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ORIGINAL PAPER

Approaches towards the enhanced production of Rapamycin by *Streptomyces hygroscopicus* MTCC 4003 through mutagenesis and optimization of process parameters by Taguchi orthogonal array methodology

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Abstract The present research was conducted to define the approaches for enhanced production of rapamycin (Rap) by *Streptomyces hygroscopicus* microbial type culture collection (MTCC) 4003. Both physical mutagenesis by ultraviolet ray (UV) and chemical mutagenesis by *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (NTG) have been applied successfully for the improvement of Rap production. Enhancing Rap yield by novel sequential UV mutagenesis technique followed by fermentation gives a significant difference in getting economically scalable amount of this industrially important macrolide compound. Mutant obtained through NTG mutagenesis (NTG-30-27) was found to be superior to others as it initially produced 67% higher Rap than wild type. Statistical optimization of nutritional and physiochemical parameters was carried out to find out most influential factors responsible for enhanced Rap yield by NTG-30-27 which was performed using Taguchi orthogonal array approach. Around 72% enhanced production was achieved with nutritional factors at their assigned level at 23 °C, 120 rpm and pH 7.6. Results were analysed in triplicate basis where validation and purification was carried out using high performance liquid chromatography. Stability study and potency of extracted Rap was supported by turbidimetric assay taking *Candida albicans* MTCC 227 as test organism.

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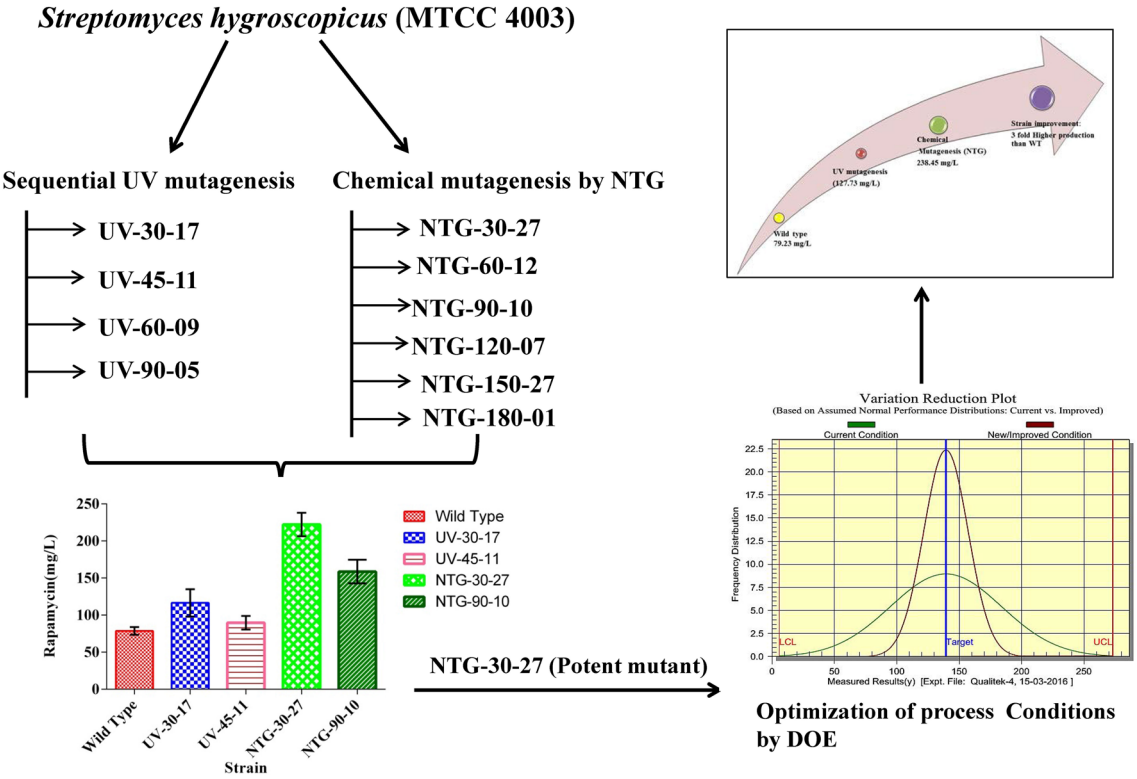
Graphical Abstract



Rapamycin yield by Wild type culture and Different mutants

Keywords Rapamycin · *Streptomyces hygroscopicus* · Sequential UV mutagenesis · Nitrosoguanidine · Turbidimetric assay

Introduction

Rapamycin (Rap), globally known as Sirolimus, is a proactive antibiotic. In contrast to the first generation antibiotics i.e. penicillin and cephalosporin, it has numerous medical applications and was first reported as antifungal agent (mainly against *Candida* spp.) isolated from soil borne actinomycete *Streptomyces hygroscopicus* by Vezina et al. (1975). It is also known for its antitumor, immunosuppressive and anti-aging activities (Weber et al. 2005; Zou and Li 2013). Harrison et al. (2009) described that it can expand the lifespan of mice which is a big research platform in medical science related to aging (Harrison et al. 2009). Rap is getting importance day by day for its effectiveness in various medical fields (Parkinson's disease etc.). However, the low yield of Rap production by the organism increases its production cost. Therefore, the key challenge for researchers is to scale up its production yield (Dutta et al. 2014a). In today's context, 1 mg sirolimus tablet (30 nos.) costs around $175 which is quite high in comparison with other generic medicines (Medindia 2016). Thus selection of potent strains for higher Rap yield can be a cost effective remedy to overcome the present challenge. Strain improvement technique is globally acceptable for getting better yield of desirable product through physical, chemical and classical mutagenesis. For idiolite (secondary metabolite) overproduction (especially for antibiotics) random mutagenesis technique is favourable than molecular approaches i.e., site-directed mutagenesis (SDM), genetic modification (error prone PCR) (Jung et al. 2011; Kennedy and Krouse 1999; Xu et al. 2005). In random approach, a very little start-up time is required to isolate fruitful mutants which are long lasting in nature (Parekh et al. 2000). It helps to screen a large number of potent mutants with higher productivity. However, researchers have also tried molecular approaches i.e., effects of positive and negative regulatory genes from entire Rap gene cluster to check its production pattern and regulation (Kuščer et al. 2007; Yoo et al. 2015). In our work, mutation study was conducted with wild type strain using physical mutagen (uv ray) and chemical mutagen (*N*-methyl-*N*-nitro-*N*-nitrosoguanidine, NTG) for enhanced production of Rap by submerged fermentation process. As a potent mutagen, NTG has achieved tremendous importance since last

