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Antagonistic Potential of *Streptomyces* Associated with the Gut of Marine Ornamental Fishes

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Abstract: Nearly 87 isolates of *Streptomyces* were found to be associated with the gut of marine ornamental fishes namely *Chaetodon collare* (Red tail butterfly) and *Archamia fucata* (Orange-lined cardinal). Among them, only seven strains showed bioactivity against *Vibrio cholerae*. The seven strains were characterized by conventional methods and the studies strongly suggested the strains belong to the genus *Streptomyces* sp. The culture extracts of the seven strains were initially screened for antibacterial activity by spot inoculation method which exhibited more than 10 mm of inhibition zone against *Vibrio cholerae*. *In vitro* screening of the submerge culture extracts showed more than 10 to 30 mm of inhibition zone against *Vibrio cholerae*. The production of bioactive substances from the active extracts was confirmed by UV spectral analysis by the absorbance peaks that ranged from 239 to 326 nm and the TLC (Rf values) ranging from 0.46 to 0.78. The results indicated that *Streptomyces* strains isolated from gut of marine ornamental fishes produced potential bioactive metabolites against *Vibrio cholerae*.

Key words: Marine ornamental fishes · Fish gut extract · Marine actinomycetes · *Streptomyces* · Bioactive compounds

INTRODUCTION

Marine organisms produce many of the pharmaceutically active natural compounds (drugs). The practical importance of antibiotics and other secondary metabolites is tremendous. They are widely used in human therapy, veterinary field, agriculture, scientific research and in countless other areas. Natural product medicines have come from various source materials including terrestrial plants, terrestrial microorganisms, marine organisms and terrestrial vertebrates and invertebrates [1]. Microbial sources are one among them synthesizing antibiotics and other secondary metabolites. Most characteristic features of secondary metabolites are their incredible array of unique chemical structures, very frequent occurrence and versatile bioactivities. Various antibiotics were mainly isolated from bacteria of different species. The search for new bioactive substances has been remarkably successful and approximately two third of naturally occurring antibiotics including many medical importance have been isolated from actinomycetes and the majority from the genus *Streptomyces* [2]. The isolation of actinomycetes from marine environments has been a fruitful area of research in the past decade. However, little is known about the diversity of actinomycetes from marine samples compared to the diverse range of actinomycetes isolated from terrestrial environments [3].

Many marine actinomycetes taxon in ocean sediments were found to have widespread, persistent populations in ocean systems and some taxa have no counterparts in terrestrial environments**.** It is becoming evident that marine habitats are an abundant source of actinomycetes for discovering natural products. Many promising bioactive compounds including antimicrobial, antitumour, immunosuppressive agents and enzymes are being discovered from marine actinomycetes [4]. The antibiotics produced are entirely new and unique when compared to those from the terrestrial ones. Most of the actinomycetes particularly *Streptomyces* from marine environment were isolated from sediments, seawater, seaweeds, mangroves, mollusks and invertebrates [5-10]. In the present study, an attempt was made to isolate *Streptomyces* strains from the gut of marine ornamental fishes and tested for their antagonistic potential against *Vibrio cholerae*.

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MATERIALS AND METHODS

Sample Collection: In the present study, marine ornamental fishes like *Chaetodon collare* (Red tail butterfly) and *Archamia fucata* (Orange-lined cardinal) were collected by Self contained underwater breathing apparatus (SCUBA) diving from Vizhinjam port, situated on the Southwest coast of Kerala about 16 Kilometres to the South of Trivandrum at 8°22' 30" N latitude and 76°59' 16"E longitude in India. The work was conducted in the Department of Aquatic Biology and Fisheries, University of Kerala, Trivandrum, India, during January-April 2009. Fishes were transported to the laboratory within the minimum possible time to avoid the external microbial contamination and excessive proliferation.

Actinomycetes Isolation: After transportation to the laboratory, fish gut were removed and homogenized with sterile water. Portions of one millilitre from each fish gut homogenized samples were subjected to a dilution of 10^{-2}. Later a quantity of one millilitre of the dilutions was mixed with 20 ml of culture medium containing sterilized glycerol asparagine agar medium (selective media) and incubated at room temperature (28 ±2°C) for seven days. The media were amended with rifampicin (2.5 µg/ml) and amphotericin B (75 µg/ml) to inhibit bacterial and fungal contamination [11].

Enumeration and Maintenance of Cultures: The *Streptomyces* strains isolated were maintained on glycerol asparagine agar slant cultures at 28±2°C [12]. The inoculum age used in all experiments was of seven days old cultures, unless otherwise stated.

