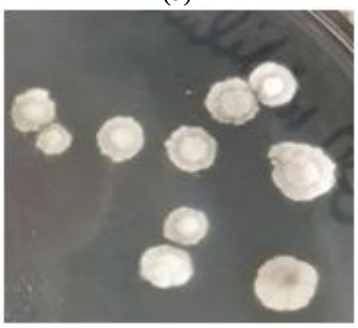
Table 5: Activity post mutation

Age (Hrs)	Initial Activity (mg/L)	Activity after treatment with EMS (mg/L)	Activity after treatment with NTG (mg/L)
120	108	134	110
144	156	220	206
168	189	320	285
192	205	410	398

The above table indicates that our culture has undergone more changes due to treatment with EMS in comparison to NTG. The maximum activity obtained post UV exposure was found to be 410 mg/L in case of EMS treated spore suspension and 398 mg/L after NTG treatment. The colonies showing maximum activity were picked up for final screening to establish stable mutants to be used for further studies. The figure below (Figure 3) highlights the changes in colonies during consecutive screenings. It was observed that introduction of mutagenesis modified the colonies to exhibit a slight tinge of grey.



(a)



(b)

(c)

Figure 3: Colony morphology (a) initially, (b) post-physical mutagenesis, and (c) after chemical mutagenesis

Medium Optimization

Plackett-Burman Design:

Mutagenesis is an important method of strain improvement. One more methodology that is employed towards enhancement in the production activity of the secondary metabolites is medium optimization. For our study, we have made use of Plackett Burman (PB) optimization procedure followed by OVAT.

The chief medium components (X_1 to X_8) comprising of 5 factors and 3 dummies were screened through 8 variable 12 run design of PB. The design along with the response recorded after 192 hours of incubation has been configured in table 6.

Table 6: Plackett Burman design and its response

Run	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	Response (mg/L)
1	1	0	1	0	0	0	1	1	428
2	1	1	0	1	0	0	0	1	400
3	0	1	1	0	1	0	0	0	38
4	1	0	1	1	0	1	0	0	650
5	1	1	0	1	1	0	1	0	528
6	1	1	1	0	1	1	0	1	655
7	0	1	1	1	0	1	1	0	489
8	0	0	1	1	1	0	1	1	215
9	0	0	0	1	1	1	0	1	500
10	1	0	0	0	1	1	1	0	638
11	0	1	0	0	0	1	1	1	350
12	0	0	0	0	0	0	0	0	238

F-test value evaluation has pointed out the significance of corn starch and dried yeast. For the runs, where the values of both these components was higher, better outcomes with the maximum being 655 mg/L for the 6th run was optained. The design has fostered well the importance of these components which have been further confirmed through experimental analysis.

One variable at a time method:

The experiments conducted based on Plackett Burman design have helped in the prediction of carbohydrate and nitrogen sources to be of eminent significance. The concentration of the variables incorporated has been further optimized using OVAT. It has been observed that maximum yield was seen in the run which involves a higher coded value for corn starch and dried yeast. However, it is important to point out here that the run in which lower values of the listed components have been incorporated, the product yield has been significantly reduced.

The effect of varying concentrations of corn starch on the yield of ascomycin are showed in Table 7.

Table 7: Effect of corn starch concentration on activity

Concentration (g/L)	Activity (mg/L)
20	337
30	489
40	510
50	578
60	670

The effect of varying concentrations of dried yeast on the yield of ascomycin were showed in Table 8.

Table 8: Effect of dried yeast concentration on activity

Concentration (g/L)	Activity (mg/L)
3.5	298
4.5	360
5.5	445
6.5	580
7.5	690

Based upon PB model, the most significant factors were found to be corn starch and dried yeast. The most appropriate concentration of both these components was further assessed by using OVAT model. In case of corn starch, maximum activity of 670 mg/L was obtained with the concentration of 60 g/L while 7.5 g/L dried yeast was found to be perfect for the designing of final production media. Thus, the concentration of various components present in the final production media have been tabulated as follows:

Table 9: Final production media

Material	Quantity (g/L)
Corn starch	60.0
Malto Dextrin	32.0
Maltose	25.0
Glycerol	24.0
Soya bean flour	40.0
Calcium Carbonate	2.5
Dried yeast	7.5
Initial pH – 7.80	

Final production activity

Post several screenings after treatment with mutagenic reagents and optimising the medium components by following various strategies, final ascomycin production activity was tested for different intervals of time and the results have been included in the table below:

Table 10: Final production activity

Age (Hrs)	pH	PMV (%)	Activity (mg/L)
24	7.0	25	-
48	8.0	25	310
72	7.5	30	320
96	7.0	35	456
120	5.7	35	490
144	5.3	38	654
168	5.4	45	690
192	5.5	50	755

During the current study, the maximum activity obtained after 192 hours of incubation of the mutated spore suspensions on the optimized media was found to be 755 mg/L. This culture broth was processed with solvents as discussed before and the final product was quantified using HPLC.