deacdes for enhancement of secondary metabolite yield in actinomycetes (Baby Rani et al. 2013; Baltz 2001; Xu et al. 2005). It is a powerful and commonly used alkylating agent (Xu et al. 2005). Base pair substitution in the genome after treatment with NTG involves methylation of guanine at the oxygen of carbon no. 6. During DNA replication, the O^6–methylguanine added pairs with thymine (rather than cytosine) which leads to GC→AT unidirectional substitution inducing mutation (Gordon et al. 1990; Parekh et al. 2000). In our study, final Rap production by submerged fermentation (SmF) with culture conditions optimized using Taguchi methodology reached 315.587 mg/L via phenotypically expressed mutated strain which is 3.98-fold higher than produced by the wild type.

Materials and methods

Chemicals

All the chemicals used were of analytical grade and purchased from Sigma-Aldrich (USA), Himedia (India), SRL (India), Merck (India) Ltd. Standard Rap (1 mg) for high performance liquid chromatography (HPLC) analysis was purchased from Merck, Germany. For HPLC assay deionized water was prepared by ultrapure water system (Arium©, 611UF, Sartorius, Germany). Being toxic, chemical mutagen NTG (5 mg) was stored properly wrapped with aluminium foil (as it is highly light sensitive) at 4 °C.

Analytical methods

Rap concentration was checked by "Agar disc diffusion assay" (Abdel-Fattah 2008; Dutta et al. 2014a; Kojima et al. 1995; Xu et al. 2005). Bigger inhibition zone against *Candida albicans* reveals higher Rap yield (Jung et al. 2011). Concentration of Rap from fermentation broth was calculated by standard curve prepared between known concentration of rap vs inhibition zone diameter (Dutta et al. 2014a). Antibiotic potency was tested by turbidimetric technique (Berridge and Barrett 1952; Joslyn and Galbraith 1950). It is a simple method and takes lesser time than agar disc technique (Lourenço and Pinto 2011). Rap concentration was determined using HPLC system (Waters™ 600, USA) equipped with UV/Visible detector (Waters 2489, USA) and a C18 hypersil column (4.69250 mm; 5 µm particle size; Waters, USA) (Dutta et al. 2014a). Mobile phase was methanol:acetonitrile (80:20) and sample was detected using UV/Visible detector at 272 nm with flow rate of 1 mL/min.

Microorganism and inoculum preparation

Streptomyces hygroscopicus (MTCC-4003) and *C. albicans* (MTCC-227) were procured in a lyophilized state from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. *C. albicans* was used as test organism for the confirmation of antibiotic produced. One loopful of culture was inoculated aseptically to sterile media composition supplied by MTCC and kept at 25 °C for 5–6 days on a rotary shaker at 120 rpm.

Production medium and cultivation conditions

The medium composition suggested by Lee et al. (1997) was followed for Rap production using statistical optimization method (Lee et al. 1997). pH of the medium was maintained at 7.2 before sterilization. 2% of inoculum (*v*/*v*) was transferred to the production medium (culture volume 50 in 250 mL conical flask) and was kept for 10 days at 25 °C in a shaker incubator (Digitech system) at 120 rpm for Rap production. Production medium contained eight trace element supplement other than carbon source, nitrogen source and buffering agents (Lee et al. 1997). Being present in a very low quantity, trace element solution (% *v*/*v*) was prepared separately and added to production media asceptically before inoculation. 1× concentration of trace element solution was prepared using the following constituents (g/L): $ZnSO_4{\cdot}7H_2O$ 0.06, $MgSO_4{\cdot}7H_2O$ 0.00256, $MnSO_4{\cdot}H_2O$ 0.012, $(NH_4)_6M_{O7}O_{24}{\cdot}4H_2O$ 0.018, $Na_2B_4O_7{\cdot}10H_2O$ 0.01, $CoCl_2{\cdot}6H_2O$ 0.01, $CuCl_2{\cdot}2H_2O$ 0.0013. $FeSO_4{\cdot}7H_2O$ 0.1 g/L was also prepared separately and sterilized by membrane filtration.

Sequential UV mutagenesis study

In our study we have used sequential UV mutagenesis approach rather than the conventional direct irradiating method. Spores were irradiated in an agar plate by UV rays in a sequential manner (i.e., incremental UV dose in a periodic fashion). Irradiated plates were kept in dark for avoiding any type of photo excision repair and to stabilize thymine–thymine (T–T) dimers (Van Houten and Sancar 1987; Witkin 1969). Figure 1 represents detailed procedure of the method. 30 W Philips lamp with 254 nm UV–C category light was used for our mutagenesis experiment. Initially each agar plates were irradiated (distance from the surface area 65 cm) with diluted spore suspensions for 30, 45, 60 and 90 s. Each plate was irradiated once for 5 days interval and the duration of the treatment was 15 s periodic increament to the initial treatment time. After incubation, a number of colonies were screened for each plate having the capability to produce

