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Characterization of Carotenoids from *Streptomyces* sp. of marine and fresh water environment

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ABSTRACT

In the present study characterization of carotenoids from selected strains of Streptomyces sp. of Tendania anhelan, Epinephelus diacanthus and Cyprinus carpio, and Osepherenemous gourami were observed. The interaction of microbes particularly Streptomyces sp. in the gut and sponges of fishes in two environmental biotopes and their ability of producing carotenoids were assessed. The UV spectrum, TLC and HPLC results showed the biosynthesis of carotenoids in various strains of Streptomyces sp., clearly indicate the presence of phytoene, phytofluene, α-carotene, β-carotene and β-isorenieratene. Carotenoids are also important due to their potential antitumor properties and because they are used as colorants in the food industry to pigment salmon, trout and poultry flesh, or to intensify the colour of egg yolk. These pigments are not essential for other carotene containing microorganisms, as is the case for fungi but they are indeed very important due to their ability to act as antioxidant agents.

INTRODUCTION

Carotenoids are pigmented compound that are widely produced by both eukaryotes and prokaryotes (Goodwin, 1980)[1]. They are synthesized by plants, algae and by some fungi and bacteria. They are involved in photosynthesis as accessory pigments, functioning as antioxidants light protection pigments and membrane stabilizers over 600 different carotenoids are known at present (Goodwin, 1980)[1]. Most consist of 40 carbon atoms. Carotenoids are lipophilic pigments with a yellow or red colour. They are essential in photoautotrophic organisms, where they are located in the thylakoid membrane participating in the light harvesting process (Siefermann Harms, 1987) [2] as well as for protection against photooxidative damage (Krinjsky, 1979)[3].

Carotenoids are essential for organisms with oxygenic photosynthesis (plants, algae, cyanobacteria) because of their protective role, which consist of both depleting the energy from chlorophyll and accepting it from other molecules such as the reactive forms of oxygen. These pigments are not essential for other carotene – containing microorganisms as is the case for fungi, but they are indeed very important due to their ability to act as antioxidant agents (Britton, 1983, Goodwin, 1986)[4,5] .

The carotenoids represent one of the most widely distributed and structurally diverse classes of natural pigments, producing light yellow to orange to deep red, colours. Eye catching example includes lycopene from tomatoes, β-carotene from carrots, and lutein from marigolds. In addition to synthesis in photosynthetic organisms carotenoids are also synthesize in some bacteria and fungi (Sandmann, 1994)[6]. These pigments have important functions in photosynthesis, nutrition, and protection against photooxidative damage.

Streptomyces sp. are chemoheterotrophic soil bacteria belonging to the order *Actinomycetales*. *Streptomyces* sp. are saprophytic filamentous gram positive bacteria inhabiting particulate soil ecosystem and marine sediments throughout the world. Secondary metabolites from *Streptomyces* sp. contribute major products for pharmaceutical uses.

Streptomyces sp. are considered exceptionally well endowed for "chemical warfare" presumably allowing them to eliminate bacterial and fungal competitors in soil ecosystem. Although thousands of antibiotics have been isolated from *Streptomyces* sp. this are thought to represent only a small fraction of the repertoire of bioactive compound (Gilard *et al.*, 1960, Broome, 1961)[7,8].

MATERIALS AND METHODS

Sample Collection

In the present investigation *Streptomyces* sp. population were isolated from gut of Marine Sponges (*Tendania anhelans*), Grouper *(Epinephelus diacanthus*) and Fresh water [*Cyprinus carpto* (common carp), *Ospherenemous gourami* (Gourami)] environment.

Isolation (Dhevendran and Annie, 1999)[9]

The gut of the fishes and the tissue of sponges were removed aseptically and transferred into the sterile flask and the serial dilution was carried out independently for each sample. 1ml of the sample from appropriate dilution was pipette out into the sterile petridish. 15ml of the Glycerol Asparagine agar medium were poured into the same petridish and mixed thoroughly by rotating the petridish both clockwise and anticlockwise directions and then incubated at room temperature (28±2°C) for 20 days.