Characterisation of the *Streptomyces* Isolates: The strains were preliminarily characterised by the method of International *Streptomyces* Project (ISP) [13]. The microorganisms were characterised by acid-fast staining and Gram's staining techniques. The isolates were also studied by employing various parameters which are detailed below.

Pigmentation of Mycelia and Spore Morphology: The cultures were grown on a Petri dish containing casein-starch-peptone-yeast extract (CSPY) agar medium with a cover slip inserted at an angle of 45°. The cover slip was removed after 7 days of incubation, air dried and observed under scanning electron microscope [14].

Utilisation of Carbon Sources: The cultures were inoculated to test tubes containing 10 ml of basal mineral salt medium to which sterilised carbon sources (xylose, arabinose, rhamnose, fructose, galactose, raffinose, mannitol, inositol, sucrose, glucose) were added to a final concentration of 1%. The tubes were incubated at 28°C and after 7 days the growth of the cultures were observed. Glucose was used as positive control.

Influence of Amino Acids: Various amino acids, namely glycine, cystine, alanine, tryptophan, and valine, were added at a concentration of 0.1% each to 5 mL of basal mineral salt medium. The medium was inoculated with the cultures and incubated at 28°C for 7 days. The obtained biomass as separated from the broth, dried and weighed. The dry weight of the biomass was expressed in grams.

Sodium Chloride Tolerance: Sodium chloride at various concentrations (1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 9 and 10%) was added to 5 mL of the basal medium. The medium was inoculated with the cultures and incubated at 28°C for 7 days. The biomass thus obtained was separated from the broth, dried and weighed. The dry weight of the biomass was expressed in grams.

Physiological and Biochemical Characteristics: were studied according to the procedures previously described [15-16].

Anti Microbial Assay

Spot Inoculation Method: Preliminary screenings for antibiotic production were carried out by earlier described method [17]. The strains were spot inoculated in glycerol asparagine medium for seven days. After seven days one ml of chloroform were added and made to stand for 40 minutes to arrest the growth of inoculated colonies and then they were over laid with 5ml of sloppy agar (0.6%) layer of nutrient agar medium previously seeded with the test organisms (*Vibrio cholerae*). They were then incubated for 24 hours at 37°C and the diameter of the inhibition zone was measured in millimetres.

***In vitro* Screening of Isolates for Antimicrobial Activity (Disc Metod):** Isolates that showed activity against the test microorganisms were inoculated in a submerged culture of 500 ml Erlenmeyer flasks containing 100 ml of the liquid medium (0.8g NaCl, 1g NH_4Cl, 0.1g KCl, 0.1g KH_2PO_4, 0.2g $MgSO_4.7H_2O$, 0.04g $CaCl_2.2H_2O$, 2g glucose, 3g yeast extract in one litre of distilled water, pH 7.3).

These cultures were grown in a rotary shaker at 200 rpm, 28°C for 120 hours under the standard condition of aeration and agitation. The resultant grownup cultures were centrifuged at 6000 rpm for 15 minutes. The culture filtrates were solvent extracted with n-butanol (1:1) in the separating funnel and shaken vigorously for 20 minutes. The upper organic layers were collected and evaporated to dryness in a vacuum evaporator at 40°C. A crude gummy extract thus obtained was weighed. The crude extracts were resuspended in ethanol at concentration of 1mg per ml for antimicrobial studies. Sterile filter paper discs 6mm in diameter (Hi-Media, India) were impregnated with 50 µl (50 µg crude antibiotic) suspension, dried and placed onto the plates previously seeded with the test microorganism (*Vibrio cholerae*). Then the plates were kept at 4°C for at least two hours to allow the diffusion of crude extracts. Then they were incubated for 24 hours at 37°C and the diameters of inhibition zone were measured [18].

Screening of Bioactive Substances from the Submerged Culture Extracts: Bioactive compounds were recovered from the culture filtrates by solvent extraction with n-butanol in the ratio 1:1 (v/v) and shaking for 1 hour [19]. The butanol phase was separated and evaporated to dryness in water bath at 80-90°C and the residue was weighed and redissolved with little volume of ethanol. The absorption spectrum was determined in UV and Visible region (200-600 nm) by using UV/VIS spectrophotometer 2101 (Systronics).