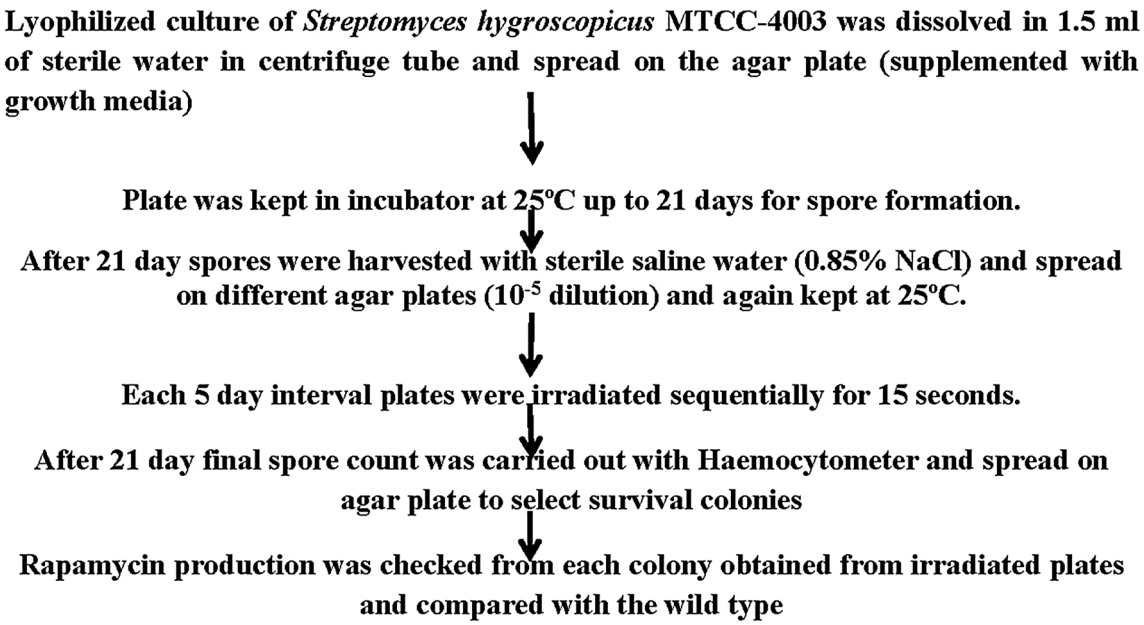


Fig. 1 Steps for sequential UV mutagenesis. Fungal spores were spread on agar plates supplimented with growth media and allowed to be penetrated by UV rays. Initially plates were exposed for 30, 45, 60 and 90 s. Then all plates were irradiated each 5 days interval and the duration of the treatment was each time the initial treatment time plus 15 s

higher Rap yield than wild type. Survival rate was calculated *once* using following formula: $S_i = \frac{X_f - X_i}{X_i}$, where as S_i is % of survival, X_i is initial viable cell count in CFU/mL and X_f is final viable cell count after mutagen dose in CFU/mL (Djurdjevic-Milosevic et al. 2011). Total spore count was carried out in Haemocytometer using following formula (spores/mL): average spore count per square of the four corner of the chamber × dilution factor × haemocytometer chamber factor (10^4/mL). At the end of sequential UV exposure upto 25 days final survival rate was around $\left[\left(\frac{2\times10^4-36\times10^4}{36\times10^4}\right)\times100\right]\% = -94\%$ where, $X_i = 36\times10^4$ and $X_f = 2\times10^4$. Here, '−' sign indicates the death rate.

Chemical mutagenesis by NTG

According to available reports, Rap yield increased significantly by high throughput screening to random selection technique, using NTG as chemical mutagen. Preliminary steps upto spore formation was similar to our UV mutagensis study as discussed earlier. NTG stock concentration was 10 mg/mL in 0.2 (M) phosphate buffer (pH 6). Spore suspension was mixed with NTG (1:1 dilution) and kept in incubator for 3 h at 25 °C and 180 rpm for proper interaction. Samples were withdrawn each 30 min and diluted 10× with autoclaved distilled water followed by 1:2 dilution with saline water. After appropriate dilution, 100 µL of spore suspension was spread on isolation medium supplemented with growth factors.

Turbidimetric assay of rapamycin

A calibration curve of standard Rap concentration against *C. albicans* was prepared to find out the concentration of extracted Rap from fermentation broth. 900 µL of *C. albicans* active culture was added to each tube having concentration of Rap (100–900 µg/mL). Tubes were then incubated for 4 h at 25 °C. After incubation, 50 µL (1:3 dilution) of formaldehyde was added to arrest the cell growth and OD was taken at 600 nm (Vieira et al. 2014).

Taguchi orthogonal design

Study was conducted to get higher fold of Rap with potent mutant using Taguchi orthogonal array approach. Statistical optimization of media components as well as physiological parameters for better production of Rap using potent strain was carried out using this design tool. It is a globally acceptable robust method for media optimization, determination of influential parameters as well as visualization of interaction between different factors with least number of execution/experiments (Basak et al. 2013a, b; Kennedy and Krouse 1999). Comparing other optimizing method, change one variable at a time (COVT) is very primitive and many experimental sets need to be carried

out with all possibilities which has been found to be time consuming and economically not feasible (Dutta et al. 2014b; Nandal et al. 2013; Prasad et al. 2005; Rao et al. 2004). Response surface methodology (RSM) does not allow to screen the contributing factors. However, Taguchi methodology promises for inclusion of categorical factors with variable ones (Nandal et al. 2013). Identification of key contributing factors and elimination of non influencial elements are very essential for large scale fermentation (Parekh et al. 2000). Very few reports are available in literature for media optimization of Rap production via Taguchi design (Ng et al. 2014). In Taguchi methodology, Design of experiments (DOE) are distributed in three different steps i.e. system, parameters and tolerance design (Rao et al. 2004). Our experiment was designed for 13 factors including both physiochemical and nutritional at three levels orthogonal array layout which results a total number of 27 experimental setup designated by L_{27} array. Result output analysis were based on the signal to noise (S/N: desirable value/undesired value) ratio in a "Bigger is better" performance charecteristics throughout the experiment (Basak et al. 2013a). Table 1 represents coded factors with their three level of variations resulting in 27 executable experimental sets. All four executable steps were performed i.e. planning, conducting, analysis and validation (Basak et al. 2013b). Statistical analysis of the parameters was carried out by Qualitek-4 software (Nutek Inc., MI, USA) package. It is a user friendly software in which large variation of data with different levels can be easily executed and analysed efficiently (Basak et al. 2013b). Based on the analysis, we finally developed an optimum culture condition method for Rap production which was achieved by pooling out of non influential factors from the experiment by setting a certain limit of contribution (95% confidence level).