Colour determination from different media

The media used for the colour determinations were Nutrient agar, Tryptophan medium, Actinomyces agar, Glucose yeast extract agar and *Streptomyces* medium. Microplates were prepared and observed every day .

Extraction and Separation of Carotenoids

Carotenoid Extraction

To the pre-weighed samples of about 5gms added with cold acetone solvent and homogenized well. Then the solvent petroleum ether was taken in a separating flask (500ml) of about 50ml and then acetone extract samples were transferred. The distilled water was slowly added along the sides of the separating flask (300ml). The two phases were getting separated. Collected the upper phase that contains petroleum ether and carotenoids. Then it was stored at 20°C.

Quantification of various

Carotenoids

Spectroscopic Analysis of Carotenoids

The samples extracted with the petroleum ether solvent were scanned between 350 and 500nm using UV- Vis Scan Spectrophotometer and it shows individual peaks for respective carotenoids with their optical densities.

Estimation of total Carotenoid

The extracted carotenoid from each experimental sample was diluted to approximate volume as to be obtaining the optical density value for that the solvent used for the carotenoid extraction were used. After proper dilution, the optical density was measured at 400-500nm. Total carotenoid in the sample was then estimated by using the formula given below.

$$\text{Total carotenoid content } (\mu g/g) = \frac{A \times \text{Volume (ml)} \times 10^4}{A^{1\%}\ 1\text{cm} \times \text{sample weight (g)}}$$

Qualitative Estimation of Carotenoid

TLC Analysis of Carotenoids

Qualitative analysis of carotenoid 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 plate, TLC plates were made. This was dried at 60°C for an hour of period. The dried plates were pre-activation base line.

After that, 3µl condensed carotenoid samples were spotted on the baseline of the TLC plates at 1.0cm interval and then allowed to dry at room temperature. Often the sample applied on TLC plates was placed in a presaturated TLC chamber contains mobile phase.(5% Methanol / Toluene in the ratio of 95.5 9v/v). Then the chromatogram was developed by providing the dark environment up to a distance of 15cm mark. Then the plate was taken out dried for few min. Using UV light torch, the developed spots were seen and taken out and marked. The distance travelled by each spot in baseline and relative R_f values were calculated. By comparing the standard R_f values for the chosen mobile phase, the carotenoids present in the samples were identified.

$$R_f = \frac{\text{Distance travelled by the substance}}{\text{Distance travelled by the solvent}}$$

HPLC analysis of Carotenoids

To the pre-weighed samples of about 5gms added with cold acetone solvent and homogenized well. Then the solvent petroleum ether was taken in a separating flask (500ml) of about 50ml and then acetone extract samples were transferred. The distilled water was slowly added along the sides of the separating flask (300ml) and observed the two phases getting separated. Collected the upper phase that contains petroleum ether and carotenoids. Concentrated the carotenoid solution at 35°C in rotary evaporator. Then it was re-dissolved in HPLC grade acetone solvent. As far as HPLC chromatographic condition C_{18} column is needed with mobile phase of mixture Acetonitrile: Methanol: Ethyl acetate (80:10:10) and at the flow rate of 1ml/ minute and it was observed in UV detector.

RESULTS AND DISCUSSION

The present investigation was an attempt to understand the distribution pattern of *Streptomyces* sp. from gut regions of fishes of two environment such as marine and freshwater regions. In marine environment, 2 *Streptomyces* sp. were isolated and noted as AQB-S and AQB Gr. In freshwater environment, 2 *Streptomyces* sp. were noted as AQB-C and AQB – G.

The coloration pattern of aerial and substrate mycelium were observed on four different media. In nutrient agar medium, the isolates from aquatic sponges and Gruper shows white in colour, common carp shows white but in Gourami, shows white color in substrate mycelium brown in aerial mycelium. In actinomyces agar medium all the four isolates were produced yellow color. In tryptophan medium AQB-S, AQB Gr and AQB C isolates producing white colour colonies but, AQB-G isolate show brown color. In Streptomyces agar medium, all the isolates producing white colour except, AQB Gr in aerial mycelium. Dhevendran and Annie (1999)[9] isolated the *Streptomyces* sp. was carried out under selective media like Glycerol asparagine agar. In that bacterial and fungal colonies were minimum in numbers because of these selective medium (Glycerol asparagine agar), which inhibits the growth of bacterial and fungal population. The occurrence of *Streptomyces* sp. colonies inhibited the growth of bacteria because it has already been proved that marine *Streptomyces* sp. synthesized antibiotics, anticancer agents, was reported by Nishino *et al.,* 1991, Dhevendran and Annie (1999a) [9].

