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Isolation of marine *Streptomyces* and the evaluation of its bioactive potential

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Nearly ten isolates of *Streptomyces* were found to be associated with two species of sponges namely *Mycale mytilorum* (Annandale) and *Tendania anhelans* (Lieberkuhn). Among the ten isolates, four strains of white series were selected and characterized by conventional methods and assessed for their antagonistic activity against fish pathogens like *Aeromonas hydrophila* and *Vibrio* sp. All the strains showed inhibitory activity against these fish pathogens. The screening of antibacterial substances by the *Streptomyces* isolates proved the production of highly polyene nature compounds which were observed using criteria like thin layer chromatography (TLC) and spectral analysis. The results of the present investigation revealed that the sponges associated *Streptomyces* were found to be promising source of antibacterial bioactive substances. It concludes that development of appropriate fermentation and downstream processing technologies would bring out new classes of antibiotic leads.

Key words: Antimicrobials, marine *Streptomyces*, marine sponges.

INTRODUCTION

Marine sponges are rich sources of structurally unique natural compounds, several of which have shown a wide variety of biological activities (De Rosa et al., 2003). It is well known that even excellent drug candidates from sponges are often not developed because those sponges are rare, difficult to collect or both. Sponges harbour a rich diversity of marine organisms in their tissues (Friedrich et al., 1999). Numerous products from marine invertebrates show striking structural similarities to metabolites of microbial origin, suggesting that micro-organisms are the true source of these metabolites or they may be initially involved in their biosynthesis (Proksch, 2002). Sponges are host organisms for various symbiotic microorganisms such as archea, bacteria, cyanobacteria and microalgae within their tissues where they reside in the extra- and intra- cellular space (Muller et al., 1981; Amann et al., 1995) . In some cases, these microbial associates comprised as much as 40% of the sponge volume and can contribute significantly to host metabolism (for example, via photosynthesis or nitrogen fixation).

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Secondary metabolite production can be ascribed to symbiotic microorganisms only when synthesis has been demonstrated in cultures isolated from the host species (Wilkinson, 1978a) and it remains possible that these compounds are simultaneously produced by the host. In many instances, the limited availability of sponge material may preclude the commercial production of bioactive compounds (Simpson, 1984). These limitations could be overcome if the need to harvest sponges from the natural environment was eliminated using large scale laboratory culture that would provide a consistent yield and extrac-tion of bioactive compounds from symbiotic bacteria. The sponge will consume a substantial part of this primary production by direct ingestion of the symbionts or in the form of excreted metabolic products such as glycerol or glycogen. It has been estimated that 80% of the total energy requirements of the sponge *Phyllospongia lammellosa* was produced by its phototrophic symbionts (Cheshire et al., 1997).

It is generally assumed that the interior of the sponge body is continuously oxygenated, due to the efficient pumping of water through the aquiferous system (Bergquist, 1978; Osinga et al., 1999) . Hence sponges are not likely to harbour anaerobic microorganisms. However, the presence of facultative anaerobic bacteria in sponges has been demonstrated (Santavy and Colwel,

1990; Santavy et al., 1990; Wilkinson, 1978b) and the recent discovery of sulphate-reducing bacteria (Schumann-Kindel et al., 1997; Manz et al., 2000) and other symbiotic archea in sponges shows that anaerobic microbial process may take place in sponge's tissues.

The search for new bioactive substances has been remarkably successful and approximately, two-third of naturally occurring antibiotics including many of medical importance, have been isolated from actinomycetes (Okazaki and Okami, 1975) and majority from the genus *Streptomyces*. Compared to terrestrial forms, marine *Streptomyces* are important sources for the discovery of novel antibiotics. *Streptomyces* has wide application for antiviral, antibacterial, antitumour, auti-helminthic, insecticidal, immuno-modulator, immuno-suppressant among others. Occurrence of enzymes, L- asparaginase, which is employed in the treatment of tumours and acute lymphatic leukemia, has been reported in bacteria, fungi, Streptomycetes (Selvakumar, 1979; Maya et al., 1992; Dhevendaran and Annie, 1999a). Thus an alternative strategy targeting the microorganisms preferably *Streptomyces* associated with sponges for the screening of its bioactivity may prove to be an effective approach to the requirement of antibiotics from Streptomycetes, since no literature is available pertaining to this aspect.

