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MUTATION STUDIES ON *STREPTOMYCES* FOR AMPHOTERICIN B YIELD IMPROVEMENT

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Abstract: *Four Streptomyces strains isolated from the soil of Western Ghats of India producing Amphotericin B were subjected to random mutagenesis by UV irradiations for yield improvement. The mutants were then screened for their Amphotericin B concentrations and their antifungal activity by agar well diffusion method. Two mutants demonstrated 13% and 16% higher concentration than the isolated strains respectively. Thus mutation can be a very useful tool to minimize production cost with the same benefits.*

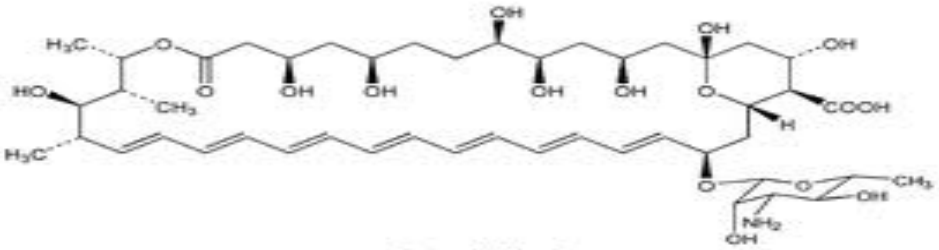
Keywords: *Amphotericin B, UV mutation, Streptomyces, strain improvement*

Introduction:

Strain improvement has a been an important part in commercial fermentation process to improve the products in terms of yield, cost effectiveness of the process, better performance etc.[1] Conventionally it is being achieved by mutation, selection or genetic recombination. These experiments are based on the understanding of the physiology of the organism, its pathway regulation and control and rate-limiting catalytic steps resulting in over expression of certain primary or secondary metabolites.[2] This is generally coupled with expertise in fermentation process on optimization of media and fermentation parameters.

Streptomyces or broadly *Actinomycetes* are gram positive bacteria known to produce several antibacterial and antifungal antibiotics, plant growth factors and other substances.[3] Amphotericin B is a classical broad spectrum anti-fungal antibiotic obtained from *Streptomyces nodosus* (Gold et al. 1955) used for over fifty years now despite its toxicity[4]. It has also been identified as antibiotic for treating prion infections and Leishmaniasis. Nephrotoxicity has been greatest limitation of this antibiotic[5] hence, there is a need to identify novel strains, which aims at improving the therapeutic index of this drug. The standard for commercial acceptance of Amphotericin B for intravenous use is it should be minimum of 75% pure with less than 5% of Amphotericin A contents. (FUNGISONE – marketed by E R Squibb & Sons Inc.)[6]

The anti-fungal activity is due to the attachment of the polyketide to the ergosterol of the fungal cell wall creating



amphotericin B

holes in the cell membrane allowing the contents to leak out resulting in cell death[7]

This study aims at carrying out random mutagenesis by UV mutation on four isolates of *Streptomyces* from the soil of Western Ghats of India for yield improvement to make it more commercially viable.

Materials and Methods

Fungus:

Control Strain: B-2371 and NRRL-WC-3694. The cultures were revived using ISP–2 medium and maintained at 8degC.

Test strains: Four isolates of *Streptomyces* from the Western Ghats with accession numbers- JJ/ Micro/Lab/**003**, JJ/ Micro/Lab/**012**, JJ/ Micro/Lab/**013** & JJ/ Micro/Lab/**038**.The cultures were also maintained on ISP-2 medium.

Protoplast preparation: The test and the control strains were grown in growth medium8 (yeast extract –1%, Dextrose –1%, Calcium carbonate – 0.01) for 48 hrs at 30°C in shaking conditions. The biomass was subjected to lysozyme in isotonic solution in for a period of 30 minutes. Sample from each of the flasks were drawn and observed under the microscope for protoplasts. After sufficient quantity being converted to protoplast, it was then separated from the other cells by filteration through glass wool. The protoplasts were washed repeatedly remove the residues of lysozyme and stored in isotonic solution.

UV irradiation: 3 ml of the protoplast suspension for each of the test strain were subjected to UV irradiation for a period of 5,10,15,20 and 25 minutes9. The suspensions were then plated on regeneration medium and incubated in dark for 5 days. The number of colonies obtained was counted and survival rate calculated. Mutants with 99 % kill rate or 1% survival rate were taken up for screening for Amphotericin B.

Fermentation medium and culture conditions: The mutants were inoculated into growing medium as mentioned above. 5% of the inoculum was transferred into production medium (Production medium: Bactopeptone – 1%, dextrose –5%, CaCO3 – 1%, MnCl2.4H2O –0.001%, FeSo4.7H2O – 0.01% pH –7.4) and incubated at 30°C for 192 hrs. 0.4 µg/ml of Streptomycin sulphate solution was added to the medium at 24 and 96 hrs10. The concentration of product was measured using spectrophotometer for Amphotericin A and B (13).