Carotenoid estimation was analyzed by three different ways such as UV spectral analysis. Thin layer chromatography (TLC) and High Performance Liquid Chromatography (HPLC). In UV visual spectrum analysis, AQB-C samples having highest β carotene is (4.88 µg/100g), In AQB – G sample the ratio of β-Isorenineratene is(3.07 µg/100g) – (Fig 1 a,b,c).

Kruqel *et al.,* (1999)[10] reported that the aromatic carotenoid was found in some *Streptomyces* sp. The biosynthesis of the aromatic carotene isorenieratene is restricted to green photo synthetic bacteria and a few actinomycetes. Among them *Streptomyces griseus* has been used to study the genes involved in this pathway. Five genes out of seven of two adjacent operons in one duster could be identified to be sufficient for the synthesis of isorenieratene. The total carotenoids was found to be maximum in *Streptomyces* sp. isolated from gut of carp fish and least accumulation was found to be in *Streptomyces* sp. isolated from gut of Tilapia fish. In marine groups fish gut, isolated *Streptomyces* sp. α-carotene was found in TLC analysis. Phytoene, β-carotene were and β-isorenieratene analysed by comparing standard Rf values. Each strain was similar pattern of

spectral data (Table 1). The TLC results showed the presence of various other carotenoids other than β-carotene. This was mainly because other carotenoids (Phytoene, phytofluene) were present in UV region not in visible region. These carotenoids picture cannot be shown because the visibility is only through UV light detector since the sample carotenoids concentration is less. These results very well correlated with the results.

As far as HPLC analysis is concerned it clearly proved the presence of these carotenoids which were earlier identified through UV spectrometer and TLC in contrast to *Streptomyces mediolani* where 3-hydroxy and 3, 3p-dihydroxy derivatives of isorenieratene one formed the carotenoeginc pathway of *Streptomyces* sp. ends with the synthesis of isorenieratene. The carotenoids include phytoene, phytofluene, α-carotene and β-carotene. In *Streptomyces* sp. carotenoid production is a widespread metabolic activity, which occurs in a constitutive, light-dependent, or cryptic manner was reported by Koyama *et al.*, 1976[11].

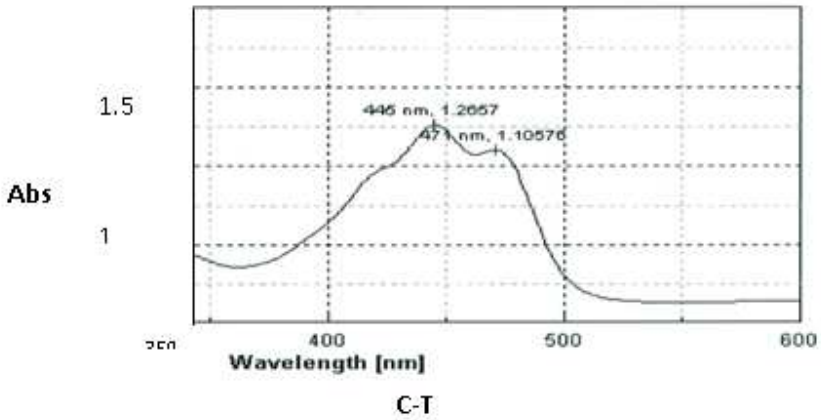
In this study HPLC analysis, phytoene, β-carotene, phytofluene and β-isorenieratene were analysed and compared with standard Rf values in all samples phytoene shows highest ratio. (Fig 2a,b,c,d). This implies the presence of a certain diversity in the molecular mechanism of carotenoid production in this group of bacteria.

Table 1- TLC analysis of selected strains of *Streptomyces* sp.