Results

Effect of UV mutagenesis on rapamycin production

Cumulative UV exposure time for each plate was given for 150 s (UV-30), 210 s (UV-45), 270 s (UV-60) and 330 s (UV-90) for 21 days. As reported earlier, bright-field micrographs (Dewinter technologies, Biowizard software, 100× magnification) revealed that the number of spore decreased with increase of UV dose as represented in Fig. 2 (Dimond and Duggar 1941). *Black spots* in the picture indicated dead cells which resulted by uncontrolled DNA damage due to extensive UV exposure. In this study *dose* indicates exposure time of UV irradiation on spore suspension. As a result around 94% killing ratio was achieved after final Haemocytometer count. Numerous colonies were obtained after primary screening of mutants, among them some were deteriorating in nature and rest maintained Rap producing activity which was preliminary checked by agar disc diffusion method as described earlier. Average Rap production by different UV mutants were as follows: UV-45-11 89.66, UV-60-9 84.33 and UV-90-5 76.33 mg/L. Comparative results revealed that among all UV induced mutants, UV-30-17 showed higher Rap yield of 127.73 mg/L.

Rapamycin overproduction with NTG

Results indicated that more than 90% of cells were killed at the end of NTG treatment and viable colonies were tested for Rap production. Exponential decrease nature of spore numbers with respect to NTG treatment time was observed. NTG-30, NTG-60, NTG-90, NTG-120, NTG-150 and NTG-180 (30, 60, 90 etc. correspond to the treatment time of NTG on spore suspension) were all viable mutants and

Table 1 Coded factors with three level of variation

Serial no.	Coded factor	Factor	Level-1	Level-2	Level-3
1	A	Fructose (g/L)	15	20	25
2	B	Mannose (g/L)	2.5	5	7.5
3	C	L-Lysine hydrochloride (g/L)	2.5	5	7.5
4	D	Ammonium sulphate (g/L) $(NH_4)_2SO_4$	2.5	5	7.5
5	E	Di-potassium hydrogen phosphate (K_2HPO_4) (g/L)	1.5	1.75	2
6	F	Sodium chloride (NaCl) (g/L)	2.5	5	7.5
7	G	Calcium carbonate ($CaCO_3$) (g/L)	2	3	4
8	H	Ferrous sulphate, heptahydrate($FeSO_4{\cdot}7H_2O$) (g/L)	0.05	0.1	0.15
9	I	Sodium sulphate (Na_2SO_4) (g/L)	0.3	0.35	0.4
10	J	Trace element (% v/v)[a]	1	1.5	2
11	K	Temperature (°C)	23	25	27
12	L	pH	6.8	7.2	7.6
13	M	Agitataion (rev/min)	100	120	140

[a]Trace element solution contains(g/L): $ZnSO_4{\cdot}7H_2O$ 0.06, $MgSO_4{\cdot}7H_2O$ 0.00256, $MnSO_4{\cdot}H_2O$ 0.012, $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ 0.018, $Na_2B_4O_7{\cdot}10H_2O$ 0.01, $CoCl_2{\cdot}6H_2O$ 0.01, $CuCl_2{\cdot}2H_2O$ 0.0013

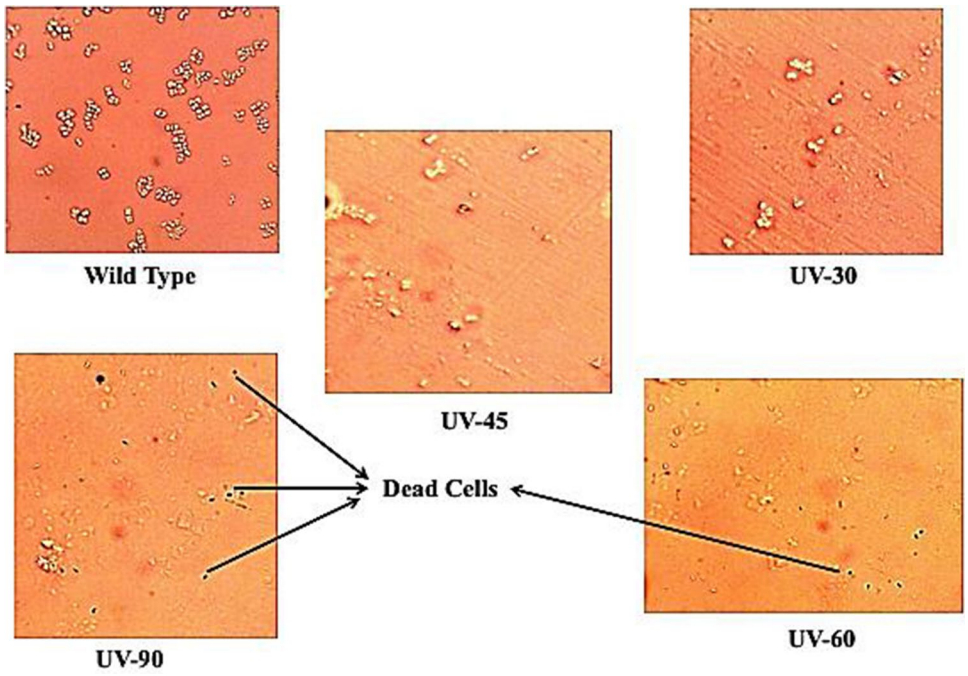


Fig. 2 Brightfield Micrograph of wild type and mutated spores of *S. hygroscopicus*. After 20th day (5th *round*) of UV penetration spore morphology and viability was examined under brightfield microscope. In wild type (untreated) culture all spores were unharmed whereas decreasing nature of spore count was observed in UV penetrated slides. Black spots appeared in UV-60 and UV-90 were indication of death cells due to uncontrolled UV exposure

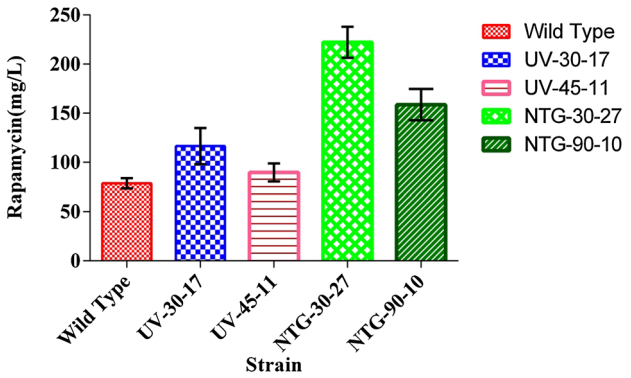


Fig. 3 Comparison of rapamycin production by different mutants. Among all viable mutants screened after triplicate analysis of rapamycin production, NTG-30-27 was found to be stable and potent one. Around 238.45 mg/L rapamycin yield was obtained by the culture followed by NTG-90-10 and UV-30-17. In view of UV mutants, UV-30-17 was highest rapamycin producer i.e. 127.73 mg/L