MATERIALS AND METHODS

In the present study two varieties of sponges were collected at a depth of 5 – 10 m by SCUBA diving from Kovalam Coast which is situated on the west coast of Kerala about 14 km to the south of Thiruvananthapuram at 8° 23' N latitude and 76° 57' E longitude in India. Samples of sponges were collected in sterile polythene bags and transported to the laboratory within minimum possible time to avoid the external microbial contamination and excessive proliferation. All the epiphytic faunas were removed.

Identification of sponges

The sponge tissues were preserved in 10% formalin and photographed. The spicules were separated from the sponge and fixation was carried out. The spicules of the species were drawn and photographed through Camera Lucida microscope.

Isolation of *Streptomyces*

The sponges extract were obtained by squeezing gently with a glass stick. A portion (1 ml) of each sponge's extract was subjected to a dilution of 10^{-2}. A quantity (1 ml) of the dilutions was mixed with 20 ml of culture medium containing sterilized glycerol asparagine agar medium (selective media) in petri dishes and incubated at room temperature (28 ± 2°C) for seven days. The total *Streptomyces* population of the two sponges were counted.

Maintenance of culture

The selected *Streptomyces* strains were maintained as glycerol asparagine agar slant cultures at 28 ± 2°C (Pridham and Lyons, 1961). The inoculums used in all the experiments were seven day old cultures, unless otherwise stated.

Characterisation of the *Streptomyces* isolates

The strains were characterised according to the method employed by collaborators in International *Streptomyces* Project (Shirling and Gottlieb, 1966). They are characterised by acid-fast staining and by Gram's staining techniques. The isolate was also studied by employing various parameters which are detailed below. In the nutritional uptake experiments given below, the culture was inoculated into the basal medium + nutrient and incubated at 28°C for 7 days. The biomass thus obtained was separated from the broth, dried and weighed. The weight of the biomass was expressed in grams.

Melanoid production

The cultures were streaked onto peptone-yeast extract-iron agar slants and incubated at 28°C for 48 h.

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.

Carbon utilization (Modified from 'Pridham and Gottlieb, 1948)

Various carbon sources namely glucose, xylose, arabinose, rhamnose, fructose, galactose, raffinose, mannitol, inositol and sucrose were separately autoclaved and mixed at a concentration of 1% each to 10 ml of basal mineral salt medium. The cultures were inoculated into the medium and the control without carbon sources was maintained. The tubes were observed for 10 to 14 days.

Amino acid influence

Various amino acids namely glycine, cystine, alanine, tryptophan and valine were mixed at a concentration of 0.1% each to 5 ml of basal mineral salt medium.

Sodium chloride tolerance

Sodium chloride at varying concentrations (1, 1.5, 2, 2.5 and 3%) was added to 5 ml of the basal medium.

Anti microbial assay

Spot inoculation method

The four strains were spot inoculated in glycerol asparagine medium for seven days. After seven days, 1 ml of chloroform was added and made to stand for 40 min to arrest the growth of inoculated colonies. It was then over laid with 5 ml of sloppy agar(0.6%) layer containing seeded microbes like *Aeromonas hydrophila, Vibrio* sp and incubated for 24 to 48 h at 37°C. Diameter of the incubation zone was recorded in millimetres.

In vitro screening of isolates for antagonisms

Isolates that showed activity against test microorganisms were inoculated in submerged culture of 250 ml Erlenmeyer flasks containing 50 ml of the liquid medium (0.8 g NaCl, 1 g NH_4Cl, 0.1 g KCl, 0.1 g KH_2PO_4, 0.2 g $MgSO_4.7H_2O$, 0.04 g $CaCl_2.2H_2O$, 2 g

Table 1. Total count of *Streptomyces* associated with two different species of sponges.