S. no	Samples	Rf values	Carotenoids
1	**AQB-S**	0.99 0.93	Phytoene, β-Carotene
2	**AQB-Gr**	0.91	α-Carotene
3	**AQB-C**	0.99 0.93	Phytoene, β - Carotene
4	**AQB-G**	0.99 0.95	Phytoene, β -Isorenieratene

S- Sponges, Gr – Grouper, C – Common Carp, G – Gourami, AQB – Aquatic Biology.

Figure 1 UV spectral analysis of Carotenoids from *Streptomyces* sp.



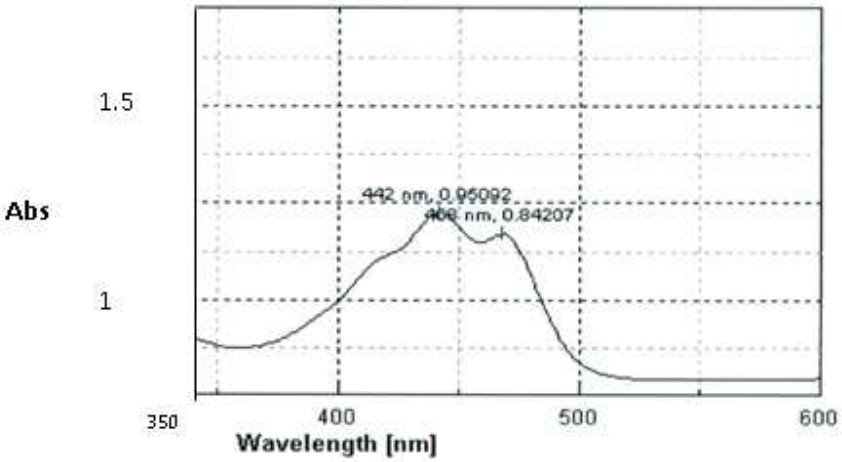

1.5
Abs
1
442 nm, 0.95092
406 nm, 0.84207
350
400
500
600
Wavelength [nm]
Gr- T

Fig. 1(b)

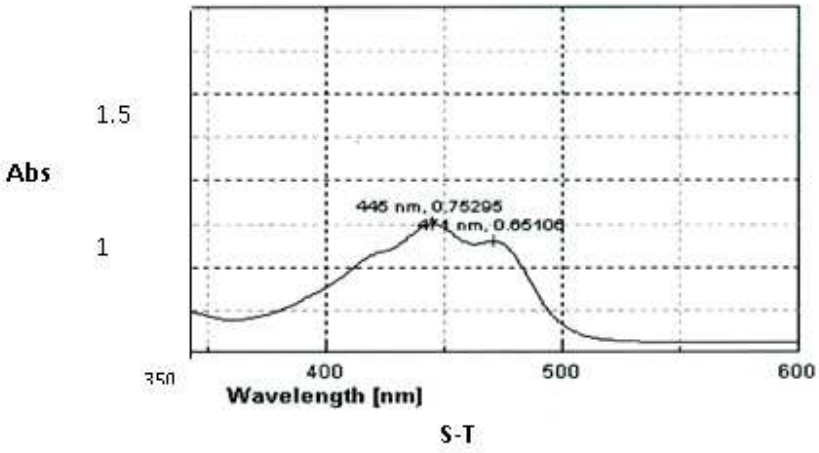

1.5
Abs
1
446 nm, 0.75295
414 nm, 0.65106
350
400
500
600
Wavelength [nm]
S-T

Fig 1 (c)