produced more Rap yield than that of the wild type. Investigation revealed that for, NTG-180 plate, only a single colony appeared indicating <1% survival rate. However, some of the colonies proved to produce no Rap. After repeated subculture, NTG-30-27 appeared to be stable, potential and highest Rap producer among numerous mutants screened individually for Rap production including UV-30-17 mutant. Rap production by NTG-30-27 was around 238.45 mg/L which was 67% (approximately threefold) higher than wild type (79.23 mg/L) and 47% higher than UV-30-17 mutant. Figure 3 indicates comparison of Rap production by different mutants with wild type. This potent mutant (NTG-30-27) was selected for statistical optimization study using Taguchi orthogonal array approach to increase the fold of rapamycin.

Rapamycin potency check by turbidimetric assay

As turbidity of *C. albicans* decreased with increasing Rap concentration, a negative slope was observed on the standard calibration curve having regression co-efficient (R^2) of 0.9961 as depicted in Fig. 1(s). For instance, Rap fermentation in the broth started on 6th day and its production slowed down on 10th day (Lee et al. 1997; Xu et al. 2005). Satisfactory reduction of turbidty OD_{600} was also found with optimized culture which started from 6th day till 10th day of fermentation. As a consequence, a significant variance was observed among the clearence of turbidity in different tubes.

Enhanced rapamycin production using Tagauchi orthogonal array

To get optimized values of process condition, Taguchi orthogonal study was conducted. Here we went through a series of experiments to execute the result. The steps were as follows, to study the main effects of different factors, survey of all possible interactions between factors concerned, followed by analysis of variance (ANOVA) for getting robust and statistically significant result and finally optimized experimental conditions that would give higher yield than actual via proper validation.

Effects of distinct factors

Table 2 describes main effects of all the factors considered for optimization experiment. The term "main effect" actually implies average value of achieved outcomes of each factors at specified levels whereas sign (+ or −) indicates whether changes from one level to other increases or decreases (Nandal et al. 2013).

Interaction between different factors

Rapamycin is synthesized via shikimic acid pathway, undergoes lots of interaction during growth and metabolism (Jung et al. 2011). 78 types of interactions (with entire possible combinations) between all the factors concerned for Rap production are described in Table 1(s). Percentage (%) of interaction between two distinct factor was designated by severity index (SI) (Basak et al. 2013b). Highest SI was found between NaCl and trace element solution (SI = 76.51%) whereas least was observed between fructose and L-lysine HCl (SI = 0.08%). It was quite interesting to note that NaCl, $FeSO_4 \cdot 7H_2O$ were less influencial at their individual level but found strongly interactive when present in the media. The term "column" in Table 1(s) represents location of the factor with assisgned value. Interaction between both the carbon sources showed significant impact on Rap production having SI of 60.3%. Though, L-lysine HCl and rpm showed relative low SI (52.76%), both gave significant effect on Rap production. Ammonium sulphate, inorganic nitrogen source present in the media showed SI value less than 50% (49.12%) when interaction with Na_2SO_4 was considered. When amino acids were not considered, $(NH_4)_2SO_4$ was found to be best nitrogen source for Rap production (El-Nasser et al. 2010; Lee et al. 1997). It was seen that K_2HPO_4 and pH has least individual impact but showed significant interaction (SI 45.53%). Researchers agreed that K_2HPO_4 is one of the best inorganic phosphate source for different antibiotic and enzyme production (El-Nasser et al. 2010; Ng et al. 2014). Effects of each factor towards Rap production at their different level was depicted in Fig. 2(s). In this case only fructose showed exponential increase pattern of Rap production with its increasing concentration. Na_2SO_4, mannose, trace element solution, $CaCO_3$, rpm increases Rap production from Level 1 to Level 2, however it drops at level 3 for each factor. Most of the factor showed highest impact at set value when acted as individual. Increase of temperature from Level 1 to 2 and subsequently at Level 3 diminished Rap production. Rap production was also hampered with $FeSO_4 \cdot 7H_2O$ concentration in the media. Similar result was obtained by the researchers claiming that $FeSO_4 \cdot 7H_2O$ possess some negative effects during secondary metabolite synthesis (El-sersy and Abou-Elela 2006; Sharma and Manhas 2013; Zhou et al. 2014). However, Rap production increased when the values of L-lysine HCl, $(NH_4)_2SO_4$, K_2HPO_4, pH and NaCl were raised from Level-2 to Level-3.

Analysis of variance (ANOVA)

Effects of distinct parameters on Rap production was investigated thoroughly by ANOVA. "F" ratio and "Percent of contribution" term are the fundamental parameters to find out most significant factors. ANOVA chart with all the contributing factors are described in Table 3. In our case, temperature was found to be most significant as it contributed 28.314% of the total value. $CaCO_3$ (15.51%) and K_2HPO_4 (13.391%) were second and third contributing factors respectively. Other factors such as fructose, mannose, Na_2SO_4, $(NH_4)_2SO_4$ showed moderate effects whereas L-lys HCl (0.614%), NaCl (1.768%), trace element solution

Table 2 Effects of all the factors considered for rapamycin production

Serial no.	Coded factor	Factor	Rapamycin production			
			Level 1	Level 2	Level 3	L2–L1
1	A	Fructose	41.763	42.208	43.224	0.445
2	B	Mannose	41.748	43.161	42.285	1.413
3	C	L-Lysine hydrochloride	42.496	42.048	42.65	−0.449
4	D	$(NH_4)_2SO_4$	43.409	41.746	42.039	−1.663
5	E	K_2HPO_4	42.748	41.155	43.291	−1.593
6	F	NaCl	42.807	41.92	42.467	−0.888
7	G	$CaCO_3$	41.075	43.388	42.731	2.312
8	H	$FeSO_4 \cdot 7H_2O$	42.637	42.303	42.255	−0.335
9	I	Na_2SO_4	42.775	43.144	41.275	0.368
10	J	Trace element	42.311	42.75	42.134	0.439
11	K	Temperature	43.376	43.266	40.553	−0.11
12	L	pH	42.472	41.848	42.874	−0.625
13	M	RPM	42.535	42.658	42.001	0.123

