INTRODUCTION

Actinomycetes have been traditionally a rich source for biologically active metabolites. Although heavily studied over the past three decades, actinomycetes continue to prove themselves as reliable sources of novel bioactive compounds. Among the well-characterized pharmaceutically relevant microorganisms, actinomycetes remain major sources of novel, therapeutically relevant natural products (Jenson et al., 2000). Actinomycetes, the filamentous bacteria, are primarily saprophytic microorganisms of the soil, where they contribute significantly to the turnover of complex biopolymers, such as lignocellulose, hemicellulose, pectin, keratin, and chitin (Vijayakumar et al., 2007). The recently proposed class actinobacteria (Stackebrandt et al., 1997) is comprised of high G+C content gram-positive bacteria and includes the actinomycetes (order Actinomycetales), whose members have an unparalleled ability to produce diverse secondary metabolites (Mincer et al., 2002).

During the past 30 years, a large number of new compounds with structures completely different from those isolated from terrestrial organisms were successfully discovered from marine sources (Elyakov et al., 1994; El-Gendy et al., 2008). Marine environment is a relatively untapped ecosystem with regards to isolation of indigenous Streptomyces, although existence of terrestrial origin has been reported (Solanki et al., 2008; Krishnaraj and Mathivanan, 2011). Actinomycetes are the main source of clinically important antibiotics, most of which are too complex to be synthesized by combinatorial chemistry and which are eco-friendly filamentous bacteria. Streptomyces and related actinomycetes continue to be useful sources of novel secondary metabolites with a range of biological activities that may ultimately find applications as anti-infectives, anti-cancer agents or other pharmaceutically useful compounds (Bibb, 2005). The value of actinomycetes to society in terms of providing useful drugs especially antibiotics and anticancer agent and to the pharmaceutical industry for revenue generating discovery platform, is indisputable (Baltz, 2007).

In recent literature revealed that at least 4,607 patents have been issued on actinomycetes related product and processes (Berdy, 2005). Out of 22,500 total bioactive secondary metabolites 10,100 (45%) are reported to be produced from actinomycetes in which 7630 from Streptomyces and 2470 from rare actinomycetes (Berdy, 2005). Recent studies are focusing on the response of antioxidant system of bacteria, which is important in terms of biotechnology, such as Streptomyces growth in various oxidative stress conditions (Thomas schweder, 2005). Antioxidants play an important role in inhibiting and scavenging free radicals, thus providing protection to humans against various infections and degenerative diseases (Sharma and Gupta, 2008). Dietary antioxidants have already been shown to be protective against chronic diseases. Some degradation processes of aerobic living organisms are mediated by reactive oxygen species, such as superoxide anion radical (O2), hydrogen peroxide radical and hydroxyl radical. Particularly O2 has been considered as a causative species to induce inflammation (Trenam et al., 1992).
Nutritional antioxidant deficiency also leads to oxidative stress, which signifies the identification of natural antioxidative agents present in the diet consumed by the human population. There are certain naturally occurring antioxidants that can give protection to liver from hepatotoxins. Modern research is now directed towards natural antioxidants from plants and microorganisms and serves as safe therapeutics (Suryavathana et al., 2010). The objective of this present study is to establish antioxidant potential of novel actinomycetes strain by evaluating both intracellular and extracellular metabolites.

MATERIALS AND METHODS

Collection of soil sample

Marine soil samples were collected from the Bay of Bengal coast of Puducherry, India. About 15 grams of soil were collected and stored at 4°C until further use. The soil samples were air dried for preventing bacterial contamination. Then the sample was serially diluted up to 10^2 dilution by adding 1 gram of soil to 10 ml of distilled water.

Isolation of Actinomycetes

About 0.1 ml of each dilution was poured and plated on Starch casein agar (SCA) by spread plate method. After three week incubation at room temperature, the plates were analyzed for white powdery colonies. These colonies were sub-cultured and maintained on International Streptomyces Project-1 (ISP-1) agar plate.

Primary screening of actinomycetes for antifungal Activity

The pure cultures of actinomycetes from the collected soil samples were then screened for their antifungal activity by cross streak method. Starch casein agar plates were inoculated with pure culture of actinomycetes, incubated at room temperature for 7 days. After adequate growth of isolates the test organism, *Rhizoctonia solani* was inoculated perpendicular to the streak line of the isolates. The plates were then incubated at room temperature for a period of 3–4 days (Augustine et al., 2005).