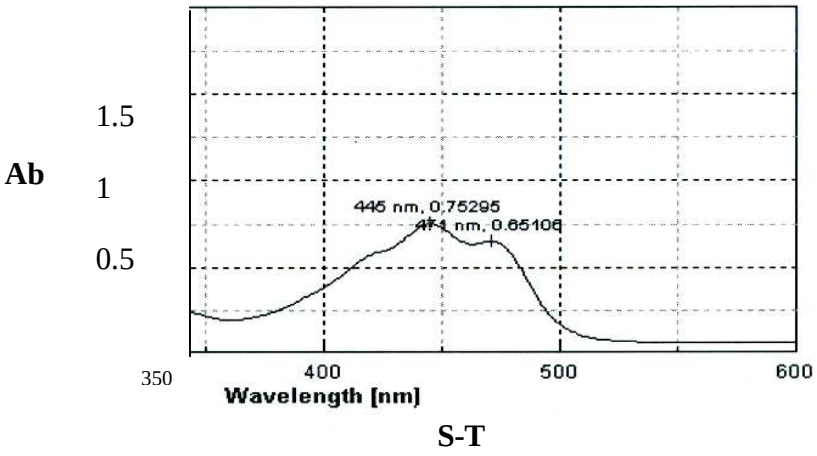

Ab
1.5
1
0.5
446 nm, 0.75295
414 nm, 0.65106
350
400
500
600
Wavelength [nm]
S-T

Figure 2 (a) HPLC analysis of Carotenoids from *Streptomyces* sp.

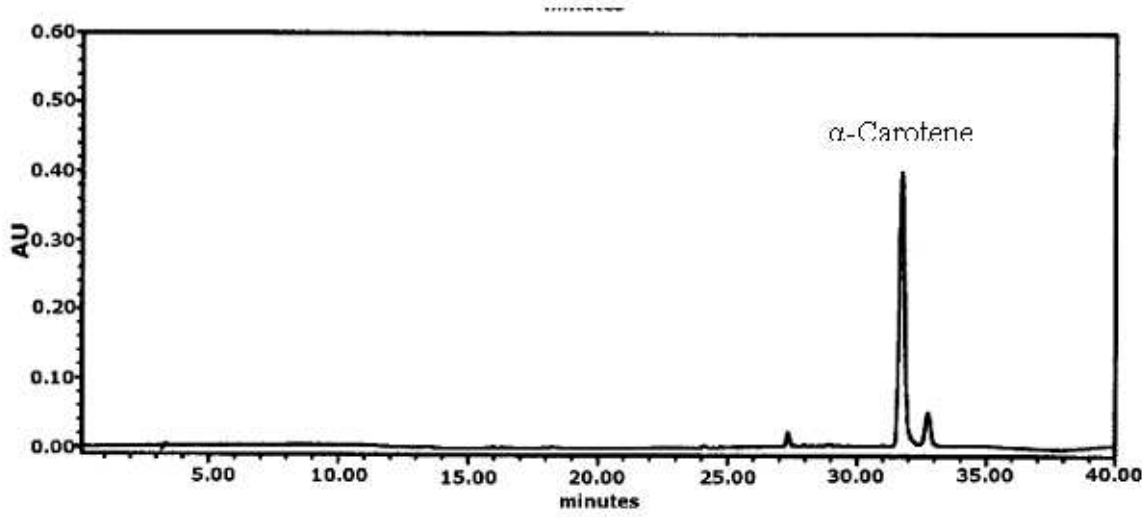


Fig 2 (b)

