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Studies on isolation of nutritional grouping streptomycetes from fishes

S. Deepa, R. Bharathidasan and A. Panneerselvam

A. V. V. M Sri Pushpam College, Poondi, Thanjavur, India

ABSTRACT

As far as fishes of three environmental biotopes – marine (Epinephelus diacanthus [grouper]), estuarine (Oreochromis mossambicus [Tilapia]) and fresh-water (Cyprinus carpio [common carp]) are concerned , which have wider association with muddy soils. The nutrient rich diversity provided the fish with all required habitats. Here we studied the interaction or association of microbes particularly Streptomyces spp in the gut of fishes of three environmental biotopes and their ability of producing antibacterial components against human pathogen Vibrio cholerae. This proves that these extracts have the effective antibacterial components production against pathogen Vibrio cholerae. The TLC 'R_f' values also exhibited the same. It ranges from 0.40 to 0.78. The bioactive components revealed the maximal U.V absorption peaks ranging from 215 to 300nm.These strains produced either a broad spectrum antimicrobial compounds with different media or nature spectrum for specific target pathogen like, Vibrio cholera. Further investigation is needed in order to determine the structure of active components. In domestic markets, no toxic bio-fungicides and dermatogens do exist. It is a new venture for the development of Biotechnological exploration and exploitation in the years to come.

Keywords: Biotopes marine, estuarine, bioactive compounds.

INTRODUCTION

For thousands of years, natural products have played an important role throughout the world in treating and preventing human diseases. Natural product medicines have come from various source materials including terrestrial plants, terrestrial microorganisms, marine organisms and terrestrial vertebrates and invertebrates (Newman *et al*, 2000). The importance of natural products in modern medicine has been discussed in recent reviews and reports (Newman *et al*, 2003, Kochn *et al*, 2005, Paterson *et al*, 2005, Balunas *et al*, 2005, Jones *et al*, 2006).. Marine, estuarine and fresh-water organisms produce many of the pharmaceutically active natural compounds (drugs). Drugs discovery research from Marine, estuarine and fresh-water organisms has been accelerating and now involves interdisciplinary research including biochemistry, biology, ecology, organic chemistry, and pharmacology (Capon, 2001; Haefner, 2003). Natural products are the carbon compounds isolated from diverse living things. These compounds may derive from primary or rather secondary metabolism of living organisms. The primary metabolites (polysaccharides, macrolides, nucleic and fatty acids) are common in all biological systems. The secondary metabolites are, however, low molecular (MW<3000) chemically and taxonomically extremely diverse compounds with obscure function, characteristic mainly to some specific, distinct types of organisms. The practical importance of antibiotics and other secondary metabolites is tremendous. They are widely used in the human therapy, veterinary, agriculture, scientific research and in countless other areas.

About 45% of presently known bioactive microbial metabolites, over 10,000 compounds were still isolated from various actinomycetales species, 34% from *Streptomyces* and 11% from rare actinomycetes. The most frequent producers, *Streptomyces* produces 7600 compounds (74% of all actinomycetes), while the rare actino products in 1970 was only 5%.

Streptomyces is a largest antibiotic producing genus in the microbial world discovered so far. The number of anti microbial compounds reported from the species of this genus per year has increased almost exponentially for about two decades. Recent reports show that this group of microorganisms still remains an important source of antibiotics. As a result of the increasing prevalence of antibiotic resistant pathogens and the pharmacological limitations of antibiotics, there is urgency for new anti microbial substances. The result of extensive screening has been the discovery of about 4000 antibiotic substances from bacteria and fungi, many of which have found applications in medicine, most of them are produced by *Streptomyces* (Korn-Wendisch and Kutzner, 1992). Most *Streptomyces* and other actinomycetes produce a diverse array of antibiotics including amino glycosides, anthracyclins, glycopeptides, β-lactams, macrolides, nucleosides, peptides, polyenes, polyethers and tetracycline's (Good Fellow *et.al*., 1988).

