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Research Article

SCREENING OF HMG CO A REDUCTASE INHIBITOR PRODUCING MARINE ACTINOMYCETES

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ABSTRACT:

The objective of the present study was screening of 3-hydroxy-3- methyl glutaryl Co A (HMG CoA) reductase inhibitor producing marine actinomycetes. A total of 65 morphologically different actinomycetes were screened for HMG CoA reductase inhibitor production in a two stage submerged fermentation and evaluated for HMG CoA reductase inhibitor activity by agar diffusion and thin layer chromatography technique using lovostatin as a control. Among 65 marine Actinomycete strains, only one strain produced HMG Co A reductase inhibitor.

Key words: Lovastatin, Marine actinomycetes, HMG Co A reductase inhibitor

INTRODUCTION

Hypercholesterolemia involves heterogeneous disorders of lipid metabolism characterized by elevated levels of plasma total cholesterol and low density lipoprotein (LDL) derived cholesterol. Cholesterol enters the body in two ways i,e. absorption from diet or endogenous biosynthesis. The interference of either process would provide an effective means of lowering plasma total cholesterol. 3-Hydroxy- 3 -methyl glutaryl Co enzyme A (HMG Co A) reductase, a rate limiting enzyme in cholesterol biosynthetic pathway, was considered a promising target of inhibition. Structurally related compounds such as compactin (ML 236B) and mevinolin (monacolin K) were discovered from fungal culture broths as potent and specific inhibitors of HMG Co A reductase [2,3,4,5] Since then, the derivatives lovastatin, pravastatin and mevinolin have been used clinically for the treatment of hypercholesterolemia [6] .

Statins are a class of organic molecules which posses polyketide structure and a hydroxyl naphthalene ring system with different side chains. Statins are produced as secondary metabolites by microorganisms like bacteria [9](*Bacillus* spp.), Fungi[10, 11,4,12] (*Aspergillus* spp, *Monascus* spps.)And Actinomycete spp.[13, 14] (*Streptomyces* spp, *Actinomadura* spp). The aim of the present study is to isolate actinomycetes from marine environment and screen for HMG Co A reductase inhibitor activity.

MATERIALS AND METHODS

Isolation of marine actinomycetes:

Marine water and sediment samples were collected from different places of Bay of Bengal, Visakhapatnam. Samples were processed on the same day of collection. One gram or one milliliter (1ml) of sample was suspended in 10 ml of sterile 5mM phosphate buffer (pH=7.0) and stirred for 1 min in a super mixer. A 10-fold serial dilution of the suspension was made. One ml of the suspension was spread on starch casein agar plate (g/l: Starch 19, Casein 0.3,KNO_3 2, NaCl 2, K_2HPO_4 2 ,$MgSO_4.7H_2O$ 0.05,$CaCo_3$ 0.02,$FeSO_4.7H_20$ 0.01, agar 20) and Oat meal agar (g/l: Oat meal 20, agar 20, trace salt solution 1ml) plate and incubated at 28^0C for 21 days.

Starch casein agar and Oat meal agar were supplemented with Cycloheximide 50 µg/ml and 5µg/ml rifampicin to inhibit fungal and bacterial contamination respectively.

Identification of Actinomycetes

Actinomycetes were recognized by their characteristic tough leathery colonies that adhered to the agar surface, branched vegetative mycelia, and when present, aerial mycelia and spore formation. Actinomycete colonies were characterized morphologically and physiologically following methods given in the International streptomyces Project (ISP) .Actinomycete isolate SS16/4 was maintained on yeast extract-malt extract agar slants at 28^0C.

Screening of HMG Co A reductase inhibitors

The selected isolate SS16/ 4 was grown on YEME (ISP 2 media) agar slant for 5-7 days. 5 ml of a spore suspension was added to a 100 ml shake flask

containing 20 ml of the seed medium (for every liter: glucose 10 g, peptone 2g, soy protein 4 g and KH_2Po_4 1 g ,P^H 7.0 +_0.2). The seed culture was incubated at 27^0 C for 24 to 48 hrs in a rotary shaker at 200 to 220 rpm. 2to 5 ml of the seed culture was transferred to a 250ml shake flask containing 50 ml of the fermentation medium (per liter: glucose 15 g, peptone 5 g, corn steep powder (C.S.P.) 5 g and soybean meal 4 g, KH_2PO_4 1 g, P^H 7.0+.0.2). The flasks were incubated at 27^0C for 5-7days on a rotary shaker at 200 rpm

Extraction

After incubation, production media was centrifuged at 1500 rpm for 15 min at 10^0C in cooling centrifuge, to separate fermented broth (supernatant) and mycelium.

Before extraction , supernatant obtained by centrifugation was acidified to different pH values of 3,5,7,9 with 1N HCl and extracted with ethyl acetate [15,12]

The mycelia mass obtained by centrifugation was stirred with ethyl acetate at room temparture for 1hr, acidified to P^H 3 with 1N HCl and extracted with ethyl acetate[15, 12]. Extracted product was centrifuged at 1500 rpm and the organic phase was collected, and concentrated in rotary evaporator (Heidolph). Concentrated product was used for further studies[16].