Crude extraction results

The broth obtained from the production flask was filtered to give 90 g of cell mass, extracted using toluene. Activity of 700 mg rich solvent concentration was found as 95 mg/g. The product was crystallized to give 650 mg harboring an activity of 920 mg/g.

HPLC results for ascomycin are in figure 4 and figure 5.

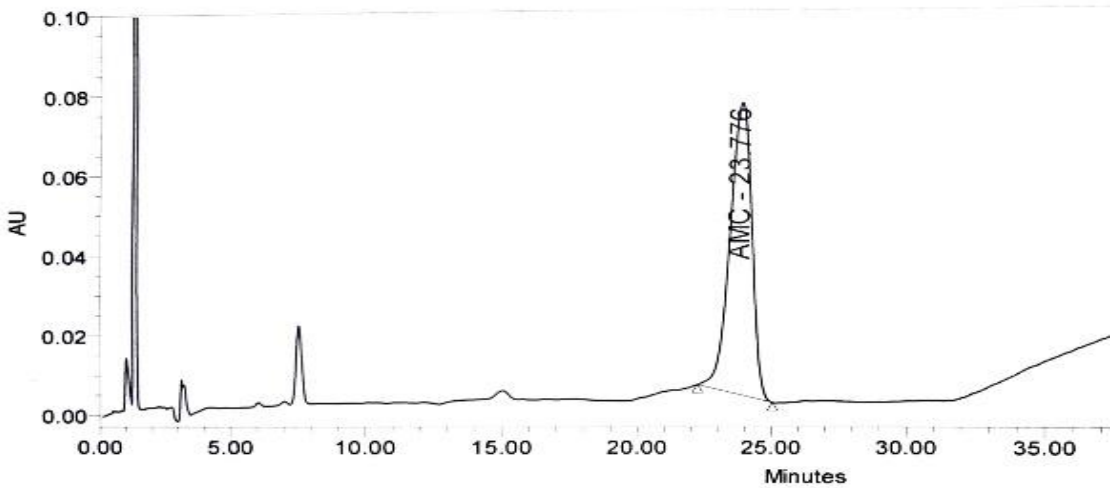


Figure 4: Ascomycin standard chromatogram

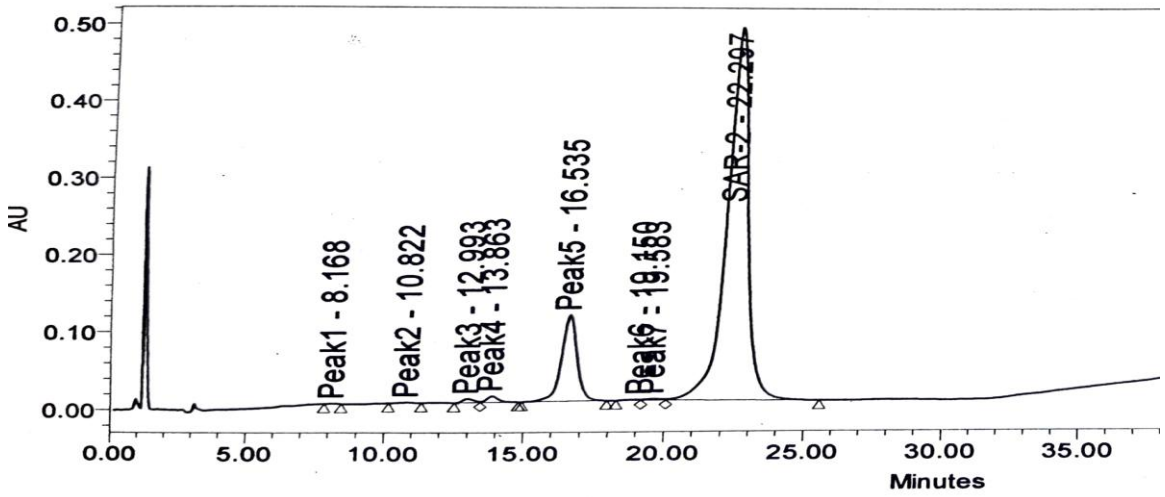


Figure 5: Product chromatogram

5.Conclusion

Ascomycin-producing strain namely *Streptomyces hygroscopicus* var. *ascomyceticus*, was procured and allowed to grow on basal media. The initial productivity was recorded post shake flask and laboratory production stage. The spores were then subjected to mutagenesis via UV exposure and EMS and NTG addition. The stable strains were selected to inoculate an optimised production media. Medium optimisation have established corn starch and dried yeast to be of notable significance and thus, highly selected values of both these components have been used to design the production media. The activities recorded at regular intervals of time have helped in assessing the impact of successive mutagenic events on the parent strain and finally an optimised media has provided an added advantage. Our approach has helped us in improvising the initial yield of 205 mg/L to 755 mg/L reinforcing an enhancement of more than 3-3.5 folds.

6.Future Scope

Introduction of mutation via UV irradiation and chemical mutagens is an old practice when it comes to studying the effect of random mutation on micro-organisms [18, 31]. We have tried to utilize these easily available genetic tools to modify the cluster of the microorganism in a more simple manner and the effect has been identified continually based on morphological classification. Finally, the sequentially modified colonies were allowed to grow in precisely optimized medium conditions which have helped us in