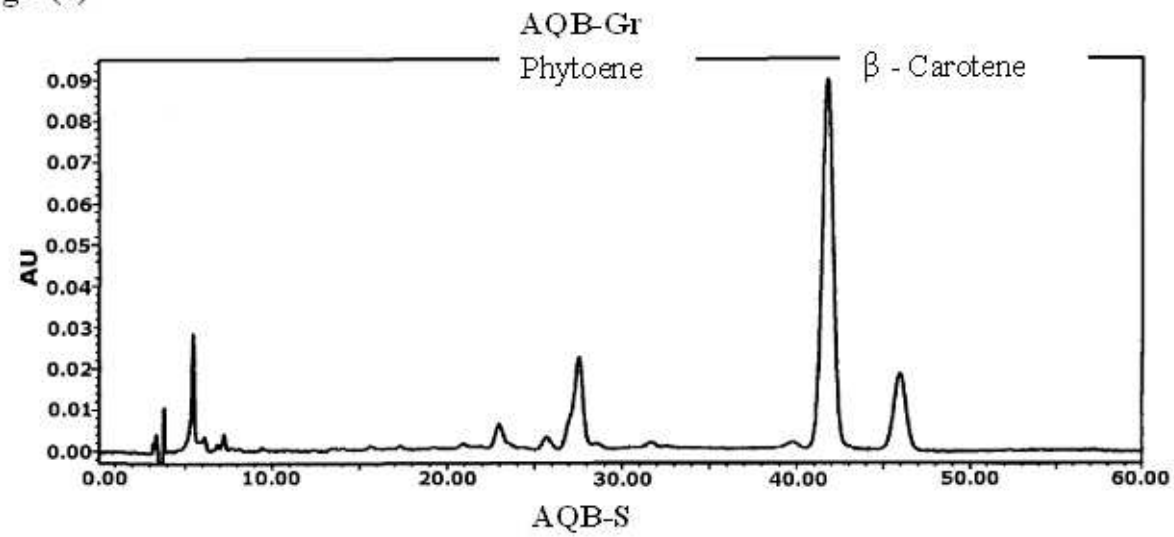
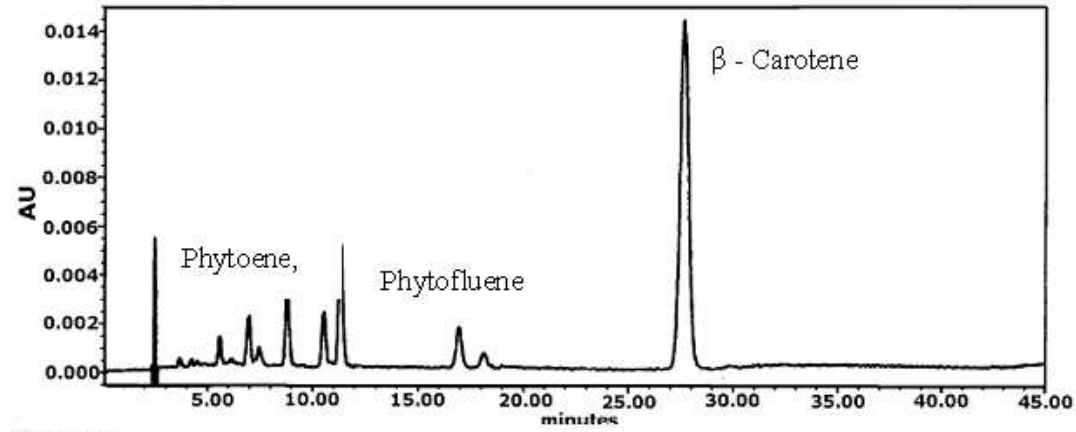
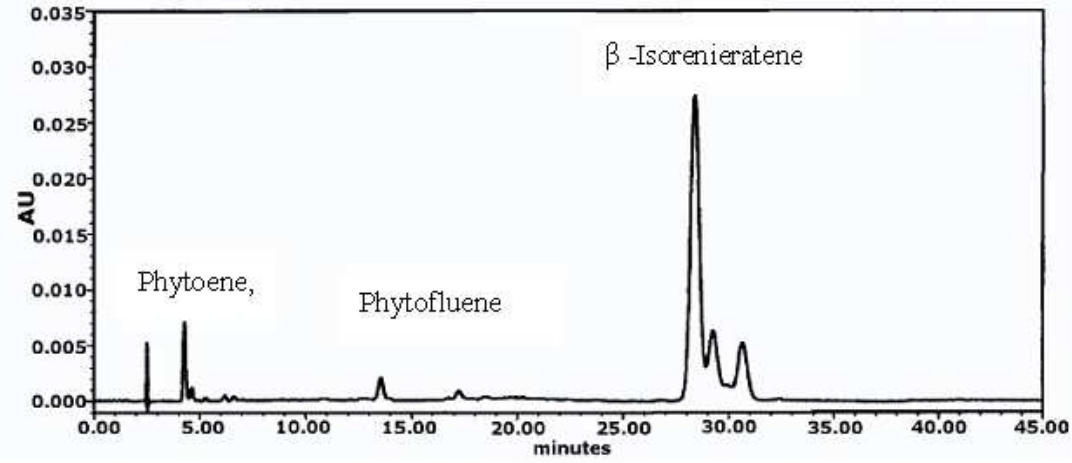


Fig 2 (c)



AQB-C

Fig 2 (d)



AQB-G

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