Sponges	Medium used	Number of *Streptomyces* colonies x 10^2 CFU / ml
Mycale mytilorum	Glycerol Asparagine Agar	6
Tendania anhelans	Glycerol Asparagine Agar	4

Table 2. Characterization of *Streptomyces* isolates associated with marine sponges.

Name of Sponges identified	*Streptomyces* isolated	Mycelial colouration		Melanoid pigmentation	Spore surface	Spore morphology
		Aerial	Substrate			
Mycale mytilorum (Annandale)	AQB – S 1	White	Yellow	-	Smooth	Rectiflexibilis
	AQB – S 2	White	White	-	Smooth	Rectiflexibilis
Tendania anhelans (Lieberkuhn)	AQB – S 3	White	Pink	+	Smooth	Rectiflexibilis
	AQB – S 4	White	orange	+	Smooth	Rectiflexibilis

+ = Pigments produced; - = Not produced.

glucose and 3 g yeast extract in one litre of distilled water, at pH 7.3). Incubation was carried out at 180 rpm, 28°C for 120 h under the standard condition of aeration and agitation. After growth, the contents of each flask were extracted with methanol and the mixture was shaken for 2 h and filtered. Filter paper disks (6 mm in diameter) were impregnated with filtrate, dried and placed in plates seeded with fish pathogen *A. hydrophila* and *Vibrio* sp. The plates were incubated at 37ºC for 48 h and examined for zone of inhibition.

Thin layer chromatographic analysis of Antibacterial compounds

The extracts were spotted on the baseline of the silica gel plates at 1.0 cm and then allowed to dry at room temperature. Then the plates were placed in pre-saturated TLC chamber which contains the mobile phase butanol-acetic acid-water (4:1:2). Then the chromatogram was developed and dried for few minutes. It was visualized under ultraviolet (UV) light and spots were marked. The R_f values for each band were measured.

Isolation of antibacterial metabolites

Antibacterial compounds were recovered from the filtrate by solvent extraction with ethyl acetate in the ratio 1:1 (v/v) and shaken well for 1 h. The ethyl acetate phase was separated and evaporated to dryness in water bath at 80 - 90°C. Residue was weighed and redissolved with little ethyl acetate. The absorption spectrum of each extract was determined in UV region by using UV/VIS spectrophotometer.

RESULTS

In the present study, two species of sponges were collected at a depth of 5 – 10 m by SCUBA diving from Kovalam coast, Kerala, India. The sponges were identified as *Mycale mytilorum* (Annandale), *Tendania anhelans* (Lieberkuhn) belonging to the class Demospongiae Sollas. For identification, the sponge tissues were preserved in 10% formalin, photographed, then spicule separated and fixed. The spicules of the two species were drawn and photographed through Camera Lucida microscope. Each species has unique spicules that form the basis of the species' classification (Thomas, 1970; Hooper and Van Soest, 2002). They have monoaxonic and tetraaxonic silicious spicules (Sollas, 1988).

The total count of *Streptomyces* isolated from *Mycale mytilorum* (6 x 10^2) and *Tendania anhelans* (4 x 10^2) are shown in Table 1, out of which four strains of *Streptomyces* (AQB S1, AQB S2, AQB S3 and AQB S4) of white series were selected for the study. The characterization of *Streptomyces* sp. was studied by following methods recommended by International *Streptomyces* Project. The colonies were slow growing, chalky, folded and aerobic. Mycelial colour pattern of four strains were totally different as shown in Table 2. Arial mycelial colour expressed by all four strains was white series. Substrate mycelia colour for each strain was totally different like yellow, white, pink and orange. This colouration may be due to other primary as well as secondary metabolites production provided by the enriched media (Dhevendaran and Annie, 1999b; Dhevendaran et al., 2004). These primary and secondary metabolites of different colouration are the rich source of certain compounds like amino acids, sugars, fatty acids, terpenes, antibiotics and so on. All the four strains were acid-fast negative and found to be Gram -positive. Two strains (AQB S3 and AQB S4) produced melanoid pigments (Table 2) . The screening of streptomycetes from various sources showed that less than 10% produced melanin pigments (Sheeja, 1994; Mathew, 1995; Devan, 1999). Spore morphology of the strains (AQB S1, AQB S2, AQB S3 and AQB S4) showed smooth spore surface and rectiflexibiles (RF) hyphae (Table 2, Figure 1). It has been observed that strains with smooth surface were maximum in the grey colour series (Lakshmanaperumalsamy, 1978; Vanajakumar, 1981).