Considering the above facts which leads to studies on (1) isolation and nutritional grouping of *Streptomyces* spp from gut of fishes of three environmental biotopes – marine (*Epinephelus diacanthus* [Grouper]), estuarine (*Oreochromis mossambicus* [Tilapia]) and fresh-water (*Cyprinus carpio* [Common carp]),and (2) their antibiogram against *V.cholerae* and its antibiotic spectrum.

MATERIALS AND METHODS

The present study was aimed at to isolate, enumerate the nutritional grouping of *Streptomyces spp* from the gut of fishes includes of three environmental biotopes – marine (*Epinephelus diacanthus*[grouper]) Vizhinjam, estuarine (*Oreochromis mossambicus* [Tilapia]) Veli lake and fresh-water (*Cyprinus carpio* [Common carp]) CARE (Centre for Aquatic and Research Extension). Then the samples were transported to the laboratory within the minimum possible time to avoid the external microbial contamination. After transportation to the laboratory fish gut were removed. Normally for nutritional grouping of microorganisms they use basal media for isolation but instead of that used selective media like Glycerol asparagine agar and some additional growth factors like amino acids, vitamins, yeast extract, sediment extract, for enrichment as well as isolation of more number of *Streptomyces* colonies.

Nutritional Grouping of *Streptomyces spp*

The selective media, Glycerol asparagine agar, which were enriched with amino acids, vitamins, yeast extract, and sediment extract. Totally 7 different media were prepared.

Enumeration and Maintenance of Cultures

In each selective media, numbers of colonies of *Streptomyces spp* were found. There selective colonies of *Streptomyces* species are sub cultured in slants by using Glycerol asparagine agar medium enriched with nutrients. Later they were kept in refrigerator (4^0C) till further analysis was to be carried out.

Characterization of selected *Streptomyces spp*

The characterization of the selected *Streptomyces spp* were carried out according to the methods employed by collaborator in International *Streptomyces* Project (ISP) (Shirling and Gottlieb, 1966)

Antibacterial Activity of Selected *Streptomyces spp*

A loop full of *Streptomyces* strains was inoculated into Glycerol asparagines broth and incubated for 28^0C at 120h done in shaker. Then it was centrifuged at 5000 rpm for 15 minutes and the cells are separated. 2ml of the cells was transferred to fermentation broth (100ml) and incubated at 28^0C for 120h in shaker at 105 rpm. After growth it was centrifuged at 10,000 rpm for 20 minutes to separate the mycelial biomass. Then the supernatant was mixed with ethyl acetate, hexane, and toluene in 1:1 proportion. Then it was shaken for 2hrs in shaker and transferred to separating funnel, solvent was separated. Again it was centrifuged at 5000 rpm for 15 minutes to remove the traces from fermentation broth. Then the solvent was dried in water bath of 80-90^0C and residue was weighed. The residue is mixed and concentrated with little ethanol. Then it was impregnated with filter paper disk (6mm diameter) and dried. Then it was placed in microbes cultivated plates and kept in 37^0C for 48h. Inhibition zone was noted and measured.

TLC Analysis of Antibacterial compounds

Quantitative analysis of antibacterial compounds in the experimental sample was carried out by using Thin Layer Chromatography (TLC). Then, applying slurry made by silica gel G for TLC grade and applied over the glass slides, TLC slides were made. This was dried at 60°C for an hour of period. The dried slides were pre-activation base was drawn on the TLC slides 1.0 cm away from the base line on the portion of the TLC slides. The distances traveled by each spot in base line and relative R_f values were calculated.

$$R_f = \frac{\text{Distance traveled by the solute}}{\text{Distance traveled by the solvent}}$$

Spectral Analysis

The residue mixed and diluted with ethanol was taken for spectral analysis at 200-400nm using UV spectrophotometer.

