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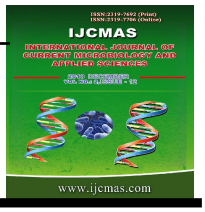

Original Research Article

Isolation and Screening of *Streptomyces* from the Western Ghats for the presence of Amphotericin B

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A B S T R A C T

45 isolates of Streptomyces from the regions of Coorg and the Western Ghats were characterized and screened for the production of Amphotericin B. Four isolates have shown promising results with lower levels of amphotericin A as compared to the standard strain of *Stretomyces nodosus*. Further studies on these isolates for optimization of product and pharmacological and toxicological studies are being carried out.

K e y w o r d s

Streptomyces; Amphotericin B; Western Ghats.

Introduction

Western Ghats of India has been a hosting a unique diversity of microorganisms known to have great potential for treating various ailments in humans and animals. Several area of the ghats have been studied by various scientists , botanist and microbiologist for bioactive lead compounds which can be nature's answer to synthetic chemistry and drug discovery projects. (Raghunathan *et al.*, 2010)

Stretomyces nodosus is the only known high producer of Amphotericin B, a widely used anti-fungal drug for chronic and systemic fungal infection identified by Gold *et al.*, (1955). In 1961 & 1962, two patents were granted to Dutcher and his team (Dutcher *et al.*, 1959) and Trejo and Bennett (Trejo, Benett 1961) respectively for large scale production of this antibiotic. This has opened newer areas of research to improve the yield, reduce toxicities, explore newer organisms for similar or better products which can result in a much more efficient and reliable product with minimum side effects. Amphotericin B is essentially a high molecular weight macrocyclic lactone, better known as a macrolide, possessing a chromophore of 7 conjugated double bonds. In addition to the large lactone nucleus, amphotericin B has other

characteristic groups including an amino sugar (Metzgen and Julio 1976)

With a great rise of several diseases like AIDS, Prion infections etc, design and development of effective drug specific to the target have become a necessity (Crueger and Crueger 2010). Although an excellent drug as antifungals, nephro-toxicity is a dose limiting factor in many patients particularly when used in combination with other potential nephorotixic agents like aminoglycosides, cyclosporins etc (www.stephen williamson.com) .Therefore, there is a need to develop new formulations, which aims at improving the therapeutic index of this drug. Amphotericin A is the byproduct in the production of Amphotericin B which is a less potent drug compared to the latter .The other challenge is to reduce the production yield of amphotericin A for better potency of the drug (janin). 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.) (Janina Brajtburg *et al.*, 1990; John and Edward 1990). Thus some of the challenges traits used for the study are Isolation of new strains of Streptomyces, identifying their potential for the drug and Production process to reduce the production of Amphotericin A as well as develop a less toxic strain.

Materials and Methods

Medium for Isolation - ISP–2 medium

Control strains - NRRL-B-2371 and NRRL-WC-3694 obtained from USDA.
Soil and humus samples were collected from the Western Ghats. After diluting the samples in isotonic solution, they were plated out on ISP-2 medium for isolation. (Fig 1) The isolated colonies were taken up for microscopic examination and further identification.

Upon primary identification to species level, they were taken up for screening for Amphotericin B production. The isolates were maintained on Glucose asparagines slants at 8 °C.

Growth medium

The cultures upon primary selection were inoculated into growing medium (yeast extract –1%, Dextrose –1%, Calcium carbonate – 0.01) for 48 hrs at 30ºC in shaking conditions. 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 30ºC for 192 hrs on incubator shaker. 0.4 µg/ml of Streptomycin sulphate solution was added to the medium at 24 and 96 hrs . The concentration of product was measured using spectrophotometer for Amphotericin A and B (Harald *et al*., 1974)

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 minutes 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 (Harald *et al*., 1974).

Results and Discussion

Out of 183 actinomycete culture isolated, 45 showed the following characteristics;

Growth on ISP-2 Agar medium

The isolates formed characteristic creamish-white colonies embedded into the medium, which sporulated well upon longer incubation turning completely white in colour. The reverse of the colony showed clear demarcations and was pigmented to pinkish brown colour.

Gram staining

Gram positive stained thin mycelium with open and closed spirals typical of streptomyces species. Streptomyces nodosus is a gram-positive bacteria, which can be identified by several methods. Along with a positive gram stain, the mycelium are very distinct when viewed under 640X magnification showing open and closed spirals The spores can also be viewed with an electron microscope to identify a semi-spiral shaped structure as seen in Fig.2 (Janina Brajtburg *et al*., 1990)

Growth on glucose

Aspargine agar medium- showed grayish coloured growth with a yellow coloured water insoluble fluid deposit at the butt of the slant . This proved the identitity to belong to streptomycetes species (www.stephenwilliamson.com)

A yellow liquid was also found to be present surrounding the colonies on the slant which when analysed were found to Amphotericin B.

All the 45 isolates upon identification were taken up for screening for production of Amphotericin B using the process of production as mentioned in the production process patent (Janina Brajtburg *et al*., 1990), as mentioned in the procedure. After 8 days of incubation , the broth was used for analysis to check the presence of Amphotericin A and B by spectrophotometric determination. (Table - 1) Four cultures proved to be promising by not only showing similar or lower activity of amphotericin A but also showing higher yields of Amphotericin B (Table -2).

183 cultures of *Actinomycetes* were isolated from various soil and humus samples from the Coorg region of the Western Ghats. 45 isolated were identified to belong to *Streptomycetes* species based on the morphology, microscopic observation upon Gram staining and cultural characteristics on Glucose asparagine and ISP-2 growth medium.