increasing the production of ascomycin by several folds. This approach can prove to be instrumental as an aid to several other complicated techniques [32] being adopted towards the yield enhancement procedure. We are further aiming to develop a better and less-arduous screening model for the identification of improved mutant strains which can be directly utilized to obtain higher production of secondary metabolites in general. The scaling-up of the currently adopted approach is under process and we hope to improvise on our results by several folds.

Abbreviations

EMS-Ethyl methanesulfonate
NTG-N-methyl-N´-nitro-N-nitrosoguanidine
PB-Plackett-Burman
OVAT-one-variable-at-a-time method
HPLC-High-performance liquid chromatography
PMV-Packed mycelial volume

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References

[1] Liu F, Wang YQ, Meng L, Gu M, Tan RY., "FK506-binding protein 12 ligands: a patent review," *Expert opinion on therapeutic patents*, vol.23 (11), pp.1435-49, 2013. https://doi.org/10.1517/13543776.2013.828695
[2] Risdian C, Mozef T, Wink J., "Biosynthesis of polyketides in Streptomyces," *Microorganisms*, vol.7 (5), pp.124, 2019. https://doi.org/10.3390/microorganisms7050124
[3] Koehn FE, Carter GT., "The evolving role of natural products in drug discovery," *Nature reviews Drug discovery*, vol.4 (3), pp.206-20, 2005. https://doi.org/10.1038/nrd1657
[4] Gaudêncio SP, Pereira F., "Dereplication: racing to speed up the natural products discovery process," *Natural product reports*, vol.32 (6), pp.779-810, 2015.
[5] Bulusu MA, Baumann K, Stuetz A., "Chemistry of the immunomodulatory macrolide ascomycin and related analogues," *Progress in the Chemistry of Organic Natural Products*, vol.94, pp.59-126, 2011.
[6] Ferraboschi P, Colombo D, De Mieri M, Grisenti P., "First chemoenzymatic synthesis of immunomodulating macrolactam pimecrolimus," *Tetrahedron Letter*, vol.50 (30), pp.4384-8, 2009. https://doi.org/10.1016/j. tetlet.2009.05.066