Table 3 Analysis of variance (ANOVA)

Sl. No.	Factors	DOF	Sum of squares	Variance	F-ratio	Pure sum	Percent
1	Fructose	2	10.085	5.042	12.943	9.306	5.822
2	Mannose	2	9.155	4.577	11.750	8.376	5.240
3	L-lysine hydrochloride	2	1.760	0.880	2.259	0.981	0.614
4	$(NH_4)_2SO_4$	2	14.172	7.086	18.188	13.392	8.379
5	K_2HPO_4	2	22.183	11.091	28.470	21.404	13.391
6	NaCl	2	3.606	1.803	4.628	2.827	1.768
7	$CaCO_3$	2	25.577	12.788	32.826	24.798	15.515
9	Na_2SO_4	2	17.635	8.817	22.633	16.856	10.546
10	Trace element (1×)	2	1.809	0.904	2.322	1.030	0.644
11	Temperature	2	46.035	23.017	59.082	45.256	28.314
12	pH	2	4.812	2.406	6.176	4.033	2.523
13	RPM	2	2.195	1.097	2.818	1.416	0.886
	Other/error	2	0.803	0.401			6.358
	Total	26	159.835				100.00

(0.644%), pH (2.523%) and RPM (0.886%) exhibited negligible contribution. Above 95% confidence level factors were pooled until degree of freedom for the noise term was almost half of the total DOF of experimental design. After pooling, $FeSO_4{\cdot}7H_2O$ was found to be insignificant for Rap production. However, it holds higher SI values with other interacting factors.

Optimum condition and validation of rapmaycin production

Optimized process conditions for the enhanced Rap production for economic feasibility is presented in Table 4. After pooling, product formation was carried out with the significant factors, where $(NH_4)_2SO_4$, temperature, $CaCO_3$ were found to be major contributors. Expected results at optimized condition was found to be 50.347% where total contribution from all factors was 7.948. Taguchi DOE suggested that, optimum Rap production can be achieved with (g/L): Fructose 25, Mannose 5, L-lys HCl 7.5, $(NH_4)_2SO_4$ 2.5, K_2HPO_4 2, NaCl 2.5, $CaCO_3$ 3, Na_2SO_4 0.35 with supplementation of sterilized trace element solution (1×). Physicochemal parameters for optimized production were found to be temperature 23 °C, pH 7.6 and agitation speed 120 rpm. As per theoretical prediction expected yield at optimum condition was 329.117 mg/L of Rap. Thus around 72% (from 238.45 to 329.117 mg/L) of theoretical enhanced yield was achieved. Figure 4 showed

Table 4 Optimized process condition and their contribution for rapamycin production

Coded factor	Factor	Values	Level	Contribution for rapamycin production from S/N ratio
A	Fructose (g/L)	25	3	0.825
B	Mannose (g/L)	5	2	0.763
C	L-Lysine hydrochloride (g/L)	7.5	3	0.251
D	$(NH_4)_2SO_4$ (g/L)	2.5	1	1.010
E	K_2HPO_4 (g/L)	2	3	0.892
F	NaCl (g/L)	2.5	1	0.409
G	$CaCO_3$ (g/L)	3	2	0.989
I	Na_2SO_4 (g/L)	0.35	2	0.745
J	Trace element (1×)	1.5	2	0.351
K	Temperature (°C)	23	1	0.978
L	pH	7.6	3	0.476
M	RPM	120	2	0.260
Total contribution from all factors				7.948
Current grand average performance				42.398
Expected result at optimum condition				50.347

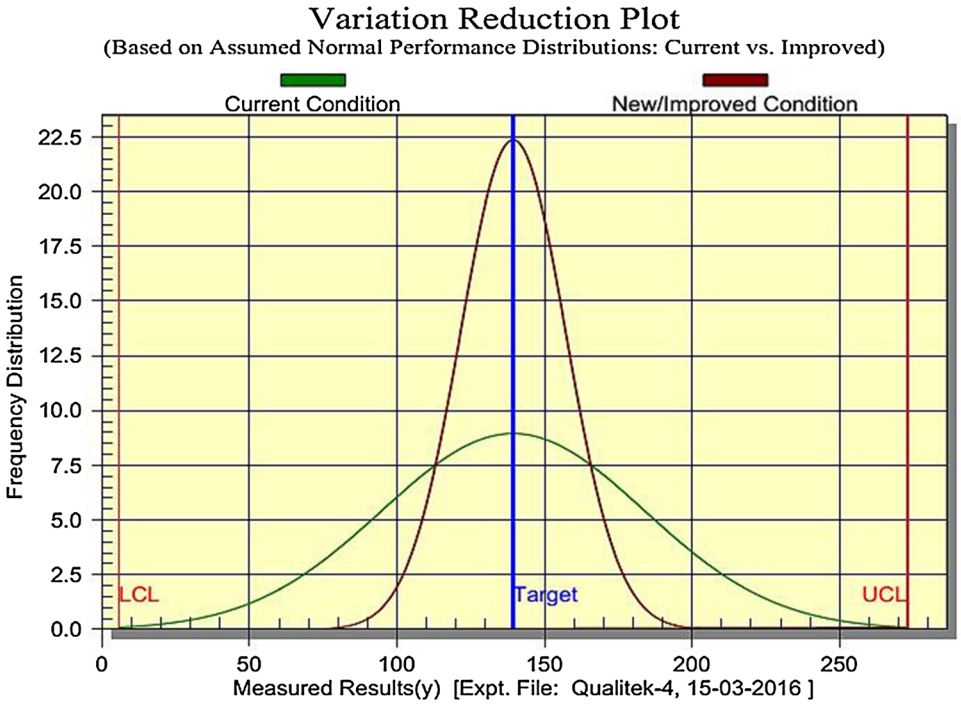


Fig. 4 Frequency distribution curve of current condition and improved condition for rapamycin production. Theoretical study after software analysis revealed that rapamycin yield may be increased from 238.45 to 329.117 mg/L (S/N ratio is 50.347 at optimum) in optimized condition. It means overall 72% enhanced production may be attained

the frequency distribution curve of current condition with improved condition. Here current condition signifies Rap yield before optimization study was being followed whereas improved condition gives the maximum Rap concentration could be achieved in optimized culture environment. Validation of experiment was carried out with all the significant factors at their reported levels and Rap production was found around 315.587 mg/L by agar disc diffusion assay. HPLC analysis was also carried out for better justification of the result. Satisfactory difference between Peak area viz. concentration was also found among untreated and mutant Rap yield as depicted in Fig. 5. As per the result obtained, our model is about 96% validated. Effect of significant factors and interaction influences are described in Fig. 6. It also defines the variation of Rap production at elected levels of different factors. Here, individual factors were shown to be more prospective towards higher Rap yield in comparison with optimized condition. One of the possible explanation may be due to interactive effect of various factors (Jha et al. 2014).