Primary screening of actinomycetes for antibacterial Activity

Isolates that showed activity against the fungal test organisms were inoculated in a submerged culture of 500 ml Erlenmeyer flasks containing 100 ml of the liquid medium of starch casein agar. These cultures were grown in a rotary shaker at 200 rpm, 28°C for 1 week under the standard condition of aeration and agitation. The resultant growth cultures were centrifuged at 6000 rpm for 15 minutes. The culture filtrates were solvent extracted with Ethyl acetate (1:2) 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 extract thus obtained was weighed. The crude ethyl acetate extracts was screened for antibacterial activity using the cup well agar diffusion method as described by Krishnaraj and Mathivanan (2011). For this purpose sterilized molten Mueller Hinton agar (MHA) was prepared gently and aseptically poured into Petri dishes and allowed to solidify. Sterile cork borer (6 mm diameter) was used to bore holes in the plate. About 100µl of the crude ethyl acetate extract at a concentration of 10 mg/ml was carefully dispensed into bored holes. This was done in duplicate. Extracts were allowed to diffuse for about 2 h before incubating. Plates were incubated at 37°C for 24 h. The presence of zone of inhibition around each well was indicative of antibacterial activity. Control experiment was carried out by loading DMSO into control well against each test organism to ensure that it does not have activity against test bacteria.

Genus level Identification of potential strain

The putative isolate was identified by various parameters such as Colony morphology, spore arrangement, Staining and Biochemical reactions (Augustine et al., 2004).

Optimization of media and cultural conditions

Modified method of Sambamurthy and Ellaih (1974) was used. To study the influence of incubation period, the culture was maintained in the production medium for up to 8 days. The broth drawn at 24, 48, 72, 96, 120, 144, 168 and 192 hrs was tested for antibacterial activity. To determine the optimal nutritional medium and cultural conditions and to identify the suitable media for growth, the isolate was inoculated in different culture media such as, Starch Casein Agar, ISP – 5 (Glycerol asparagine agar), Glucose asparagine agar, Kuster’s agar, Maltose yeast extract agar and the growth was investigated.

Bulk fermentation and preparation of crude ethyl acetate extracts

Based on the results of primary screening, the isolate *Streptomyces* sp. ABTRI12 was selected for the fermentation and assessment of antibiotic production. Fermentation for production of antibiotic and subsequent extraction of the antibiotics was done as described by Ilic et al. (2007) with modification. Starch casein broth was prepared and about 20 ml was dispensed into 100-ml Erlenmeyer flask capacity, sterilized, allowed to cool and inoculated with 0.5 ml *Streptomyces* isolate suspension and incubated at 28°C for 48 h at 200 rpm. About 500 ml of SC-broth was prepared in 1 L Erlenmeyer flask and inoculated with the 48 h old pre-culture of Streptomyces isolate and incubated for 6 days at 28°C and 200 rpm. At the end of the incubation period, the culture was harvested by centrifugation at maximum speed for 15 min. The culture supernatant was extracted with double the volumes of ethyl acetate (1:2 v/v) and vaporized to dryness in a rotary evaporator at 50°C. The extract was stored for further studies.

Antioxidant Activity

Estimation of Radical Scavenging Activity (RSA) Using DPPH Assay

The RSA activity of secondary metabolite was determined using DPPH assay according to Alagumani et al., (2010), with small modification. The decrease of the absorption at 517 nm of the DPPH solution after addition of the antioxidant (secondary metabolite) was measured in a cuvette containing 2960 µl of 0.1 mM ethanolic DPPH solution was mixed with 40 µl of 20 - 200 µg/mL of crude extract. Blank containing 0.1 mM ethanolic DPPH solution without secondary metabolite and vortexed thoroughly, the setup was left at dark at room temperature. The absorption was monitored after 20 min. Ascorbic acid (AA) and Butylated hydroxytoluene (BHT) were used as references. The ability to scavenge DPPH radical was calculated by the following equation.
% of DPPH radical scavenging activity = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100

Abs_{control} is the absorbance of DPPH radical + ethanol; Abs_{sample} is the absorbance of DPPH radical + crude extract. Measurements were performed in triplicate. Absorbance values were corrected for radical decay using blank solutions. The IC_{50} (concentration providing 50% inhibition) was calculated graphically using a calibration curve versus percentage of inhibition.