[7] Lipozencić J., "Pimecrolimus--a safe and effective local immunomodulator in the treatment of inflammatory skin diseases," *Acta dermatovenerologica Croatica: ADC*, vol.13 (1), pp.63-9, 2005.
[8] Qi HS, Xin X, Li SS, Wen JP, Chen YL, Jia XQ., "Higher-level production of ascomycin (FK520) by *Streptomyces hygroscopicus* var. *ascomyceticus* irradiated by femtosecond laser," *Biotechnology and Bioprocess Engineering*, vol.17 (4), pp.770-9, 2012. https://doi.org/10.1007/s12257-012-0114-2
[9] Shirai A, Tsunoda H, Tamaoki T, Kamiya T., "Topical application of cyclosporin A induces rapid-remodeling of damaged anagen hair follicles produced in cyclophosphamide administered mice," *Journal of dermatological science*, vol.27 (1), pp.7-13, 2001. https://doi.org/10.1016/S0923-1811 (01) 00097-4
[10] Lecluse LL, Piskin G, Bos JD., "The use of patch tests in determining hypersensitivity to etanercept and infliximab, *Archives of dermatology*, vol.144 (8), pp.1070-1, 2008.
[11] Katz L, Baltz RH., "Natural product discovery: past, present, and future," *Journal of Industrial Microbiology and Biotechnology*, vol.43 (2-3), pp.155-76, 2016. https://doi.org/10.1007/s10295-015-1723-5
[12] Paul C, Graeber M, Stuetz A., "Ascomycins: promising agents for the treatment of inflammatory skin diseases," *Expert opinion on investigational drugs*, vol.9 (1), pp.69-77, 2000. https://doi.org/10.1517/13543784.9.1.69
[13] Muramatsu H, Mokhtar SI, Katsuoka M, Ezaki M., "Phylogenetic analysis of immunosuppressant FK506-producing streptomycete strains," *Actinomycetologica*, vol.19 (2), pp.33-9, 2005. https://doi.org/10.3209/saj.19.33
[14] Margassery LM, Kennedy J, O'Gara F, Dobson AD, Morrissey JP., "A high-throughput screen to identify novel calcineurin inhibitors," *Journal of microbiological methods*, vol.88 (1), pp.63-6, 2012. https://doi.org/10.1016/j. mimet.2011.10.012
[15] Sierra-Paredes G, Sierra-Marcuño G., "Ascomycin and FK506: pharmacology and therapeutic potential as anticonvulsants and neuroprotectants," *CNS neuroscience & therapeutics*, vol.14 (1), pp.36-46, 2008. . https://doi.org/10.1111/j.1527-3458.2008.00036. x
[16] Marsland AM, Soundararajan S, Joseph K, Kaplan AP., "Effects of calcineurin inhibitors on an in vitro assay for chronic urticaria," *Clinical & Experimental Allergy*, vol.35 (5), pp.554-9, 2005. https://doi.org/10.1111/j.1365-2222.2005.02242. x
[17] Van Leent EJ, Gräber M, Thurston M, Wagenaar A, Spuls PI, Bos JD., "Effectiveness of the ascomycin macrolactam SDZ ASM 981 in the topical treatment of atopic dermatitis," *Archives of Dermatology*, vol.134 (7), pp.805-9, 1998. https://doi.org/10.1001/archderm.134.7.805
[18] Sambyal K, Singh RV.,"Bioprocess and genetic engineering aspects of ascomycin production: a review," *Journal of Genetic Engineering and Biotechnology*, vol.18 (1), pp.1-11, 2020. https://doi.org/10.1186/s43141-020-00092-0
[19] Koch K, Newborg MF, Hanson DC, Cooper K, Shepard RM, Biehl ML, Biggers MS, Ramchandani M, Schulte G., "The C-32 triacetyl-L-rhamnose derivative of ascomycin: a potent, orally active macrolactone immunosuppressant," *Journal of medicinal chemistry*, vol.38 (8), pp.1255-8, 1995. https://doi.org/10.1021/jm00008a001
[20] Ayer J, Young HS., "Pimecrolimus for psoriasis," *Expert opinion on pharmacotherapy*, vol.14 (6), pp.767-74, 2013. https://doi.org/10.1517/14656566.2013.775247
[21] Kirby B, Griffiths CE, "Psoriasis: the future," *British Journal of Dermatology*, vol.144, pp.37-43, 2001. https://doi.org/10.1046/j.1365-2133.2001.144s58037. x
[22] Qi H, Zhao S, Fu H, Wen J, Jia X., "Enhancement of ascomycin production in Streptomyces hygroscopicus var. ascomyceticus by combining resin HP20 addition and metabolic profiling analysis," *Journal of Industrial Microbiology and Biotechnology*, vol.41 (9), pp.1365-74, 2014. https://doi.org/10.1007/s10295-014-1473-9
[23] Arai T, Koyama Y, Suenaga T, Honda H., "Ascomycin, an antifungal antibiotic," *The Journal of Antibiotics, Series A, vol.1*5 (6), pp.231-2, 1962. https://doi.org/10.11554/antibioticsa.15.6_231
[24] Meadows RP, Nettesheim DG, Xu RX, Olejniczak ET, Petros AM, Holzman TF, Severin J, Gubbins E, Smith H, Fesik SW., "Three-dimensional structure of the FK506 binding protein/ascomycin complex in solution by heteronuclear three-and four-dimensional NMR," *Biochemistry*, vol.32 (3), pp.754-65, 1993. https://doi.org/10.1021/bi00054a004
[25] Chaudhary AK, Dhakal D, Sohng JK., "An insight into the "-omics" based engineering of streptomycetes for secondary metabolite overproduction," *BioMed research international*, 2013. https://doi.org/10.1155/2013/968518
[26] Driggers EM, Hale SP, Lee J, Terrett NK., "The exploration of macrocycles for drug discovery - An underexploited structural class," *Nature Reviews Drug Discovery*, vol.7 (7), pp.608-24, 2008. https://doi.org/10.1038/nrd2590
[27] Song K, Wei L, Liu J, Wang J, Qi H, Wen J., "Engineering of the LysR family transcriptional regulator FkbR1 and its target gene to improve ascomycin production," *Applied microbiology and biotechnology*, vol.101 (11), pp.4581-92, 2017. https://doi.org/10.1007/s00253-017-8242-4
[28] Zhang Y, Chen H, Wang P, Wen J., "Identification of the regulon FkbN for ascomycin biosynthesis and its interspecies conservation analysis as LAL family regulator," *Biochemical Engineering Journal*, vol.151, pp.107349, 2019. https://doi.org/10.1016/j. bej.2019.107349
[29] Revill WP, Voda J, Reeves CR, Chung L, Schirmer A, Ashley G, Carney JR, Fardis M, Carreras CW, Zhou Y, Feng L., "Genetically engineered analogs of ascomycin for nerve regeneration," *Journal of Pharmacology and Experimental Therapeutics*, vol.302 (3), pp.1278-85, 2002.