Discussion

In the present study sequential UV mutagenesis was proved to be a novel and effective method for enhancing Rap yield as compared to direct penetration/irradiation technique with minumum damage to cell morphology and genotypic activity. Initially it increased the Rap yield of 1.61-fold than that of wild type culture. It has been noticed that survival rate of mutants decreased with higher mutagen (both physical and chemical) dose (Fig. 2). However, present investigation supports that chemical mutagen (NTG) was found to be more effective than physical mutagen (UV ray) with respect to strain improvement as reported in the previous studies (Naveena et al. 2012). Research proposes that frequency of mutant generation via NTG (10^{-4}) is higher than UV (10^{-7}) (Baltz 2014). Being actinomycetes, *S. hygroscopicus* has GC rich genome (GC around 72%) (Baltz 1998, 2014). Hence, GC→AT transition mutation was further operative, powerful and constant by NTG treatment. Rapid rate (90%) of transition was also observed in *Escherichia coli* and preferably dependent on O^6-alkyl-guanine formation (Baltz 2014; Miller 1983).

Predictable factors optimized concentrations were enhanced in Level-2 whereas L1 and L3 are lower and higher values than L2 accordingly. Difference between average values of different levels (L2–L1) signifies comparative influence of the factors. Higher the difference, stronger is the influence. As reflected in Table 2, L2–L1 value is highest for $CaCO_3$ (2.312) folowed by mannose (1.413) which supports stronger influence of these factors for enhanced Rap yield. However, $(NH_4)_2SO_4$ and K_2HPO_4 showed negative effect. Table 3 also reflects higher influence of $CaCO_3$ followed by mannose, fructose and trace element solution. Previous research also suggested that $CaCO_3$ have positive influence on antibiotic production by UV induced mutants (Darken et al. 1960). Though in our study, optimum pH was found to be around 7.6 (described in Table 4) which is slightly

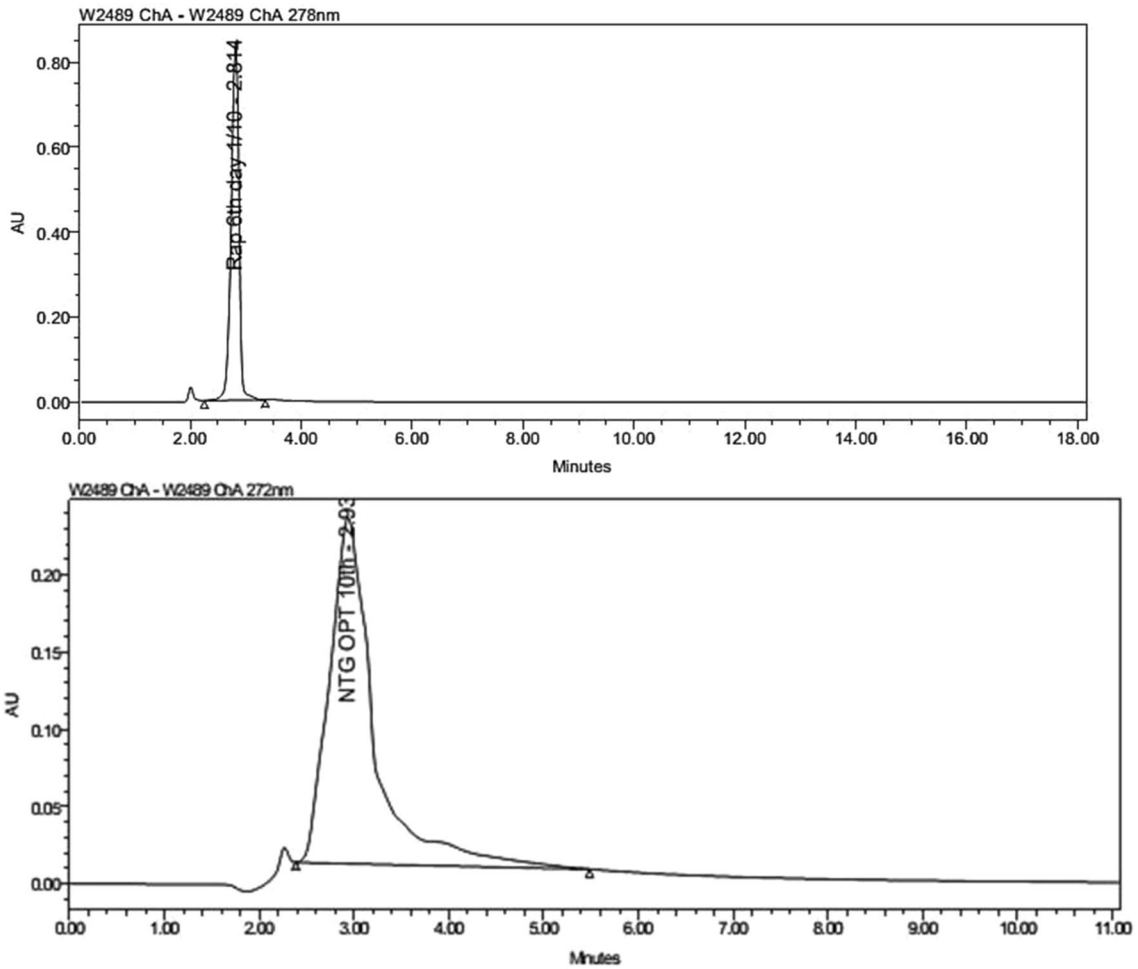


Fig. 5 HPLC chromatogram of rapamycin produced by wild type and mutant (NTG-30-27) of *S. hygroscopicus*. Rapamycin was extracted by centrifugation after 6th day of fermentation using methanol as a co-solvent. For both the cases Retention time (RT) was nearby same as standard rapamycin stock solution purchased from Merck India (data not shown). Area under the curve was concentration of rapamycin produced and was calculated from the calibration curve using the formula: Y = 42,506 X, where Y = area under the curve and X = known Rapamycin conc. Regression coefficient (R^2) of the calibration curve was around 0.995487