RESULTS AND DISCUSSION

The present investigation was an attempt to understand the distribution pattern of *Streptomyces spp* in the micro-environment of gut regions of fishes of three environmental biotopes – marine (*Epinephelus diacanthus* [grouper]), estuarine (*Oreochromis mossambicus* [Tilapia]) and fresh-water (*Cyprinus carpio* [common carp]). The primary isolation of *Streptomyces* was carried out under selective media like Glycerol asparagine agar (Dhevendaran and Annie, 1999a). This is the first time by choosing selective media instead of basal media and other six nutritional enriched media for isolation of *Streptomyces* was attempted successively. Although the nutritional classification of terrestrial bacteria has been established (Lochhead, 1952; Rouaft, 1968) a systematic approach to understand.

Tables 1,2 and 3 showed the number of microbial colonies in selective media from gut of fishes of three environmental biotopes – marine (*Epinephelus diacanthus*[grouper]), estuarine (*Oreochromis mossambicus* [Tilapia]) and fresh-water (*Cyprinus carpio* [common carp]). In that bacterial and fungal colonies were minimum in numbers because of these selective media (Glycerol asparagine agar) have glycerol in the medium which inhibit the growth of bacterial and fungal population (Zobel, 1946; 1963). But maximal growths of *Streptomyces spp* were found particularly in the selective media enriched with amino acids and vitamins. This maximal occurrence of *Streptomyces* colonies inhibited the growth of bacteria because it has already been proved that marine *Streptomyces* synthesized antibiotics, anticancer agents, L-asparaginase enzyme as reported earlier (Nishino *et al*., 1999).

Tables 4,showed mycelial colour characteristics of selected strains of *Streptomyces* in different selective media like Glycerol Asparagine agar. The colouration pattern of aerial and substrate mycelia were totally different in each media with enrichment this may be due to that the enriched media provides certain nutrients for triggering of genes for the conversion as well as expression of other metabolic products. Which in turn lead to different aerial and substrate mycelial colourations. This colouration difference may be due to other secondary metabolites production provided by the enriched media (Dhevendaran and Annie, 1999; Dhevendaran *et al., 2004).* These secondary metabolites of different colouration are the rich source of certain compounds like amino acids, sugars, fatty acids, terpenes,etc.

Table 7 exhibited the antibacterial activity. The antibacterial activities of some *Streptomyces* strains were found to be active against test organism like *Vibrio cholerae*. The maximum inhibition zone (5-8mm) occurred in strains obtained from gut of fishes. Then TLC was carried out Table 8 for the ethyl acetate, hexane, and petroleum ether extract samples of the selected strains. The R_f values ranged from 0.40 to 0.78. The similar results were observed by Illic *et al* (2005) their bioactive regions were detected on TLC plates and the R_f values were ranges from 0.70 to 0.88.

The U.V spectral data for the ethanol extract of selected active strains from fermented broth are shown in Table 9. Maximum absorbance peaks ranged from 215 to 300nm. The range of peaks was observed from 200 to 400nm and the characteristics of absorption peaks indicate a highly polygene nature. These strains produced either a broad spectrum antibacterial compound or several compounds with different activities. The spectral data are consistent with those obtained by Swaadoun *et al* (1999). It is quite obvious that the *Streptomyces spp* are inherent in marine ornamental fish, synthesizing commercially valuable bio active compounds.

TABLE-1 -Number of *Streptomyces* colonies obtained after Nutritional grouping of from gut of marine fish (*Epinephelus diacanthus*[Dot Grouper]).

Strains	Number of Colonies	Selected strains
GA + G	4	1
GA + V	7	1
GA + S	5	1
GA + Y	4	1
GA + Y + S	8	2
GA + AA + V	9	1

- GA-Glycerol asparagine agar, G- Control, AA- Amino acid, V- Vitamins, S- Sediment extract, Y - Yeast extract.

TABLE-2 -Number of *Streptomyces* colonies obtained after Nutritional grouping of from gut of estuarine fish (*Oreochromis mossambicus* [Tilapia]).

