APPLICATION OF BRINE SHRIMP BIOASSAY FOR SCREENING CYTOTOXIC ACTINOMYCETES

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Abstract

Ethyl acetate extracts of ten Actinomycetes isolates were subjected to a bioscreening study to detect cytotoxic activity by the brine shrimp lethality bioassay. Brine shrimp lethality test was conducted on each of the extracts at six different concentrations 31.25, 62.5, 125, 250, 500 and 1000 µg.ml⁻¹. The extracts of the actinomycete isolates PCL-1, SU1, SU13, and SU4 showed IC 50 in < 500 µg/ml. The isolates SU2, SU5 and SU3 showed IC 50 in > 500 µg/ml and the isolates SU 6, SU 8, and SU 9 showed the IC 50 in > 1000 µg/ml. These results suggested that more specific bioassays should be encouraged on these actinomycetes extracts in order to confirm these conclusions.

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1. Introduction:

Microorganisms have shown to be an attractive source of natural compounds for the pharmaceutical and other industries. Far from the thousands of microorganisms which have been found to produce antibiotics, of which 2 / 3 is produced by the actinomycetes, and some actinomycetes are also used to produce vitamins, enzymes, and also used for sewage treatment. Actinomycete from marine environment produces drugs for cancer, a major problem that people face today. Chemotherapy is one of the main treatments used to combat cancer. A great number of antitumor compounds are natural products or their derivatives, mainly produced by microorganisms. In particular, actinomycetes are the producers of a large number of natural products with different biological activities, including antitumor properties. 60% of approved drugs are derived from natural compounds (¹²) and many have been extracted from actinomycetes (³). The in vivo lethality in a simple zoological organism, such as the brine shrimp lethality test (BST), developed by (⁴) might be used as a simple tool to guide screening and fractionation of physiologically active plant extracts, where one of the simplest biological responses to monitor is lethality, since there is only one criterion: either dead or alive. This general bioassay detects a broad range of biological activities and a diversity of chemical structures. One basic premise here is that toxicology is simply pharmacology at a higher dose, thus if we find toxic compounds, a lower, non-toxic, dose might elicit a useful, pharmacological, perturbation on a physiologic system (⁵). However, it has been demonstrated that BST correlates reasonably well with cytotoxic and other biological properties (⁵). Brine shrimp have been previously utilized in various bioassay systems. There have been many reports on the use of this animal for environmental studies (⁶,⁷,⁸) screening for natural toxins (⁹,¹⁰) and as a general screening for bioactive substances in plant extracts (¹¹). Considering that a major challenge today is the discovery of marine sources with promising activities and the isolation of active principles, we have applied in this work the brine shrimp test (BST) for general activity screening of several extracts of actinomycetes from the costal sediments.
2. Materials and Methods

2.1. Isolation and maintenance

Thirty six actinomycetes were isolated from 6 marine sediment sample collected from Ennore, Muttukad, Verampattinum. For the isolation, starch casein agar with the addition of rifampicin (2.5 mg/ml) and fluconazole (75 mg/ml) was used to minimize bacterial and fungal contaminations, respectively. The strain was sub-cultured onto starch casein agar slant (medium with 50% sea water) incubated at 28°C for 5 - 7 days to achieve good sporulation was preserved in 20% glycerol at -80°C.

2.2. Taxonomy

Taxonomic characteristics of the isolate were determined by cultivation on various media as described by [13]. Morphological characteristics were observed after incubation of the culture at 28°C for 14 days on Oatmeal agar (ISP 3). The carbon source utilization, NaCl tolerance, optimum pH, optimum temperature for growth was determined as per the ISP protocol [14].

2.3. Fermentation

A full grown slant culture of the isolates on starch casein agar (composition: 1.0% soluble starch, 0.03% Casein, 0.2% KNO3, 0.2% NaCl, 0.005% K2HPO4, 0.002% CaCO3, 0.001% FeSO4.7H2O and 2.0% agar) was transferred into Erlenmeyer flasks (2 x 250 ml) containing 50 ml of seed medium (composition: 1.0% Soybean meal, 1.0% corn steep solids, 0.5% glucose and 0.5% CaCO3 with pH 7.0) and incubated for 3 days at 28°C on a rotary shaker (220 rpm). A 10% level of this inoculum was transferred into 200 ml of production medium in 1 L EM flasks. The medium composition is: 1.0% soyabean meal, 0.5% corn steep solids, 1.0% soluble starch, 0.5% glucose and 0.7% calcium carbonate with pH 7.0. The inoculated production flasks were incubated for 72 h at 28°C on a rotary shaker (220 rpm).

2.4. Extraction of cell free crude extracts

The culture broth was centrifuged at 4000 rpm for 10 min, at 10°C and clear culture supernatant was separated. It was extracted twice with ethyl acetate and washed with 500 ml water at pH 7.0. The ethyl acetate layer was concentrated in vacuum at 35°C to get the crude ethyl acetate extract.

