Journal of Biotechnology Letters
ISSN: 0976-7045 & E-ISSN: 0976-7053, Volume 4, Issue 1, 2013, pp.-68-71.
Available online at http://www.bioinfopublication.org/jouarchive.php?opt=&jouid=BPJ0000250

MUTAGENESIS OF THE LIPSTATIN PRODUCER *Streptomyces toxytricini* ATCC 19813

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Received: November 16, 2012; Accepted: January 21, 2013

Abstract- Lipstatin is a potent and irreversible inhibitor of pancreatic lipase activity. It was first isolated from *Streptomyces toxytricini*. The popular antiobesity drug orlistat (trade names Xenical and Alli) is a saturated derivative of lipstatin. Orlistat (marketed under the trade name Xenical) also known as tetrahydrolipstatin, is a drug designed to treat obesity. In an attempt to improve lipstatin production, mutation and screening of the parent culture have been carried out. More than thousand survivors were obtained after mutagenesis by UV and NTG of culture. Twenty colonies were selected and checked for lipstatin production. Through UV mutation, maximum lipstatin production was 2.34 mg/g, while through NTG treatment it was 2.88 mg/g at 264 Hrs. Thus it may be concluded that maximum lipstatin production was obtained through NTG treatment.

Keywords- Mutation, Lipstatin, *Streptomyces toxytricini* ATCC 19813, NTG and UV

Citation: Luthra U., Kumar H. and Dubey R.C. (2013) Mutagenesis of the Lipstatin Producer *Streptomyces toxytricini* ATCC 19813. Journal of Biotechnology Letters, ISSN: 0976-7045 & E-ISSN: 0976-7053, Volume 4, Issue 1, pp.-68-71.

Introduction

Streptomycetes are Gram-positive filamentous bacteria having remarkable capabilities to produce drug of diverse chemical structure and biological activity [10]. Recent reports indicate that streptomycetes can contain extensive amounts of reiterated DNA, a trait common to eukaryotic but not to prokaryotic organisms. Thus the streptomycetes may occupy an evolutionary position more advanced than the common unicellular eubacteria.

Improvement of streptomycetes for overproduction of industrial product has been the hallmark of fermentation processes. Fermentation economics are driven by the profitability of a marketed product. Product value is based on manufacturing cost per unit of product [9]. Natural isolates usually produce commercially important products in very low titer. Therefore, many experimental attempts are made to increase the productivity of the chosen organism. Enhancement in yields may be achieved by optimizing the culture medium and growth conditions, but this approach will be limited by maximum ability of the organisms to synthesize the product [13]. The potential productivity of the organism is controlled by its genome and, therefore, the genome must be modified to increase the yield. The modified organism requirement would then be examined to provide conditions that would fully exploit the increased potential of the culture. Thus, the strain improvement study involves the continual genetic modification of the culture, followed by evaluation of its cultural requirements. The microorganisms increase in yield a little upon providing optimum growth conditions. Therefore, it is necessary to modify their genomes to increase the productivity of the microorganisms. The culture medium and nutritional requirements also get changed slightly when the genetic structure of the microorganism is changed. Hence, they are modified according to the new requirements to ensure maximum yield of product. Genetic manipulation of the microorganism can be done by inducing mutations in the microorganisms and selecting natural variants.

Modification and improvement of the strain through mutation are typically achieved by subjecting the genetic material (*in vivo* or *in vitro*) to a variety of physical or chemical agents [1,8]. After inducing a mutation, survivors from the population are randomly picked and tested for their ability to produce the metabolite of interest. Screening a large number of mutated organisms usually identifies improved mutants. Moreover, it offers a significant advantage over the genetic engineering route by yielding gains with minimal start-up time and sustaining such gains over years, despite the lack of scientific knowledge of the biosynthetic pathway or genetics of the producing microbe [4]. The cost of lipstatin is one of the factors determining the economics of a drug production process and can be reduced by finding optimum conditions for their production, by the isolation of high potent mutants. Thus, realizing the immense utility of microbial lipstatin, the present investigation was carried out. Here, we report effect of UV and NTG mutagen on lipstatin producing strain *Streptomyces toxytricini* ATCC 19813.