However, smooth spores in other strains have also

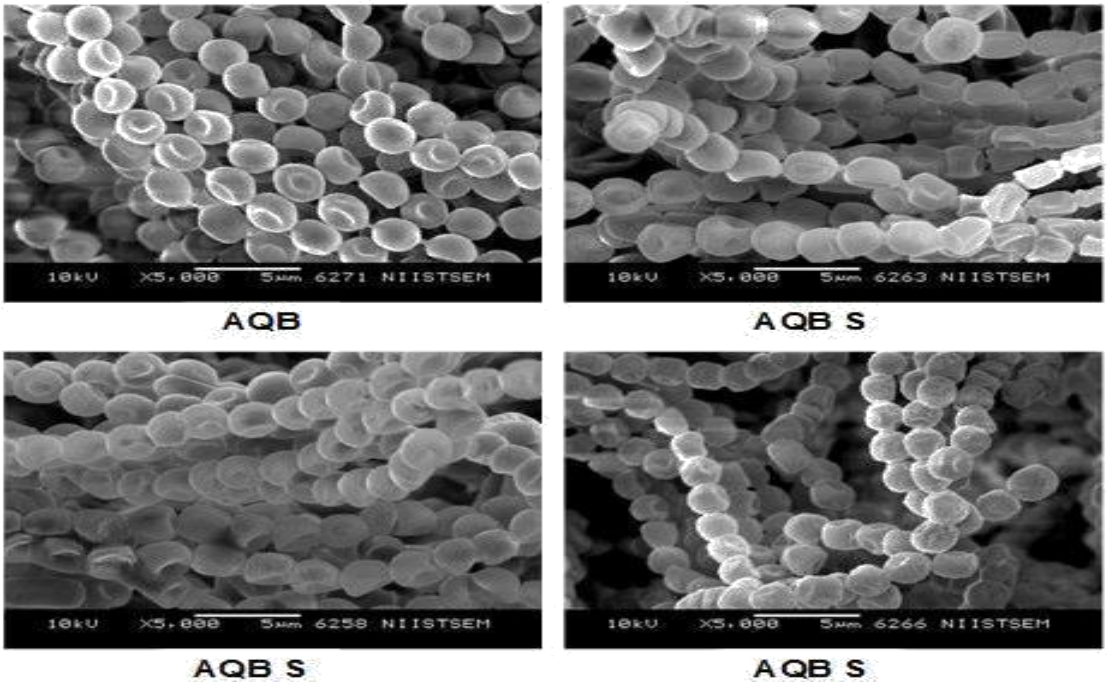


Figure 1. Scanning electron micrograph of *Streptomyces* strains (AQB S1, AQB S2, AQB S3, AQB S4).

Table 3. Utilization of carbon sources on the growth of *Streptomyces* strains.

Strains	Carbon sources									
	Glucose	Xylose	Arabinose	Rhamnose	fructose	Galactose	Raffinose	Mannitol	Inositol	Sucrose
AQB – S 1	++	++	+	+	±	-	-	+	+	-
AQB – S 2	++	++	+	-	-	+	+	-	-	±
AQB – S 3	++	++	+	+	+	+	-	-	+	±
AQB – S 4	++	++	-	-	±	+	-	+	-	-

++ = Strongly positive utilization; + = Positive utilization; ± = Utilization doubtful; - = Utilization negative.