Further these 45 isolates were taken up for screening for presence of Amphotericin B. Out of the 45 isolates, all showed production of of Amphotericin A and B but only 4 isolates showed higher yields over the test strains of *Streptomyces. nodosus* Control- 1 & 2 (NRRL-B-2371 and NRRL-WC-3694).The entire exercise was repeated 3 times to check the consistency and stability of the yields obtained. The greater advantage of these isolates is the reduction in the Amphotericin A levels which can help in making a better potency Amphotericin B with less of Amphotericin A contamination.

Table.1 Number of Actinomyctes isolated from the study area (Time interval 24 and 96 Hours)

S No	Flask No	Absorbances in nm			
		304 nm	**318 nm**	**382 nm**	**405 nm**
		Amphotericin A		**Amphotericin B**	
1	Control - 1	**0.686**	**0.485**	**0.153**	**0.086**
2	Control-2	**0.592**	**0.337**	**0.098**	**0.065**
3	Isolate No 1	0.422	0.293	0.105	0.051
4	Isolate No 2	0.680	0.455	0.148	0.086
5	Isolate No 3	**0.405**	**0.356**	**0.178**	**0.127**
6	Isolate No 4	0.402	0.385	0.146	0.112
7	Isolate No 5	0.353	0.224	0.079	0.028
8	Isolate No 6	0.296	0.324	0.065	0.054
9	Isolate No 7	0.428	0.361	0.121	0.098
10	Isolate No 8	0.551	0.432	0.055	0.032
11	Isolate No 9	0.497	0.326	0.102	0.065
12	Isolate No 10	0.556	0.421	0.162	0.091
13	Isolate No 11	0.412	0.291	0.112	0.076
14	Isolate No 12	**0.501**	**0.405**	**0.142**	**0.103**
15	Isolate No 13	**0.301**	**0.288**	**0.169**	**0.123**
16	Isolate No 14	0.282	0.311	0.073	0.069
17	Isolate No 15	0.398	0.348	0.134	0.109
18	Isolate No 16	0.543	0.445	0.065	0.063
19	Isolate No 17	0.354	0.287	0.112	0.033
20	Isolate No 18	0.477	0.378	0.105	0.027
21	Isolate No 19	0.512	0.443	0.098	0.076
22	Isolate No 20	0.482	0.346	0.076	0.046
23	Isolate No 21	0.399	0.290	0.092	0.059
24	Isolate No 22	0.461	0.365	0.117	0.082
25	Isolate No 23	0.577	0.378	0.127	0.039
26	Isolate No 24	0.565	0.403	0.155	0.046
27	Isolate No 25	0.607	0.389	0.105	0.071
28	Isolate No 26	0.437	0.377	0.045	0.037
29	Isolate No 27	0.490	0.321	0.059	0.022
30	Isolate No 28	0.456	0.346	0.071	0.038
31	Isolate No 29	0.615	0.398	0.032	0.012
32	Isolate No 30	0.578	0.326	0.061	0.025
33	Isolate No 31	0.612	0.447	0.046	0.016
34	Isolate No 32	0.553	0.387	0.095	0.078
35	Isolate No 33	0.478	0.391	0.114	0.080
36	Isolate No 34	0.435	0.327	0.127	0.092
37	Isolate No 35	0.598	0.390	0.102	0.085
38	Isolate No 36	0.536	0.391	0.069	0.047
39	Isolate No 37	0.455	0.345	0.031	0.011

40	Isolate No 38	**0.342**	**0.282**	**0.189**	**0.132**
41	Isolate No 39	0.437	0.278	0.046	0.013
42	Isolate No 40	0.577	0.367	0.073	0.041
43	Isolate No 41	0.562	0.318	0.087	0.040
44	Isolate No 42	0.444	0.329	0.043	0.062
45	Isolate No 43	0.591	0.340	0.087	0.055
46	Isolate No 44	0.482	0.385	0.072	0.028
47	Isolate No 45	0.679	0.316	0.107	0.078

Table.2 Growth of Actinomyctes

S.No	**Sample**	**Absorbance in nm**			
		304 nm	**318 nm**	**382 nm**	**405 nm**
		Amphotericin A		**Amphotericin B**	
1	Control -1	0.686	0.485	0.153	0.086
2		0.671	0.458	0.146	0.069
3	Control-2	0.592	0.337	0.098	0.065
4		0.576	0.319	0.079	0.049
5	Isolate No-3	0.405	0.356	0.178	0.127
6		0.388	0.341	0.165	0.134
7		0.415	0.362	0.163	0.132
8	Isolate No-12	0.501	0.405	0.142	0.103
9		0.487	0.447	0.137	0.112
10		0.493	0.413	0.134	0.108
11	Isolate No-13	0.301	0.288	0.169	0.123
12		0.316	0.291	0.154	0.117
13		0.321	0.273	0.149	0.126
14	Isolate No-38	0.342	0.282	0.189	0.132
15		0.356	0.295	0.194	0.141
16		0.361	0.278	0.199	0.126

Isolate 3 and 38 showed an average increase in 17 & 41% yield compared to the control strains at 382 and 405nm respectively with an average decrease in 44 & 32 % of Amphotericin A contamination at 304 and 318 nm respectively.

Thus it can be well concluded from the above experiments that Western Ghats are indeed a rich source of microbial biodiversity holding within it immense novelty and potentiality of identifying new isolates for production of life saving drugs. Further studies on the pharmacological and toxicological studies need to be carried out to see if these strains can also reduce the toxicity levels when delivered on a prolonged basis, thereby making them potential candidates for reduced nephotoxicity. Mutation studies are also needed to be designed for yield improvement.

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