Materials and Methods

Streptomyces Strain, Medium and Culture Characterization

The actinomycetes *Streptomyces toxytricini* (ATCC 19813) was used in the present study. The culture was maintained on yeast malt extract (YM) medium. The pH was adjusted to 7.2 before auto-

claving the medium at 121°C for 15 min. The cells were grown in 250 ml flask containing 30 ml of medium and incubated in orbital shaking incubator at 200 rpm and 28°C for 48±24 Hrs. For inoculum preparation, the culture was grown in YMB medium (yeast extract 4.0g/l, malt extract 10.0g/l, D-glucose 4.0g/l) at 28°C and 200 rpm for 24 Hrs. 2% of lab inoculum at the age of 32 Hrs. was transferred into the seed media (soya flour 10.0g/l, glycerol 10.0g/l, yeast extract 5.0g/l) and flask was incubated at 28°C at 180 rpm up to 48 ± 24 Hrs. or till growth appears. The production medium PM1 (glycerol 22.5g/l, soyabean flour 35.0g/l, soya lecithin 15.0g/l, soya oil 25.0g/l, PPG 0.50g/l) was inoculated with 5% (v/v) inoculum. Feeding was done in production flask from log 48 to 120 Hrs. at 24 Hrs. interval. Samples were analyzed at different hours 144, 168,192, 216 and 240 Hrs. for pH, PMV and activity.

Lipstatin Assay

Lipstatin activity in the culture broth was determined by HPLC. The culture broth of 5.0 gm was taken in 50 ml volumetric flask with 30 ml acetone and sonicates it for 10 minutes and make up the volume with acetonitrile. The resulting extracted solution was injected into the HPLC (Waters 2496) having C-18 column (Hypersil ODS, 5u C18 (150mm X 4.6 mm) for the estimation of lipstatin. Concentrations of lipstatin were calculated by comparison of peak areas with those standard lipstatin and subsequently lipstatin activity was calculated. Biomass was measured in terms of percentage mycelial volume (%) [3].

Strain Improvement

The general procedure was to treat 10^9 spores with a mutagen for a period of time which gave 0.1 to 1.0% survivor when grown on YMA media. Then single colonies were picked from plates of the desired survivor population for further analysis [12]. Specific procedures for each mutagen were as follows:

UV-Mutagenesis

The method of UV mutagenesis is based on Miller [7]. During UV mutagenesis the culture was exposed to short wavelength UV light from a distance of 60 cm for various time intervals. The selected strain of *S. toxytricini* was serially diluted with sterile water up to 10^{-5}. Sterile *Streptomyces* isolation agar was prepared and poured into sterile Petri dishes. 1.0 ml suspension from each culture tube (10^{-1} to 10^{-3}) was spread on the surface of *Streptomyces* isolation agar plate. Then, the plates were exposed to UV irradiation (254 nm) for 5, 10, 15, 30, 60, 90, 120, 150 and 180 seconds in dark and then incubated at 28°C for 48 Hrs.

N-methyl-N'-nitro-N-nitrosoguanidine (NTG) Mutagenesis

Spore suspension was prepared and centrifuged at 1,000 ×***g*** for 10 min. The spore pellets were resuspended in 1 ml of buffer or water per slant by agitation on a Wrist shaker. NTG (3 mg/ml) was dissolved in cold (4°C) buffer. 1ml of spore suspension was added to 5 ml of NTG solution, the mixture was agitated momentarily, and immediately incubated in a water bath at 37°C for 2-3 Hrs. 3 ml samples were withdrawn and centrifuged at 5000 rpm for 10 minutes. The pellets were resuspended in 3 ml of water and again centrifuged as above. Control was devoid of NTG in buffer or distilled water. Treated and control spore suspensions were plated immediately after mutagenesis for viable count and stored in the refrigerator. 1ml of appropriate dilutions of the spore suspensions were plated (usually in duplicate) in selective medium. Colonies appearing on plates were counted after incubation at 28°C for 10 days [2].

Isolation of Stable Mutants

After 7-10 days incubation, the plates were observed for the survivor of mutants. The mutant colonies were picked up based on the morphological characteristics of the colony and screened for the lipstatin production. The strains were sub cultured and stored in refrigerator at 4°C and rechecked for the lipstatin production. Many strains showed no lipstatin production due to the dark repair mechanism, *i.e.* unstable mutants. The mutation procedure was repeated as described above, to get the stable lipstatin producing mutant strains. The stable mutant strains were cultured in broth medium and compared for the maximum production of lipstatin. The mutant strain showing higher activity was selected for further study. Subsequent fermentation analysis was carried out to evaluate the metabolite titer of the obtained mutants and screened out the expected high lipstatin producing mutant strain.