Thin Layer Chromatographic Analysis of Antibacterial Compounds: The extracts were spotted on the baseline of the silica gel plates (60 F_{254}, Merck) (stationary phase) at 1 cm and then allowed to dry at room temperature [20]. The plates were placed in TLC chamber pre-saturated with the mobile phase butanol: acetic acid: water (4:1:2). The chromatogram was developed and visualized under UV light and the spots were marked. The R_f values for each spot was measured.

RESULTS AND DISCUSSION

In the present investigation, an attempt was made to understand the distribution pattern of *Streptomyces* in the micro-environment of gut regions of marine ornamental fishes and their antibacterial activity against *Vibrio cholerae* were assessed. The marine ornamental fishes that selected include *Chaetodon collare* (Red tail butterfly) and *Archamia fucata* (Orange-lined cardinal) were collected from Vizhinjam port situated on the south west coast of Kerala, India. Primarily the fish gut was removed and the extracts of them were prepared for the isolation of *Streptomyces* using selective media, glycerol asparagine agar. Nearly 87 cultures of *Streptomyces* were isolated from the gut of the two marine ornamental fishes which are shown in Table 1. Similar isolation and screening of *Streptomyces* from the gut of estuarine fish and shell fish of India were reported earlier [21].

Among the 87 isolates, seven strains were selected based on their bioactivity against *Vibrio cholerae* for the preliminary characterization by the methods recommended by International *Streptomyces* Project (ISP). The colonies were slow growing, chalky, folded and aerobic. Aerial mycelia colour pattern of the strains were found to be white and grey and substrate mycelial colour were totally different for the strains. All the strains were acid-fast negative and found to be Gram-positive. In an observation using the scanning electron microscope, spore morphology showed smooth spore surface and rectiflexibiles (RF) hyphae (Table 2).

Table 1: Number of *Streptomyces* colonies associated with gut of marine ornamental fishes

Origin of isolates associated with gut of marine ornamental fishes	Total number of strains isolated
Chaetodon collare (Red tail butterfly)	62
Archamia fucata (Orange-lined cardinal)	25
Total	87

Table 2: Characterization and carbon utilization of *Streptomyces* strains (+ Positive results, - Negative results, ± Doubtful results, RF-Rectiflexibilis, S-Smooth)

Streptomyces strains	Color of aerial mycelium	Color of vegetative mycelium	Spore surface	Spore chain	No carbon source (negative control)	D-xylose	L-arabinose	Rhamnose	D-fructose	D-galactose	Raffinose	D-mannitol	Inositol	Sucrose	D-glucose (positive control)
AQBCC06	White	Red	RF	S	-	+	-	+	-	-	-	+	+	-	+
AQBCC20	Grey	Yellow	RF	S	-	-	+	-	+	-	-	-	+	-	+
AQBCC24	White	Pale yellow	RF	S	-	+	-	+	+	+	-	+	-	±	+
AQBCC40	White	Yellow	RF	S	-	+	+	-	-	+	-	-	+	±	+
AQBAF51	Grey	White	RF	S	-	-	+	-	-	-	-	+	-	-	+
AQBAF54	Olive green	White	RF	S	-	+	+	+	+	+	-	+	+	±	+
AQBAF75	White	Light grey	RF	S	-	+	-	-	+	-	-	+	-	±	+

Table 3: Sodium chloride tolerance of the strains at varying concentrations

	Dry weight of biomass (g / 5 ml)												
	Percentage of Sodium chloride (%)												
Stains	0	1	1.5	2	2.5	3	4	5	6	7	8	9	10
AQBCC06	0.041	0.049	0.051	0.071	0.080	0.091	0.096	0.105	0.115	0.121	0.095	0.083	0.076
AQBCC20	0.027	0.036	0.048	0.079	0.085	0.101	0.109	0.117	0.125	0.131	0.113	0.098	0.081
AQBCC24	0.044	0.057	0.061	0.070	0.078	0.091	0.103	0.109	0.118	0.127	0.104	0.089	0.070
AQBCC40	0.050	0.063	0.067	0.077	0.083	0.093	0.107	0.112	0.121	0.130	0.112	0.090	0.069
AQBAF51	0.025	0.032	0.036	0.042	0.050	0.051	0.063	0.079	0.101	0.092	0.078	0.064	0.056
AQBAF54	0.019	0.025	0.031	0.033	0.060	0.064	0.075	0.087	0.104	0.089	0.071	0.059	0.054
AQBAF75	0.028	0.032	0.034	0.054	0.060	0.067	0.078	0.091	0.112	0.099	0.087	0.072	0.061

Table 4: Physiological and biochemical characteristics of *Streptomyces* strains (+ positive results, - negative results)