Strains	Number of Colonies	Selected strains
GA + T	9	1
GA + V	7	1
GA + AA + V	6	2
GA + Y	9	1
GA + Y + S	7	1
GA + S	6	1
GA +AA	5	1

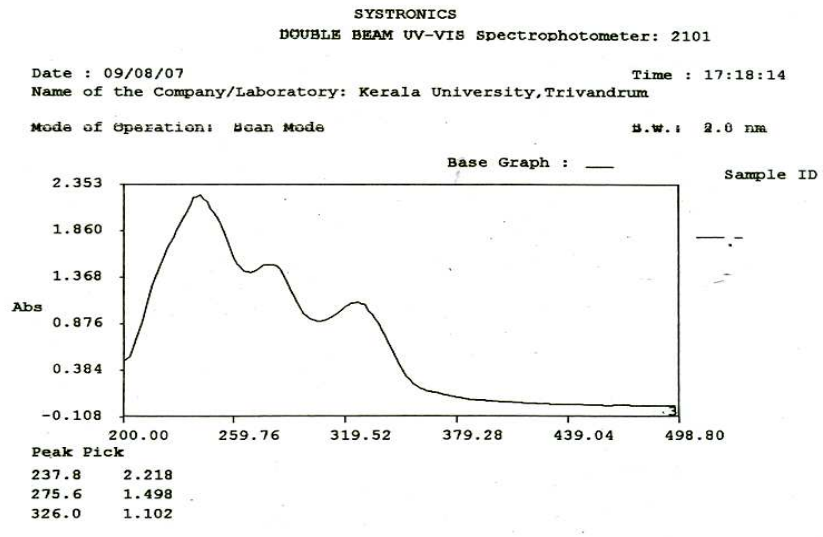
GA- Glycerol asparagine agar, T- Control, AA- Amino acid, V- Vitamins, S- Sediment extract, Y - Yeast extract.

TABLE-3 - Number of *Streptomyces* colonies obtained after Nutritional grouping of from gut of fresh-water (*Cyprinus carpio* [Common carp]).

Strains	Number of Colonies	Selected strains
GA + Y	5	1
GA + AA	4	1
GA + S	7	1
GA + AA + V	5	1
GA + Y	4	1
GA + Y + S	7	2
GA + Y_1	3	\1

GA- Glycerol asparagine agar, Y- Control, AA- Amino acid, V- Vitamins, S- Sediment extract, Y - Yeast extract.

FIG- 1 - U.V. Spectral analysis of antibacterial components isolated from *Streptomyces spp* associated in gut of marine fish (*Epinephelus diacanthus*[Dot Grouper]).



1. GA + G

Table 4 -Qualitative analysis of antibacterial components isolated from selected *Streptomyces spp* associated in gut of 1)marine (*Epinephelus diacanthus*[grouper]), 2)estuarine (*Oreochromis mossambicus* [Tilapia]) and 3)fresh-water (*Cyprinus carpio* [common carp]) fishes by using TLC

Strains of fish 1	R_f values
GA + G	0.46 0.40
GA + V	0.77
GA + S	0.45 0.41
GA + Y	0.54 0.50
GA + Y + S **a)** **b)**	 0.60 0.51 0.67
GA + AA + V	0.61

Strains of fish 2	R_f values
GA + T	0.63 0.69
GA + V	0.68
GA + AA + V **a)** **b)**	 0.70 0.80
GA + Y	0.77
GA + Y + S	0.67
GA + S	0.78
GA +AA	0.61 0.69

Strains of fish 3	R_f values
GA + Y	0.61
GA + AA	0.51
GA + S	0.76 0.49
GA + AA + V	0.51 0.46
GA + Y	0.53 0.46
GA + Y + S **a)** **b)**	 0.65 0.77
GA + Y_1	0.69

GA- Glycerol asparagine agar, G, T, Y- Control, AA- Amino acid, V- Vitamins, S- Sediment extract, Y - Yeast extract

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