Inoculum of each culture (wild and mutant) was prepared and incubated at 28°C under shake conditions (220 rpm) for 48 Hrs. Subsequently the inoculum was transferred to seed and then to production media PM1, and incubated at 28°C under shake conditions (220 rpm). Samples were analyzed after 120, 168, 216, 244 and 264 Hrs. for activity through HPLC. Five flasks were inoculated for each culture. All tests were performed in triplicate.

Results and Discussion

S. toxytricini colonies were elevated and covered with white aerial mycelia and spores. Diffused melanoid pigments were sometimes observed. On YM plates colonies were irregular, flat, covered with white aerial mycelia as described by Kmpfer [5].

On the basis of optimized production ingredients, optimized seed, and physical parameters, the new production medium PM1 has been designed and checked for lipstatin production. *S. toxytricini* was inoculated in lab inoculum, 2% of 32 Hrs. gown lab inoculum was transferred into seed media. Later 10% of matured seed was transferred to optimized production medium PM1 for Lipstatin production. HPLC assays show that the organism start producing lipstatin from 72 Hrs. which increased with regular time. The maximum amount of lipstatin produced was 2.182 mg/g titers in PM1 production medium at 264 Hrs.

Strain Improvement

For all strain improvement experiments, *S. toxytricini* was inoculated in selected lab inoculums. At 32 h, 2% lab inoculum was transferred into the seed medium. Then 10% of seed inoculum was transferred into the production medium PM1 at 24 Hrs. of seed in lipstatin production medium.

The genetic modification of the strain was carried out by UV irradiation and/or chemical method (NTG). The viable culture after mutagenesis were picked and tested for the lipstatin production. The improvement in the genetic modification was tested by the ability of the mutant strains to produce the lipstatin in large quantities. Variation in morphological characteristic after mutation is shown in [Fig-1]. In control plates colonies were small, circular covered with aerial mycelium, flat, powdery growth with central and periphery off-white in color with middle grayish ring, rough white margins as shown in [Fig-1A].

In strain improvement study through UV irradiation and/or chemical method (NTG), N number of colonies was screened for lipstatin production out of which a total of 20 colonies were selected on the basis of showing good lipstatin activity. Colony No. 17 showed the maximum lipstatin production at different interval of 120, 168, 216,

244 and 264 Hrs. resulting in 0.63, 0.83, 1.69, 2.08 and 2.34 mg/g activity through UV mutation. Colony No. 7 showed 0.56, 0.67, 1.33, 1.96 and 2.28 mg/g activity, colony No. 16 showed 0.74, 0.87, 1.71, 2.17, 2.31 mg/g activity and colony No. 20 showed 0.67, 0.89, 1.79, 2.09 and 2.29 mg/g of activity, respectively after different hours of incubation [Table-1]. Similar activity was observed in all the strain. In UV-irradiated plates the size of colonies were increased, circular covered with aerial mycelium, flat, powdery growth with central and periphery yellow in color with middle pinkish ring, rough light yellow margins as shown in [Fig-1B] [11].

Table 1- Effect of U.V. mutation on Lipstatin production for different colonies in shake flask (Activity (mg/g) at different Time (Hrs.))

Colonies No.	120 Hrs.	168 Hrs.	216 Hrs.	244 Hrs.	264 Hrs.
1	0.59	0.77	1.44	1.99	2.2
2	0.68	0.8	1.51	2	2.08
3	0.61	0.74	1.4	1.95	1.99
4	0.55	0.62	1.42	2.09	2.23
5	0.58	0.66	1.45	2.1	2.22
6	0.59	0.69	1.35	1.95	2.27
7	0.56	0.67	1.33	1.96	2.28
8	0.6	0.78	1.44	2.05	2.2
9	0.52	0.6	1.21	1.83	2.03
10	0.65	0.8	1.5	1.88	2.08
11	0.64	0.78	1.39	1.92	2.07
12	0.47	0.59	1.34	1.93	2.04
13	0.7	0.87	1.67	2	2.01
14	0.59	0.79	1.62	2.03	2.06
15	0.54	0.63	1.54	2.06	2.12
16	0.74	0.87	1.71	2.17	2.31
17	0.63	0.83	1.69	2.08	2.34
18	0.62	0.79	1.63	1.96	2.22
19	0.57	0.71	1.66	1.97	2.19
20	0.67	0.89	1.79	2.09	2.29