Parameters	AQBCC06	AQBCC20	AQBCC24	AQBCC40	AQBAF51	AQBAF54	AQBAF75
Starch hydrolysis	+	+	+	-	-	+	+
Production of H_2S	-	-	-	+	+	-	-
Degradation of cellulose	+	+	+	-	-	+	+
Liquefaction of gelatin	-	-	+	+	+	+	+
Coagulation of milk	+	-	+	+	+	+	-
Peptonization of milk	+	-	+	+	+	+	-
Degradation of urea	-	-	-	-	-	+	+
Citrate utilization	-	+	+	+	-	+	+
Indole production	-	-	+	+	+	+	+
Catalase	+	+	+	+	-	-	-

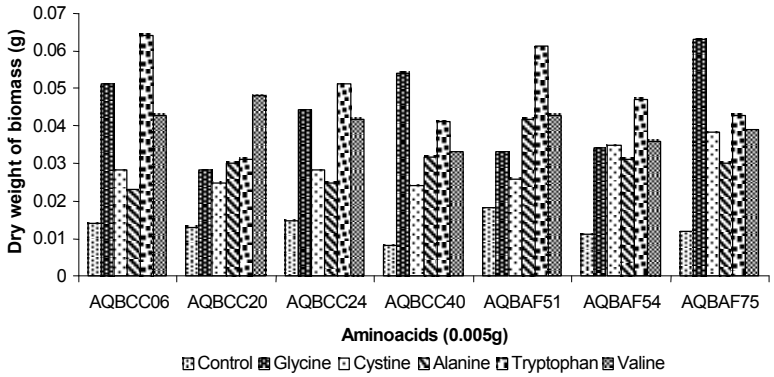

Fig. 1: Influence of amino acids on the growth of the seven strains

All the strains possess spore diameter of 2ìm. The strains showing typical morphology of Streptomycetes when analyzing the shape and spore chains under scanning electron microscope as reported earlier [14].

The nutritional characteristics of the strains were studied using criteria like carbon utilization, amino acids influence and sodium chloride tolerance. The utilization of carbon sources were displayed in table 2. All strains grew well in basal mineral salt media containing glucose but did not assimilate raffinose. On basal mineral salt medium with sucrose the growth of some strains were weak and absent for the other. The amino acids, glycine and tryptophan seemed to be positively influence the growth of the strains with different degrees except the strain AQBCC20 which showed better growth in media containing valine (Fig. 1). On using sodium chloride at concentration of 6 and 7%, the strains showed profuse growth. The isolated strain from the gut of the *Chaetodon collare* exhibited the maximal biomass at the concentration of 7% whereas the strains from *Archamia fucata* showed maximal biomass at 6% (Table 3). All the strains were able to grow in 22-45°C and pH 4-10. The strains were able to liquefy gelatine except AQBCC06 and AQBCC20. Solidification of milk and peptone cannot be done by the strain AQBCC20 and AQBAF75, while the others can do so. Strains AQBCC40 and AQBAF51were able to produce hydrogen sulphide

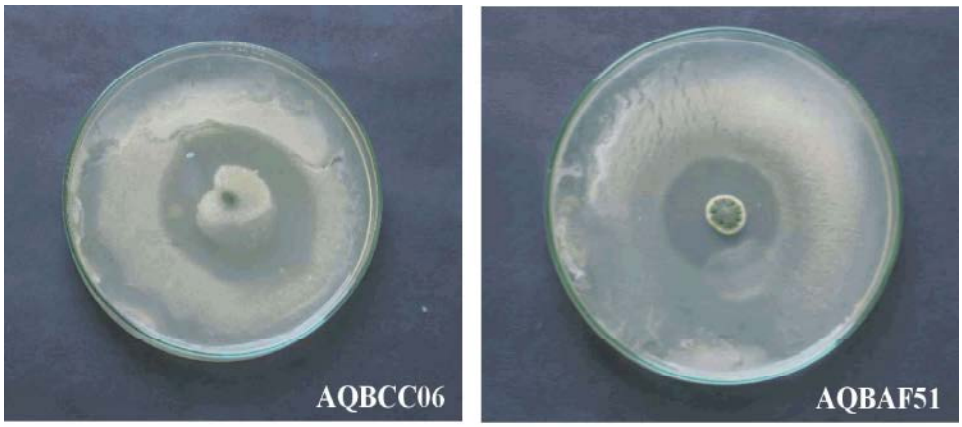

Fig. 2: Inhibition zone of the strains against *Vibrio cholerae* by Spot inoculation method isolates from the gut of the fishes *Chaetodon collare* and *Archamia fucata*.