been reported (Trenser et al., 1968). The *Streptomyces* strains formed abundant mycelium with glucose and xylose, moderate growth in medium containing arabinose, rhamnose, galactose, raffinose, mannitol and inositol, whereas growth was doubtful on media with fructose and sucrose (Table 3). *Streptomyces plicatus* isolated from the fish, *Gerres filamentoses* showed more affinity towards glucose (Koshy et al., 1997). Streptomycetes from the gut of ornamental fish, *Barbus schwanefeldi*, showed maximum growth in mannitol and xylose (Jagadamika devi, 2005). The amino acids, cysteine (0.085 g), tryptophan (0.081 g) and glycine (0.071 g) seemed to positively influence the growth of the isolated *Streptomyces* strains (Figure 2). These amino acids are the precursors for the production of the secondary metabolites, namely antibiotics (Dhevendaran et al., 2004). At 2 and 2.5% sodium chloride concentration, there was profuse growth of strains (Figure 3). The dry weight of the biomass ranged from 0.062 to 0.102 g whereas at 0% of sodium chloride concentration, it showed 0.047 g only. It has been reported that some sodium chloride sensitive actinomycetes had increased tolerance to higher concentrations during successive cultivations and the filtrate of the tolerant strain had a significantly different UV spectrum, suggesting the pro-duction of new metabolites (Okazaki and Okami, 1975).

The antagonistic properties of *Streptomyces* strains (AQB S1, AQB S2, AQB S3 and AQB S4) against fish pathogens like *A. hydrophila* and *Vibrio* sp. were investigated. These four strains showed zone of inhibition between 10 to 35 mm (Table 4) . Among the four strains, AQB S2 and AQB S4 showed highly active group and others exhibited moderate active group by spot inoculation method. The maximal bio-activity occurred in *Streptomyces* associated with sponges. As far as the *in vitro* screening of antagonism is concerned, the filtrate extracted with methanol showed maximal zonation of 18 and 20 mm by the strains AQB S3 and AQB S4 against

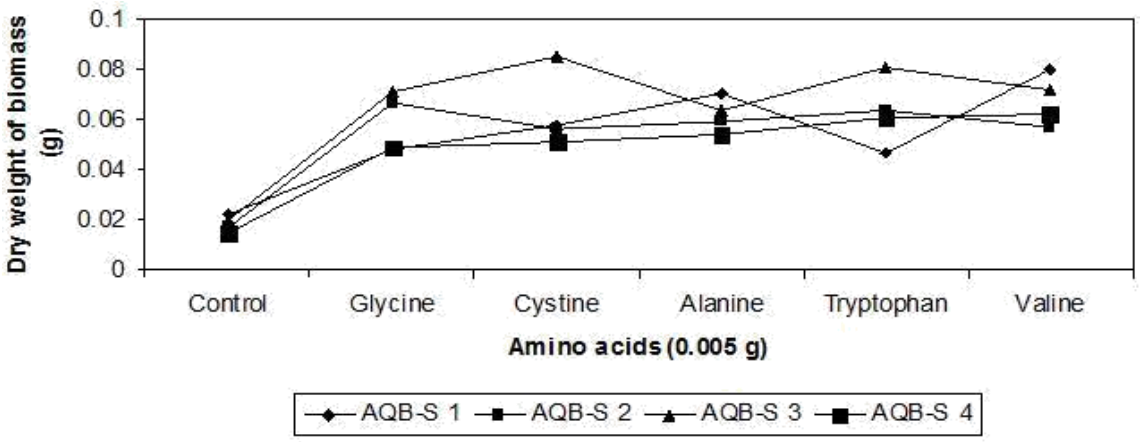


Figure 2. Influence of amino acids on the growth of selected *Streptomyces* sp.