Colony No. 15 showed maximum lipstatin production at 120, 168, 216, 244 and 264 Hrs. resulting in activity of 0.97, 1.40, 2.19, 2.85 and 2.88 mg/g through NTG treatment, while colony No. 10 showed 0.69, 1.11, 1.88, 2.45, 2.61 mg/g activity. Colony No. 13 showed 0.86, 1.16, 1.72, 2.27, 2.60 mg/g activity and colony No. 19 showed 0.85, 1.39, 2.14, 2.77 and 2.80 mg/g activity, respectively at different log Hrs. [Table-2]. In NTG treated plates colonies were large, circular covered with aerial mycelium, flat, velvety growth, central yellow brownish color with light yellow color rough margins as shown in [Fig-1C].

Table 2- Effect of NTG treatment on Lipstatin production for different colonies in shake flask (Activity (mg/g) at different Time (Hrs.))

Colonies No.	120 Hrs.	168 Hrs.	216 Hrs.	244 Hrs.	264 Hrs.
1	0.79	1.21	1.81	2.23	2.42
2	0.52	1.09	2.02	2.14	2.23
3	1	1.23	1.7	2.04	2.14
4	0.5	1.12	1.49	1.95	2.26
5	0.44	1.03	1.56	2.05	2.36
6	0.79	1.18	1.88	2.5	2.55
7	0.81	1.1	1.72	2.25	2.3
8	0.87	1.04	1.67	2.38	2.36
9	0.99	1.2	1.84	2.43	2.55
10	0.69	1.11	1.88	2.45	2.61
11	0.84	1.37	1.97	2.5	2.45
12	0.85	1.25	1.74	2.21	2.37
13	0.86	1.16	1.72	2.27	2.6
14	0.98	1.24	1.78	2.42	2.5
15	0.97	1.4	2.19	2.85	2.88
16	0.55	1.16	2.01	2.62	2.52
17	0.69	1.22	1.87	2.55	2.56
18	0.77	1.29	2.05	2.51	2.56
19	0.85	1.39	2.14	2.77	2.8
20	0.79	1.3	2.1	2.48	2.38