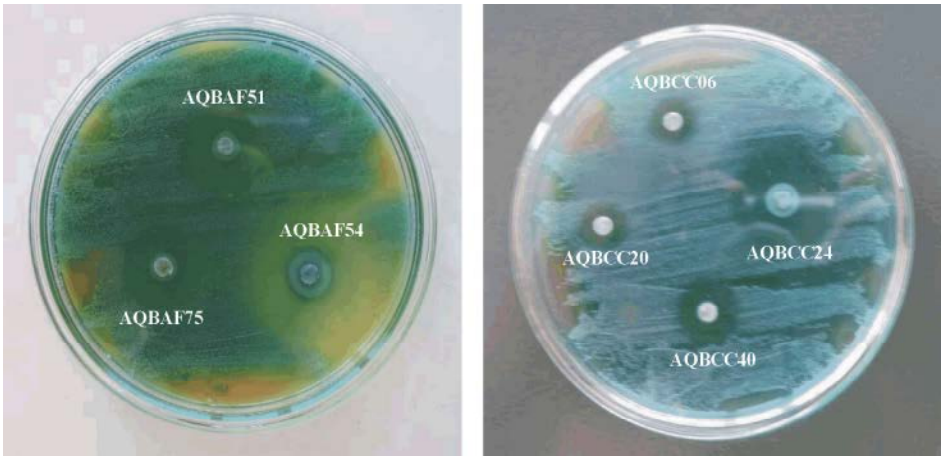

Fig. 3: Zone of inhibition of cultural extracts of strains against *Vibrio cholerae* by Disc method

but unable to hydrolyze starch and cellulose. Degradation of urea was effectively done by AQBAF54 and AQBAF75. Positive utilization of citrate was confirmed in the strains AQBCC20, AQBCC24, AQBCC40, AQBAF54, AQBAF75, other than AQBCC06, AQBAF51. Indole production was not seen in the strains AQBCC06 and AQBCC20, while the rest does. Lastly the catalase activities were effectively seen in all strains isolated from the gut of *Chaetodon collare*, while the strains from the other fish gut did not exhibit. The results of physiological and biochemical characteristics of the strains were displayed in Table 4. The identification of the *Streptomyces* is a very complex process. The *Streptomyces* classification system was mainly dependent on characteristics like the form of spores and use of carbon. The nutritional uptake, physiological and biochemical characteristics clearly proved under the classification of *Streptomyces* as reported earlier [13, 15].

Preliminary identification of antagonism against pathogen was done by spot inoculation method, in which the strains showed more than 10 mm of inhibition zone against *Vibrio cholerae* (Fig. 2). Later, In-vitro screening of the culture extracts was carried out by disc method which resulted in the occurrences of more than 10 to 30 mm diameters of inhibition zones against *Vibrio cholerae* (Fig. 3). There are some reports on actinomycetes strains from marine sediments against shrimp pathogens like *Vibrio* spp [22-23]. Recently there are reports on the isolation of *Streptomyces* associated with marine sponges and its bioactive potential against bacterial and fungal pathogens [10]. The screening of *Streptomyces* from Veli estuarine Lake, along Kerala coast, India, was studied previously [24]. The results almost correlated with the previous findings in which they investigated the antimicrobial activity of 74 *Streptomyces* isolates from soil [18]. Isolation and screening *Streptomyces* from the forest areas of Assam for antimicrobial metabolites were also earlier investigated [25].

Table 5: UV absorption and Rf values of cultural extracts of *Streptomyces* strains

Strain	Maximum (nm)/ Absorbance values	Shoulder (nm)/ Absorbance values	R_f values
AQBCC06	297.6 - 1.205	-	0.64
AQBCC20	245 – 2.309	273.8 – 2.334	0.51, 0.62
AQBCC24	248.6 – 2.475	367.4 – 1.784	0.60, 0.78
AQBCC40	295.3 – 1.079	-	0.63
AQBAF51	239.6 – 2.219	279.2 – 1.761	0.46, 0.53
AQBAF54	326.4 – 0.578	299.2 – 0.569	0. 69, 0.54
AQBAF75	320 – 2.370	270.4 – 0.824	0.69, 0.62