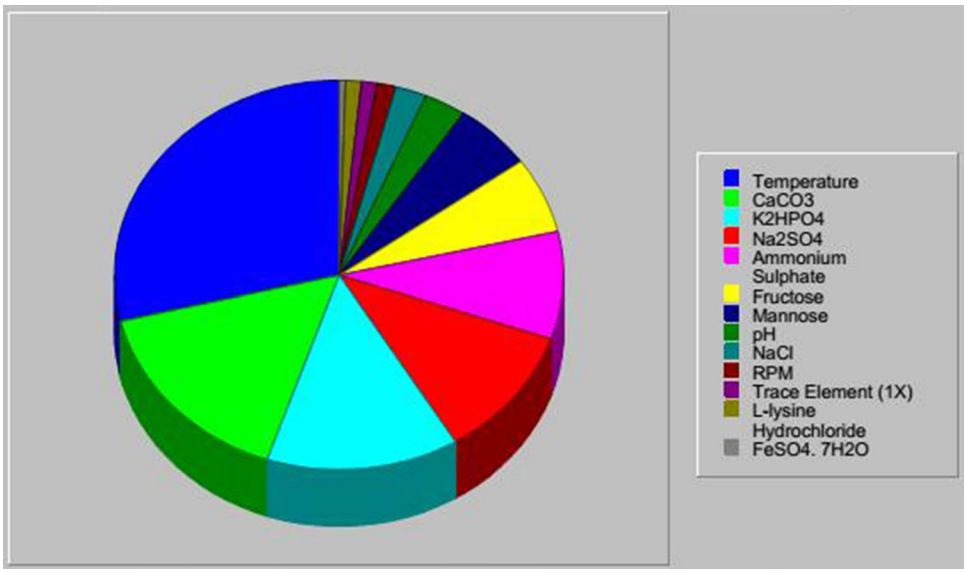


Fig. 6 Relative influence of factors and their interactions. Here distinct factors were separately significant for rapamycin enhancement in comparison with optimized condition. Temperature was found to be higly effective followed by $CaCO_3$ and K_2HPO_4. However, $FeSO_4{\cdot}7H_2O$ was found to be insignificant contributors for enhanced rapamycin yield as it was pooled after ANOVA analysis by setting up 95% confidence level

alkaline, it was found favourable for antibiotic production (Singh et al. 2009). Darken et al. (1960) suggested that, Ca^{2+} ion acts as a sequestering agent for increasing antibiotic yield when $CaCO_3$ concentration is high in the media (Darken et al. 1960). In few cases, researchers have reported that $CaCO_3$ can also acts as negative regulator in Rap production (Kim et al. 2014). As carbonate ion (CO_3^{2-}) is a determining factor for pH, increasing its value can affect Rap synthesis. Trace element and rpm were found to be significant factors as well. Greater yield of Rap with higher inhibitory activity against test organism is stimulated by the presence of trace elements in the fermentation media (Rakesh et al. 2014). It is predicted that every component present in the trace element are equally important, as in the ionic form they act as a co factor for various enzymes which are responsible for regulatory effect of Rap synthesis genes.

Better utilization of the suitable carbon source is necessary for antibiotic production (Sanchez et al. 2010). Table 4 represents that 2.5% fructose supplemented with 0.5% mannose is suitable for Rap production. This result is well in accordance with the findings of Lee et al. (1997). Among carbon sources, mannose was found more influential than fructose. However we have considered medium IV as formulated by Lee et al. (1997) for our optimization study and found fructose and mannose combination effect for Rap synthesis (Lee et al. 1997). L2–L1 value for mannose is 1.413. Our result strongly supports in comparison with the findings by other researchers which described that mannose is the most effective fermentable carbon source for Rap production by *Streptomyces* spp. (Kojima et al. 1995; Sallam et al. 2010). L-lysine acts as a potent precursor molecule for the pipecolic acid which finally gets incorporated in the complex macrolide structure of Rap (Cheng et al. 1995). However, lysine fails to support sufficient growth of cell when it acts alone in the culture medium. Result also revealed the fact that, inorganic phosphate is one of the essential component for antibiotic production (Martín and Demain 1980). It was obvious from the pattern of result that Rap production is autonomous of singular influence. Our finding is similar with previous research carried in bioreactor for retamycin production by Martins et al. (2004), indicating that higher oxygen transfer does not play any significant role on enhanced antibiotic production (Martins et al. 2004). Temperature effect (best production at 23 °C) was also found by researchers in case of Lovastatin production by *Aspergillus terreus* (Lai et al. 2005). According to them, subordinate temperature can act as environmental factor which helps to express the genes for enhanced secondary metabolite production. One possible explanation is that non metal ion SO_4^{2-} in the medium acts as impurities that hinders Rap production (Kiran et al. 2014).

The above results clearly signifies that, both nutritional and physicochemal factors are equally important for improved Rap yield.

Conclusion

Enhanced production of Rap was achieved via sequential UV mutagenesis (physical mutagen) technique and chemical mutagensis by NTG. Sequential UV mutagenesis proved to be an useful technique for enhanced yield whereas chemical mutagenesis produced potent mutant for Rap production. Higher survival rate of spore (in case of UV-30-17 and NTG-30-27) after mutagen treatment corresponds to higher yield. One possible explanation is that genotypic change in vegetative stage of mutant influences phenotypic expression. Statistical optimization for the determination of influencial factors and culture condition was carried out via Taguchi orthogogal array approach. Theoretical studies revealed that around 72% of enhanced production can be achieved via optimized condition where we got 3.98-fold higher Rap with potent mutant (NTG-30-27) than the wild type culture.

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