Thin Layer Chromatography(TLC)

TLC was used to detect the presence of HMG Co A reductase inhibitor in the extract.30 µl of concentrated product was applied to precoated silica gel plate. After the chromatogram was developed in the mobile phase (dicholarmethane and ethyl acetate 70:30 v/v) it was stained with iodine vapour and observed under UV light. Three lovastatin standard spots were applied for comparison of the Rf values [16,17]. The lovastatin standard compound was provided by Dr.Reddys laboratories. The lovastatin stock standard was prepared from the pure lactone form of the compound and dissolved in acetonitrile at a concentration of 500µg/ml. 1:10 dilution of the stock solution in the same diluent was used as working standard [16].

Bio-assay of HMG Co A reductase inhibitor using Neurospora crassa:-. *Neurospora crassa* was grown for 7-10 days on PDA slants at 28^0C; spores were harvested with sterile water. Twenty µl of spore suspension of *Neurospora crassa* was transferred into a 40×40 mm size sterilized petri plate with PDA medium (g/l : Potatoes 200g ,Dextrose 20g ,agar 15g). After solidification, wells were made using 8mm diameter borer[18].

Bio-assay of HMG Co A reductase inhibitor using *Candida albicans*: - *Candida albicans* was grown for 12 hrs on PDA slants at 28^0C, spores were harvested with sterile water. 20µl of spore suspension of *Candida albicans* was taken into 40×40 mm size sterilized petri plate with PDA medium. After solidification wells made by 8mm diameter borer[19.]

RESULTS AND DISCUSSION

The aim of study was to screen the HMG Co A reductase inhibitor producing marine actinomycetes. A total of 65 marine actinomycetes were screened for HMG Co A reductase inhibitor activity.

The fermentation broths obtained from 65 marine isolates were examined by TLC. Lovastatin was used as the standard compound having HMG Co A reductase inhibitor activity. Only one of the isolate SS16/4 was detected by chromatography to contain spots comparable to the standard, shown in table-1.

Table -1. Rf values of standard and test samples in different solvents

Solvent	Rf of standard (Lovastatin)	Test Organism SS16/4
A	0.67	0.67
B.	0.55	0.55
C	0.50 .	0.50 .

A: Dichloromethane: ethyl acetate (70:30v/v)

B.Ethyl acetate: Hexane: acetic acid (70:30:6)

C.Acetonitrile:hexane.acetic acid (30:70:6)

Bio-assay method for determing of HMG Co A reductase inhibitor activity:

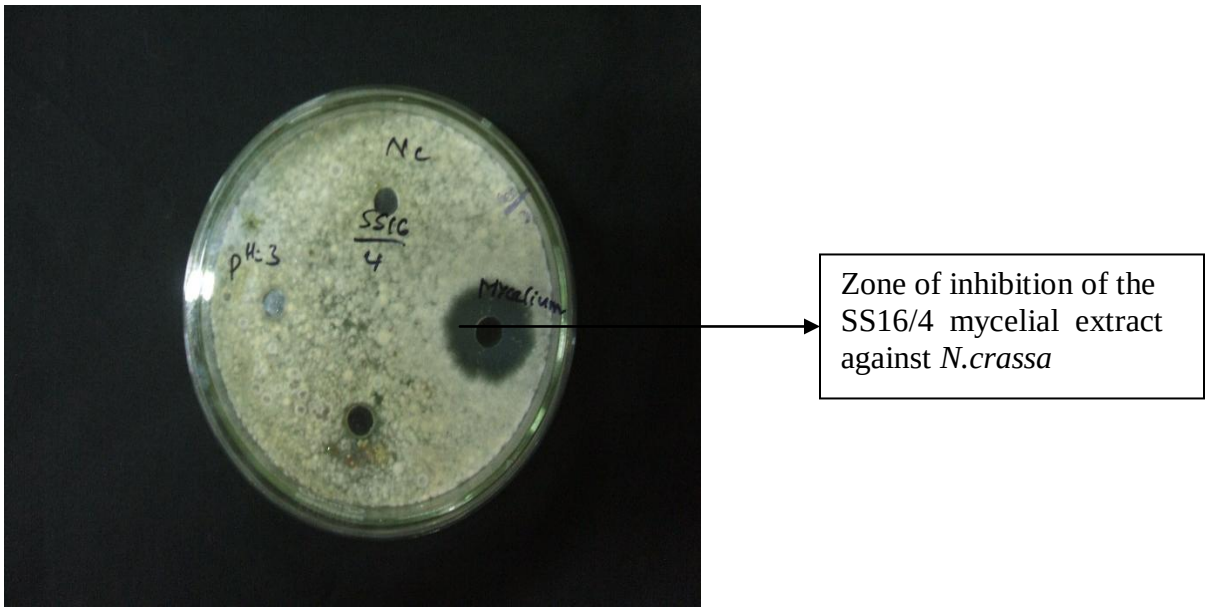

Figure-1 Bio-assay using *Neurospora crassa* as test organism

The wells in the Petri plates were bored sufficiently apart to prevent coalescence of the zones of inhibition of the extract and lovastatin. Ethyl acetate was used as the control and it did not show any inhibition zone. The assay was carried out for the broth (extra cellular) and mycelium (intracellular) separately and inhibition zones were recorded. Zones of inhibition for SS16/4 are shown in fig 1.