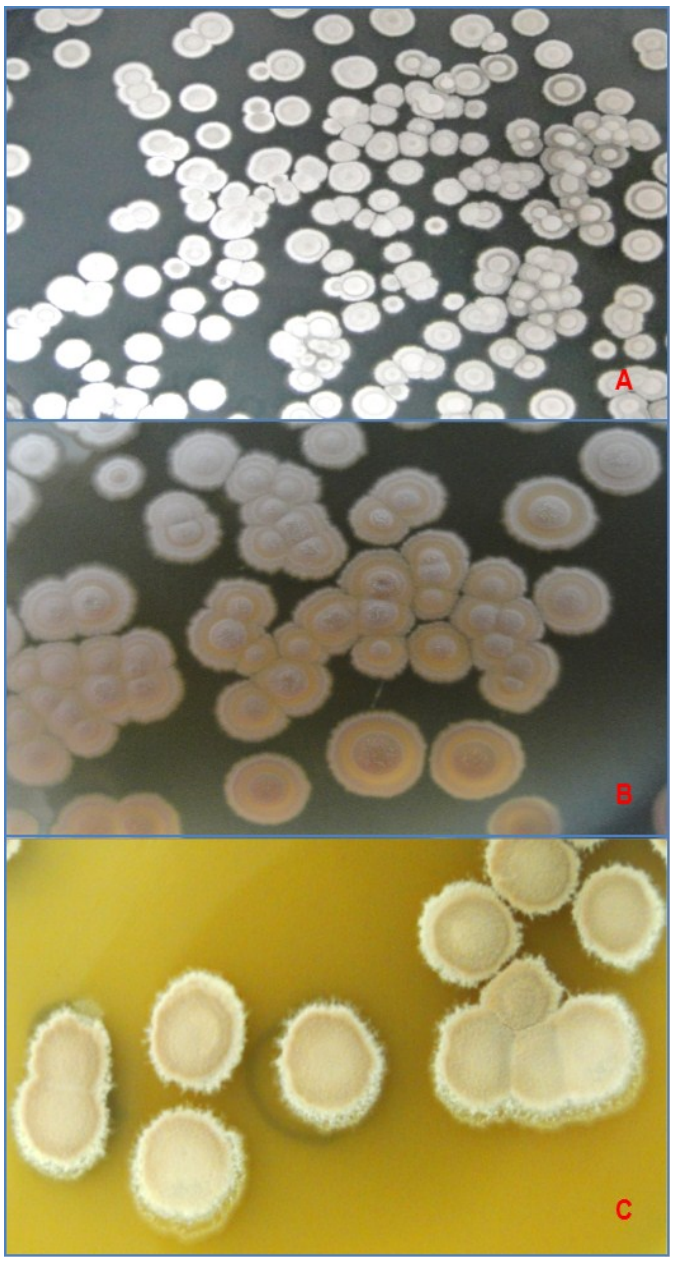

Fig. 1- Variation in morphological characteristic of *S. toxytricini* after using various method of mutation (A) Control; (B) UV mutation; (C) NTG.

Further colony no. 15 was again quantitatively tested for their reproducibility of lipstatin production. For this the colony no.15 was grown in production medium PM1 and then incubated at 28°C, 220 rpm and pH 7.2 for specified incubation period. In process parameters (*i.e.* pH, PMV, oil %) and lipstatin content was measured at different hour. The results of process parameters and their activity are shown in [Table-3]. HPLC assays show that the organism start producing lipstatin from 72 Hrs. which increased with regular time. The maximum amount of lipstatin produced was 2.75 mg/g titers with 6.66 pH, 62% PMV and 3% oil in PM1 production medium at 264 Hrs. [Table-3].

Liutskanova [6] also used conventional mutagenesis (UV irradiation) and exposure to nitrosoguanidine to produce and regenerate protoplasts, for increasing the antibiotic activity of a *Streptomyces fradiae* strain producing tylosin. Variants exceeding the activity of

the initial procedure strain by 0.5 - 28.3% were obtained. The high potent variants were produced by a combined exposure to UV and nitrosoguanidine, as well as upon regeneration of protoplasts formed from the cells of clones produced by UV irradiation. Unstable inheritance of the trait of increased tylosin production was demonstrated.

Table 3- Analysis of process parameter in PM1 medium

Time (Hrs.)	pH	PMV (%)	Oil %	Activity (mg/g)
72	6.41	35	4	0.101
96	6.66	44	4	0.161
120	6.78	55	5	0.383
144	6.96	60	5	0.647
168	7.07	65	4	0.851
192	7.12	68	4	1.652
216	6.73	70	4	1.954
240	6.79	65	3	2.64
264	6.66	62	3	2.75

Wang [11] reported the glycopeptide antitumor antibiotic zorbamycin from *Streptomyces flavoviridis* ATCC 21892 through strain improvement by using UV radiation. Two high-producing strains of zorbamycin, *S. flavoviridis* SB9000 and SB9001, were isolated. Under the optimized conditions, these two strains produced about 10 mg/l of zorbamycin, which was about 10-fold higher than the wild -type ATCC 21892 strain, as estimated by HPLC analysis. Cheng [2] have been carried out mutation and screening of the parent culture to improve rapamycin production. More than thousands of survivors were obtained after mutagenesis by NTG (3mg/ml) and UV (30W, 15cm, 30 seconds) of spore suspensions. None of them showed improved production of rapamycin.

The genetic modification of the strain was carried out by UV irradiation and/or chemical method (NTG). Out of 20 Colonies, Colony No. 17 showed the maximum lipstatin production (2.34 mg/g) through UV mutation, Colony No. 15 showed the maximum lipstatin production (2.88 mg/g) through NTG treatment at 264 Hrs. Thus it may be concluded that maximum lipstatin production was obtained through NTG treatment.

Acknowledgements

The author (UL) thanks for Ipca Laboratories Ltd. for the financial support and provided lab to do complete research work.

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