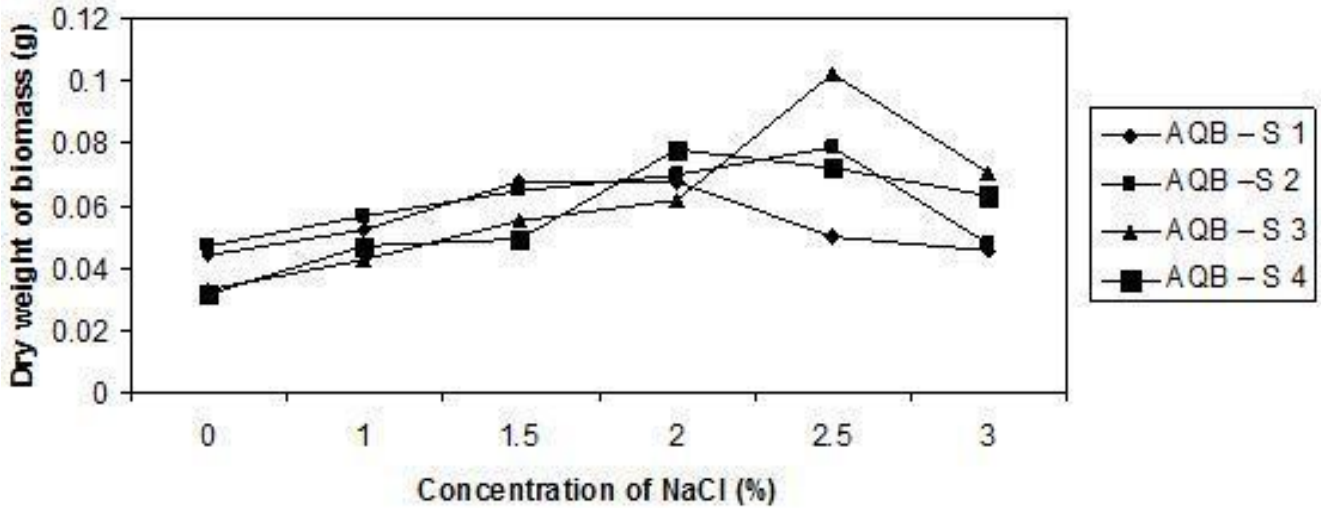


Figure 3. Tolerance of sodium chloride on the biomass of selected *Streptomyces* sp. isolated from sponge tissue.

Table 4. Antimicrobial activities of four strains of *Streptomyces* against *Aeromonas hydrophila* and *Vibrio* sp. (spot inoculation method).

Strains	Zone of Inhibition (mm)	
	A. hydrophila	*Vibrio* sp.
AQB S 1	10	20
AQB S 2	30	35
AQB S 3	22	15
AQB S 4	35	25

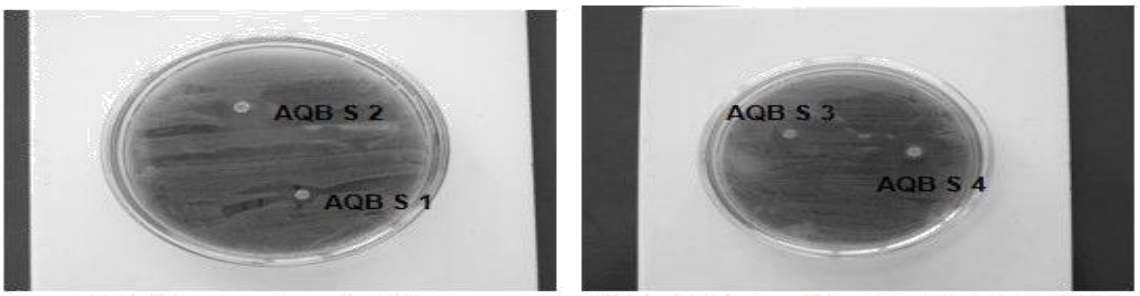
A. hydrophila and the maximal zonation 17 and 18 mm showed by AQB S2 and AQB S4 against *Vibrio* sp (Figure 4). The above results correlated with the previous findings in which the antimicrobial activity of 74 *Streptomyces* isolates from soil were investigated (Sahin and Ugur, 2003). As far as the qualitative analysis by TLC is concerned, two bioactive regions were detected and the R_f values are 0.71 to 0.89 (Table 5). Similar results were observed by Ilic et al. (2005).