The optimum amount of lovastatin required to produce a zone of 10mm was 50µl(1mg/ml) and these concentrations were used to validate each *N.crassa* bioassay method. The period of incubation of *N.crassa* was critical for this test, as incubation beyond the specified period resulted in overgrowth and the boundaries of inhibition zone could not be clearly measured. Best results were obtained with plates incubated for 16-18 hrs at 28^0C. The zones of inhibition of the test extracts and standard were measured and recorded, as shown in table2.

Standard lovastatin inhibition zone = 10 mm

Ethyl acetate inhibition zone = 0 mm

Table-2. The activity of mycelial pellet and culture filtrate of SS16/4 against *N. crassa*

Isolate	Inhibition zones (mm)	
	P^H 3(Mycelium)	P^H 3(broth)
SS16/4	20	10

In the bioassay plate method using *C.albicans* as the test organism, lovastatin causes inhibition of growth at a concentration of 50µl (1mg/ml). The broth (extra cellular) and mycelium (intracellular) extracts of SS16/4 were assayed separately .The plates were incubated for 10-16 hrs and zones of inhibition were measured[19] as shown in fig2 and table3.

Standard lovastatin inhibition zone = 15 mm

Ethyl acetate inhibition zone = 0 mm

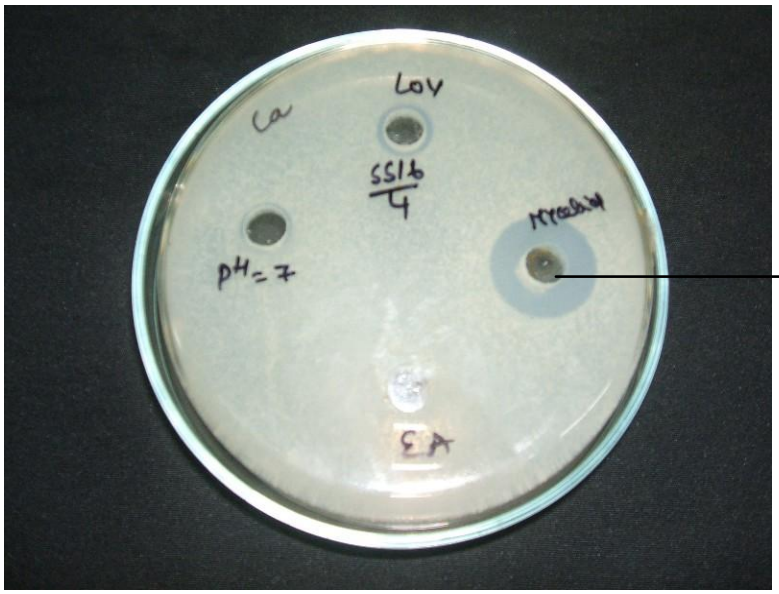

Figure-2 Bio-assay using *Candida albicans* as test organism

Table-3. The activity of mycelial pellet and culture filtrate of SS16/4 against *C.albicans*

Isolate	Inhibition zones in (mm)	
	P^H 3 (Mycelium)	P^H 3 (Broth)
SS16/4	22	10

DISCUSSION

The aim of study was to screen HMG Co A reductase inhibitor producing marine actinomycetes. A total of 65 different marine actinomycetes were screened .Among them, only one strain SS16/4 showed positive results in TLC which was later confirmed by bioassay plate method.

Disregarding small modifications in fermentation conditions this study differs from those of Shindia [20] and Gunde Cimerman et al [21] in which they used methanol extraction of acidified broth followed by TLC analysis. Where as ethyl acetate extracts of acidified fermentation broth and mycelium were used for TLC and Bio-assay plate technique in our study. By using ethyl acetate to extract HMG Co A reductase inhibitor from acidified fermentation broth and mycelium it was possible to extract a significant amount of the inhibitior. Further it was observed that when the mycelium was extracted at P^H 3 large inhibition zones were observed against *N.crassa* and *C.albicans* (Figure-1 and 2). However, when the broth was extracted at different P^H values of 3,5,7,9, only small zones of inhibition wa observed for the broth with P^H 3 (Table-2 and 3).

The nutritional and cultural conditions employed in this study were appropriate for screening HMG Co A reductase inhibitor production by the isolate SS16/4.Optimizing the medium may be necessary for improving the yields of the inhibitor for its characterization.

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