Analysis: After 192 hrs of incubation, the production medium was analysed for presence of amphotericin A and B. In Two microfuge tubes, 1 ml of 20% production medium in Dimethyl sulphoxide was pipetted. The tubes were vigorously shaken for 60 mins and centrifuged at 10,000 rpm for 10 minutes.1 ml of 20% supernatant in methanol was transferred into fresh microfuge tubes, mixed well and centrifuged again. The supernatant solution was analyzed using spectrophotometer between 250 and 450 nm. The absorbances obtained were plotted on the standard graph for Amphotericin B 11.

Antifungal activity: Disc diffusion test was carried out according to the reference document (CLSI M44A, 2002) document guidelines using Mueller Hinton agar plates with 2% dextrose and 0.5% methylene blue. Pure culture of *Candida albicans* was plated by swab culture technique. Standard discs were saturated with supernatant extracts of Amphotericin B obtained from the above mentioned mutants, dried and placed in the

centre of the plate. The plates were incubated at 37degC for 48 hrs. The zones of Inhibition were measured and tabulated. The antifungal activity of the mutants was compared with test and control strains .12 the mutants were categorized as sensitive if the zone of inhibition was greater than 15 mm or moderately sensitive.

Results

UV mutation for the four isolates yielded 236 mutants.

The mutants were first subjected to the screening screening for antifungal property on MHA plates using disc diffusion method using *Candida albicans* as test culture for antifungal susceptibility. 17 mutants were found to be sensitive and were selected based on the zone of inhibition. These mutants were then grown in the regular shake flask fermentation method, extracted Amphotericin B and estimated the levels using HPLC method. Two mutants showed 23% and 28% increase over the parent respectively.

S No	Mutant No/ control No	Absorbance in nm			
		Amphotericin A		Amphotericin B	
		304 nm	318 nm	382 nm	405 nm
1	JJ/micro/lab/003/M14	0.417	0.339	0.128	0.113
2	JJ/micro/lab/003/M28	0.453	0.321	0.168	0.137
3	JJ/micro/lab/12/M39	0.499	0.403	0.141	0.102
4	JJ/micro/lab/012/M44	0.456	0.376	0.112	0.100
5	JJ/micro/lab/012/M68	0.425	0.289	0.160	0.118
6	JJ/micro/lab/012/M79	0.513	0.366	0.123	0.078
7	JJ/micro/lab/012/M84	0.522	0.389	0.112	0.057
8	JJ/micro/lab/012/M86	0.476	0.423	0.108	0.060
9	JJ/micro/lab/012/M93	0.455	0.428	0.123	0.107
10	JJ/micro/lab/012/M112	0.487	0.389	0.128	0.087
11	JJ/micro/lab/013/M146	0.422	0.319	0.065	0.049
12	JJ/micro/lab/013/M169	0.401	0.336	0.186	0.145
13	JJ/micro/lab/013/M183	0.449	0.389	0.119	0.107
14	JJ/micro/lab/038/M191	0.679	0.472	0.123	0.035
15	JJ/micro/lab/038/M207	0.548	0.521	0.101	0.071
16	JJ/micro/lab/038/M214	0.631	0.446	0.134	0.039
17	JJ/micro/lab/038/M220	0.669	0.478	0.142	0.049
18	JJ/micro/lab/003	0.405	0.356	0.178	0.127
19	JJ/micro/lab/012	0.501	0.405	0.142	0.103
20	JJ/micro/lab/013	0.301	0.288	0.169	0.123
21	JJ/micro/lab/038	0.342	0.282	0.189	0.132
22	Control	0.686	0.485	0.153	0.086

Discussion

Four isolates found to be promising during the screening process were selected and subjected to random mutation on their protoplasts as protoplasts are known to be more susceptible to mutation and have proved to give better results on yield

improvement studies. 236 mutants were obtained from these (36, 98, 55 and 47 respectively)

The mutants were confirmed as Streptomycetes based on their gram staining characteristics. All the mutants were found to be Gram positively stained thin mycelium with open and closed spirals typical of streptomyces species.

They were then screened for antifungal activity by Disc diffusion method and 17 mutants were found to be sensitive with zone of inhibition greater than 15mm as per the CLSI guidelines.
When tested for quantification of the amount of Amphotericin A and b produced by the conventional fermentation method, 2 mutants were found to be promising with a 13 and 16% increase in amphotericin B levels as against the parent isolate from the Western ghats.

The Mutant 169 showed 21.5% and 68% increase in Amphotericin B values at 382 and 405 nm respectively in comparision to the control strain of *Streptomyces nodosus*.

From the above experiments, it can be well concluded that mutation is definitely an excellent tool for yield improvement studies. Streptomycetes has shown good susceptibility to mutation as well has enhanced the activity of the byproduct, Amphotericin B. there is also a significant reduction of Amphotericin A production improving the efficacy of the product. Although one type of mutation, UV mutation has been carried out, several other methods such as point mutation, side directed mutations can be experimented on these cultures and improve the yield further. Further studies on the pharmacological and toxicological studies need to be carried out to see if these mutants can also reduce the toxicity levels when delivered on a prolonged basis, thereby making them potential candidates for reduced nephotoxicity.

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