The UV spectral data for the ethyl acetate extract of the selected strains from fermented broth are shown in Figure 5. Maximum absorbance peaks range between 215 to 320 nm (Table 6) and the characteristics of absorption peaks indicate production of highly polyene natural compounds. These strains produced either a broad-spectrum antibacterial compound or several compounds with different activities. The spectral data were consistent with those obtained previously (Swaadoun et al., 1999).

DISCUSSION

There are more than 6,000 described species of sponges.

Zone of inhibition (mm) against *Aeromonas hydrophila*: AQB S1 - 5, AQB S2 – 15, AQB S3 – 18, AQB S4 – 20



Zone of inhibition (mm) against *Vibrio* sp: AQB S1 - 4, AQB S2 – 17, AQB S3 – 8, AQB S4 – 18

Figure 4. Antibiogram of sponges associated *Streptomyces* sp. against *A. hydrophila* and *Vibrio* sp (Disc method).

Table 5. Qualitative analysis of antibacterial components by using thin layer chromatography.

Strains	R_f values
AQB S 1	0.71
AQB S 2	0.85
AQB S 3	0.88
AQB S 4	0.89

Table 6. Characteristics of UV absorption spectra of ethyl acetate extract of fermented broth.

Strain	Maximum (nm)	Shoulder (nm)
AQB S 1	226, 270	330
AQB S 2	221, 320	270
AQB S 3	240, 270	-
AQB S 4	215,271	290, 330

These inhabit a wide variety of marine and freshwater (somewhat more restricted) systems and are found throughout tropical, temperate and polar regions (Hooper and Van Soest, 2002). Sponges have been the focus of much recent interest due to two main (and often interrelated) factors: (i) they form close associations with a wide variety of microorganisms and (ii) they are a rich source of biologically active secondary metabolites. Interactions between sponges and microorganisms occur in many forms. To a sponge, different microbes can represent food sources (Reiswig, 1971, 1975; Pile et al., 1996), pathogens / parasites (Lauckner, 1980; Hummel et al., 1988; Bavestrello et al., 2000; Webster et al., 2002), or mutualistic symbionts (Wilkinson, 1983, 1992). Microbial associates can comprise as much as 40% of sponge tissue volume (Vacelet, 1975), with densities in excess of 109 microbial cells per ml of sponge tissue (Webster and Hill, 2001; Hoffmann et al., 2005), several orders of magnitude higher than those typical for sea water. The marine sponges which were identified have high concentration of secondary metabolites (Faulkner, 1984). In some reports, this is due to the filter feeding mechanism which leads to passage of microbes inside the sponges thus resulting to deposition of secondary metabolites (Imhoff and Stöhr, 2003).

An attempt on sponges associated with *Streptomyces* was carried out in the present study. Nearly ten strains were isolated from two species of sponges. Of these, four strains of *Streptomyces* were selected for the study. Then the strains were classified and identified at the genus level. It has been reported that only 6% of sponge associated *Streptomyces* can be cultivable (Selvin et al., 2004).