[30] Wang C, Wang J, Yuan J, Jiang L, Jiang X, Yang B, Zhao G, Liu B, Huang D,"Generation of Streptomyces hygroscopicus cell factories with enhanced ascomycin production by combined elicitation and pathway-engineering strategies," *Biotechnology and bioengineering*, vol.116 (12), pp.3382-95, 2019. https://doi.org/10.1002/bit.27158
[31] Yu Z, Shen X, Wu Y, Yang S, Ju D, Chen S, "Enhancement of ascomycin production via a combination of atmospheric and room temperature plasma mutagenesis in Streptomyces hygroscopicus and medium optimization," *AMB Express*, vol.9 (1), pp.1-5, 2019.
[32] Yu Z, Lv H, Wu Y, Wei T, Yang S, Ju D, Chen S., "Enhancement of FK520 production in Streptomyces hygroscopicus by combining traditional mutagenesis with metabolic engineering," *Applied microbiology and biotechnology*, vol. (23), pp.9593-606, 2019. https://doi.org/10.1007/s00253-019-10192-8
[33] Wang P, Yin Y, Wang X, Wen J., "Enhanced ascomycin production in Streptomyces hygroscopicus var. ascomyceticus by employing polyhydroxybutyrate as an intracellular carbon reservoir and optimizing carbon addition," *Microbial cell factories*, vol.20 (1), pp.1-6, 2021.
[34] Goyal M, Baranwal M., "In Silico Approach to Analyze the Biochemical Pathways of Bacterial Metabolite Synthesis," *In Silico Approach for Sustainable Agriculture*, Springer, Singapore, pp.141-159, 2018.
[35] Qi H, Lv M, Song K, Wen J., "Integration of parallel 13C-labeling experiments and in silico pathway analysis for enhanced production of ascomycin," *Biotechnology and bioengineering*, vol.114 (5), pp.1036-44, 2017. https://doi.org/10.1002/bit.26223
[36] Yan L, Zhang Z, Zhang Y, Yang H, Qiu G, Wang D, Lian Y., "Improvement of tacrolimus production in Streptomyces tsukubaensis by mutagenesis and optimization of fermentation medium using Plackett–Burman design combined with response surface methodology," *Biotechnology Letters*, pp.1-14, 2021. . https://doi.org/10.1007/s10529-021-03144-8
[37] Deshmukh DV, Puranik PR., "Statistical evaluation of nutritional components impacting phycocyanin production in Synechocystis sp.," *Brazilian Journal of Microbiology*, vol.43, pp.348-55, 2012. . https://doi.org/10.1590/S1517-83822012000100041
[38] Luthra U, Dubey RC., "Medium optimization of lipstatin from Streptomyces toxytricini ATCC 19813 by shake flask study," *International Journal of Microbiology Research*, vol.4 (7), pp.266, 2012.
[39] Luthra U, Kumar H, Tripathi A, Talele S, Gupte V., "Study on growth and ascomycin production by Streptomyces hygroscopicus subsp. Ascomyceticus," *Biocatalysis and Agricultural Biotechnology*, vol.19, pp.101159, 2019. https://doi.org/10.1016/j.bcab.2019.101159

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