2.5. Screening for anticancer activity

2.5.1. Artemia lethality assay

The Artemia lethality assay was conducted according to [15] with minor modifications. This procedure determines lethal concentrations of active compounds in brine medium. The activities of a broad range of active compounds are manifested as toxicity to the shrimp. This method is rapid, reliable and has been used for over thirty years in toxicological studies. A positive correlation exists between brine shrimp lethality and human carcinoma.

2.5.2. Hatching the shrimp

Dried cysts of Artemia salina was incubated in natural sea water (1g.1-1) at 28-30°C under constant aeration for 48 hrs. After 48 hours, the phototrophic nauplii were collected by pipette from the lighter side of the hatching chamber and used for the assay.

2.5.3. Crude sample production

In the present study, screening of anticancer activity was done using 10 isolates among 38 isolates. Crude sample for Artemia lethality assay were produced by adopting the submerged fermentation technique in a rotary shaker using ISP-2 medium described by [16]. After 13 days of cultivation, the mycelia and culture filtrate were separated by centrifuging at 10,000 rpm for 30 minutes. The culture filtrate was extracted with ethyl acetate at pH 5.

2.5.4. Sample preparation

Different concentrations of stock solutions were prepared by dilution with dimethyl sulphoxide (DMSO) so as to obtain six different concentrations 31.25, 62.5, 125, 250, 500 and 1000 µg.ml-1 of actinomycetates extracts. 10 Artemia naupli were added into each with different concentration of extracts and 0.2% of DMSO instead of extract as control. After 24 hrs, dead shrimp was counted using microscope. The percentage of mortality was calculated as follows:

\[
\text{% Mortality} = \frac{\text{Percentage of survival in the control} - \text{Percentage of survival in the treatment}}{\text{Percentage of survival in the control}} \times 100
\]

The percentage of mortality was transferred to profit analysis software and the IC 50 was calculated.

3. Results

In the course of screening for cytotoxic actinomycetes, 36 actinomycetes were isolated from 6 different marine sediment samples of Ennore, Muttukadu, Verampattinum. Fig: 1 shows some of the isolated actinomycetes.

Figure: 1 Isolated Actinomycetes

The cultural characteristics of the strain are summarized in Table 1. The isolates PCL-1 exhibits grey colored aerial mycelium, pale yellow substrate mycelium with extended spiral spores on the aerial mycelium. The isolates SU-1 and SU2 are with white aerial and butter white substrate mycelium, the spore of the former is spiral and compact spiral for the later. SU-3 and SU-4 shows grey colored aerial mycelium with white colored substrate mycelium with spiral and compact spiral spores respectively. The isolate SU5, SU6, and SU 8 shows grey colored aerial mycelium and yellow, grey, black colored substrate mycelium respectively. The isolate SU 13 shows flexuous spores on greenish white...
colored aerial mycelium with pale yellow substrate mycelium. The melanin pigment was produced only by the isolate SU4. A diffusible pigment was produced by the isolate SU 8 only.

The biochemical characteristics of the strain are summarized in Table 2. Starch was utilized by all the isolates. Mannitol was utilized well by the isolate SU1, SU2, SU4, SU5, the isolates SU2, SU6, SU8 and SU9 were not utilizing lactose.
All the isolates were growing well at the optimum pH of 7-11 and the temperature of 20-40°C. The isolates SU 1 and SU 13 were shows presence of growth at 50°C and all are tolerate 7 to 20 % of NaCl.

The extracts studied in this work showed significant lethality against brine shrimp, the LC50 results of the ten actinomycetes evaluated in this screening are listed in Table 3. The ethyl acetate extract of the isolates PCL-1, SU1, SU13, and SU4 shows IC 50 in < 500 µg/ml. The isolates SU2, SU5 and SU3 showed IC 50 in > 500 µg/ml.

4. Discussion

The brine shrimp lethality assay is considered to be one of the most useful tools for the preliminary assessment of biotoxicity and the bioassay with cytotoxic activity against some human solid tumors. Meyer (1982) reported that, if the brine shrimp lethality assay displayed LC50 1000 µg/ml of natural derived products was known to contain physiologically active principles. The extracts studied in this work showed significant lethality against brine shrimp, which has been successfully used as a simple biological test to guide the fractionation process of biological extracts in order to detect antitumor compounds. The extracts of PCL-1, SU1, SU13, SU4, SU2, SU5 and SU3 were showed LC50 values less than <1000 µg.ml-1 than the other extracts, so this could be used as a potential anticancer drug. These extracts can be regarded as a promising candidate for a actinomycetes derived antitumor compound. This bioassay has good correlation with the human solid tumors cell lines (17). However, further and more specific bioassays are necessary in order to confirm these conclusions.

5. Conclusion

The biological product which showed > 1000 µg/ml cytotoxicity can be regarded as a promising antitumor compound. The present study reveals seven cytotoxic actinomycetes, which showed > 1000 µg/ml cytotoxicity against artemia salina among the ten tested isolates. These organisms must be studied further for getting new antitumor compounds.

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References


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