The *Streptomyces* strains (AQB S1, AQB S2, AQB S3

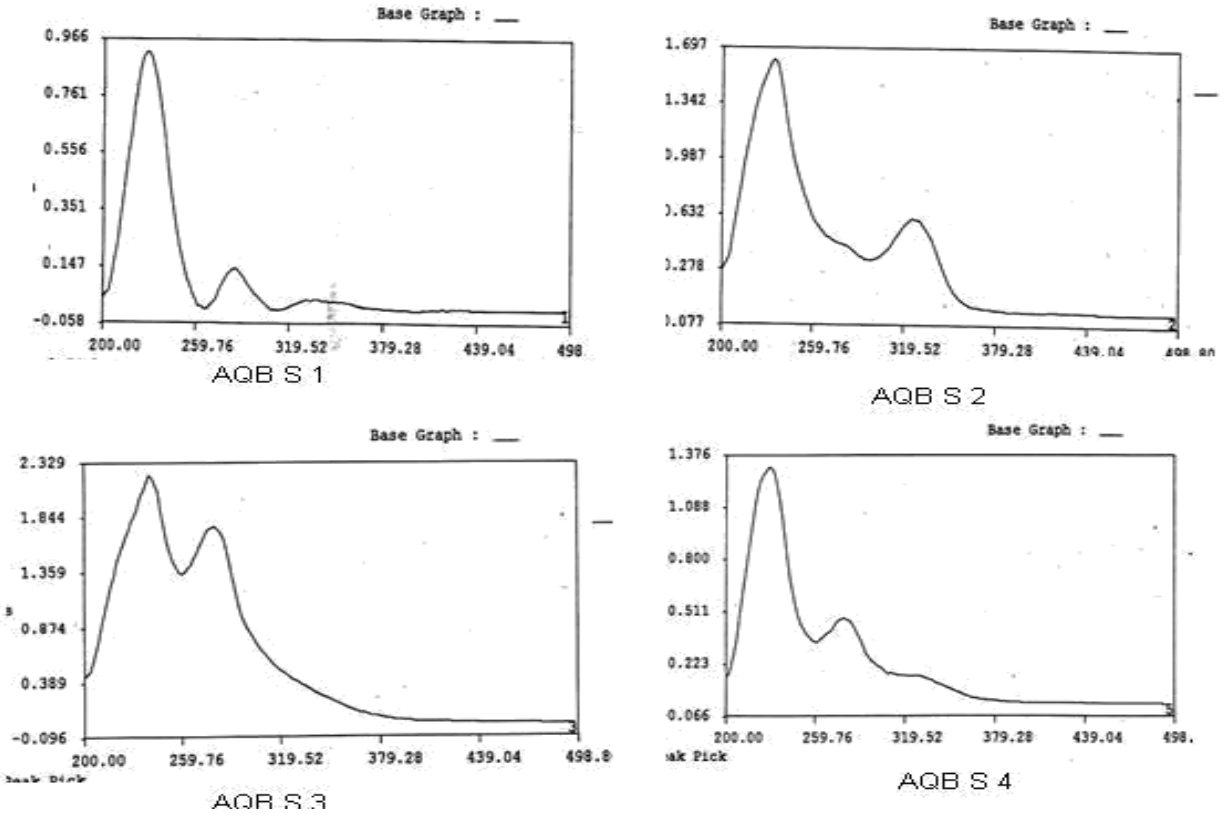


Figure 5. UV Spectrum of active components in ethyl acetate extract.

and AQB S4) showed antagonism against fish pathogens like *A. hydrophila* and *Vibrio* sp. Earlier, 26 marine endosymbiotic actinomycete strains, isolated from the Bay of Bengal (coast of India), were screened for antagonistic and antimicrobial activity against pathogenic bacteria and fungi (Gandhimathi et al., 2008). Antimicrobial activities of marine bacteria associated with sponges from the waters off the coast of South East India were also studied (Anand et al., 2006). Isolation and screening of *Streptomyces* from the forest areas of Assam for antimicrobial metabolites were also investigated (Thakur et al., 2007). The screening of *Streptomyces* sp. from *Veli Lake* was done (Suja Devan, 1999). The spectral data of the extract is between 215 to 320 nm and these results very well correlate with previous report (Swaadoun et al., 1999). Interestingly, in at least some cases, the antibacterial compounds appear to be produced by associated microorganisms rather than by the sponge (Schmidt et al., 2000; Bewley and Faulkner, 1998; Piel et al., 2004).

This study concludes that the sponge's associated with *Streptomyces* isolates were found to be potential source of novel antibacterial bioactive substances. If these symbiotic *Streptomyces* strains from which some bioactive metabolites are derived can be cultured, the *Streptomyces* strains could be used in a mass production of the bioactive compounds. Continued investigations of sponge-associated microbial derived compounds and their biotechnological and ecological implications should guarantee vigorous interest in sponge-microbe associations for some time to come.

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