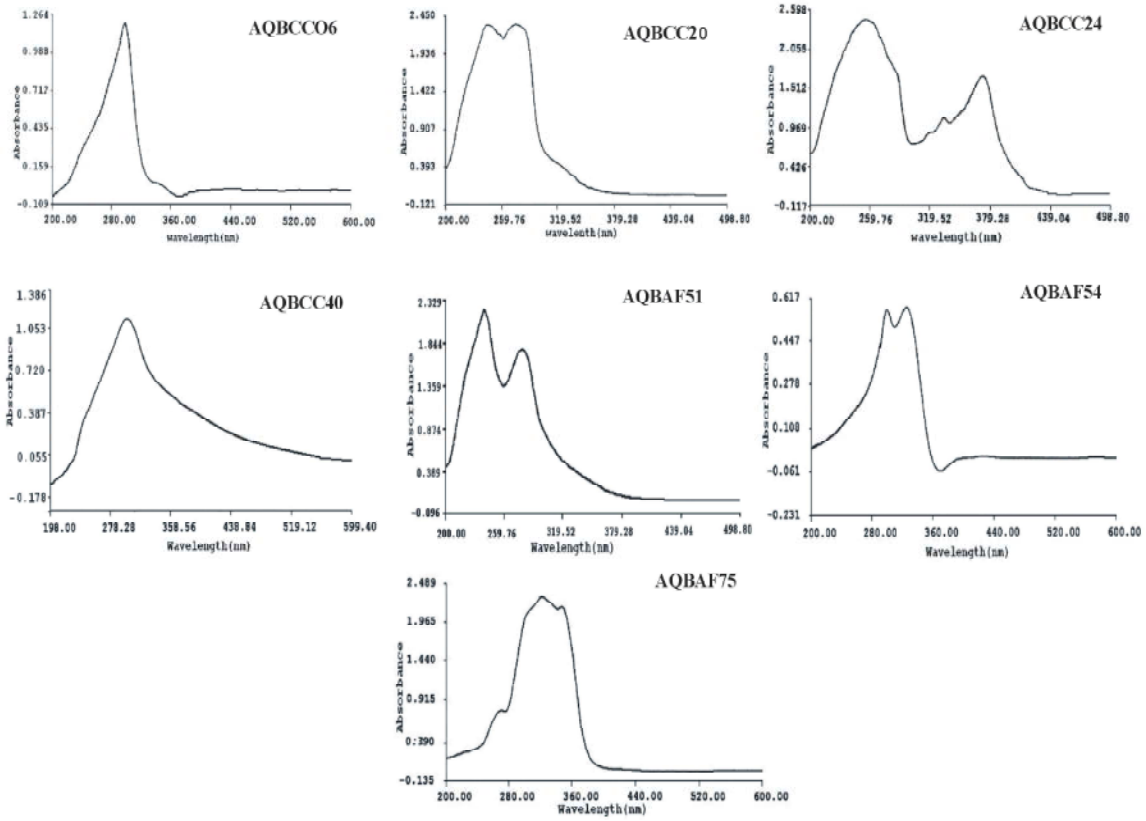

Fig. 4: UV spectra of the culture extract of *Streptomyces* isolates from the gut of the fishes *Chaetodon collare* and *Archamia fucata*.

The screenings of confirmed the production of bioactive substances from the submerged culture extracts by UV spectrum, which resulted in absorbance peaks ranged from 239 to 326 nm and by TLC analysis, the R_f values were ranged from 0.46 to 0.78 (Table 5, Fig. 4). This indicates the isolates produce different bioactive compounds which inhibit the fish and shellfish pathogens. The spectral data are in agreement with those obtained earlier [18], in which reported the maximum absorbance peaks was ranged between 212 and 260 nm and the characteristics suggest the compounds to be mostly polyene nature [19]. The cultural extracts obtained from *Streptomyces* isolated from Serbian soil showed two bioactive regions were detected on the TLC plate (R_f0.70 and 0.88) and UV spectral data of the active compounds in methanol showed peaks at 217 and 221 nm [26]. A similar result was obtained from the cultural extracts of marine sponges associated *Streptomyces* was reported [10].

The interaction or association of microbes particularly *Streptomyces* species with the gut of fishes were quite unique. The result of active extracts from the antibacterial studies, UV spectral and thin layer chromatographic analysis revealed that these strains were effective producers of bioactive metabolites against specific target pathogens. Further investigations were

needed in order to determine the structure of active components. Up to our knowledge, this is the first report on the isolation of *Streptomyces* from the gut of the marine ornamental fishes. The preparation gained from the investigated isolates would have been a great advantage over the existing commercial preparations. With increasing advancement in science and technology, there were greater demands for novel bioactive components from various sources.

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