Thin Layer Chromatographic Analysis of 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 (Hwang et al., 1994). The plates were placed in TLC chamber pre-saturated with the mobile phase as 10% ethyl acetate in hexane. The chromatogram was developed and visualized under UV light and in the iodine chamber, and then the spots were marked. The R_{f} values for each spot was measured.

Characterization of crude extracts

Infrared spectroscopy

The infrared (IR) spectra of the crude extracts were measured (as KBr discs) between 400 – 4000 cm^{-1} on Perkin Elmer 2000 FT-IR spectrophotometer. The important IR bands, such as ν(C=N), ν(O-H), ν(C-H), ν(C=C), ν(N-H), ν(C=O) and (C-H) symmetric and asymmetric stretching, and stretching frequencies were studied to determine the presence of functional group in the ethyl acetate crude extracts (Ogunmwonyi et al., 2010).

RESULTS

Isolation of marine actinomycetes

In the course of screening for novel antibiotics, 84 actinomycete isolates were isolated from different marine samples, collected at the Bay of Bengal coast of Puducherry, India. The occurrence and distribution of different actinomycete genera in different marine samples is shown in Table 1. Out of 84 actinomycetes, 61 isolates were identified as belonging to the genus Streptomyces, family Streptomycetaceae (spore chain with coiling and branching); 8 as Micromonospora, family Micromonosporaceae (clusters of spore chain, single conidia on substrate mycelia); 5 as Nocardia (morphology ranging from fugacious substrate mycelium only to Streptomyces-like); 4 as Saccharopolyspora (very long chains of conidia on the aerial mycelium) and 6 as Streptoverticillium (whorls of straight chain of conidia formed).

Primary screening of actinomycetes for antifungal Activity

All the 84 isolates obtained were screened by cross streak method and among them only 6 isolates exhibited significant antifungal activity. In this cross streak method no growth of the test organisms, Rhizoctonia solani was observed across the streak lines of the 6 isolates is shown in Table 2.

Primary screening of actinomycetes for antibacterial Activity

The result of the screening of the ethyl acetate crude extracts of the selected six putative Streptomyces for antibacterial activities is shown in Table 3. These extracts showed activities against a 6 test bacteria. ABTRI 46 and ABTRI 68 extracts showed activity against five test bacteria with zones of inhibition ranging from 14 to 20 mm, while ABTRI 10, ABTRI 27 and ABTRI 49 extracts showed activity against five test bacteria with a zone of inhibition ranging from 16 to 22 mm. Isolate ABTRI 12 extract was active against all the six test bacteria respectively with zones of inhibition ranging from 19 – 26 mm diameter.

Table 2: Antifungal activity of selected streptomycetes in the primary screening

<table>
<thead>
<tr>
<th>Isolates No</th>
<th>R. solani - Zone of inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABTRI 10</td>
<td>18</td>
</tr>
<tr>
<td>ABTRI 12</td>
<td>24</td>
</tr>
<tr>
<td>ABTRI 27</td>
<td>20</td>
</tr>
<tr>
<td>ABTRI 46</td>
<td>19</td>
</tr>
<tr>
<td>ABTRI 49</td>
<td>22</td>
</tr>
<tr>
<td>ABTRI 68</td>
<td>21</td>
</tr>
</tbody>
</table>

Genus level Identification of potential isolate

The actinomycete isolate recovered from the soil sample was identified as Streptomyces species based on microscopic, biochemical and staining characteristics are shown in Table 4. The characteristic spore arrangement is also in agreement for placing the isolate in the genus Streptomyces.

Table 4: Characteristics of actinomycetes isolates recovered from soil sample

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Streptomyces sp ABTRI12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony</td>
<td>Dark grey with white colony</td>
</tr>
<tr>
<td>appearance</td>
<td>Dark grey pigmentation</td>
</tr>
<tr>
<td>Spore arrangement</td>
<td>spiral</td>
</tr>
<tr>
<td>Gram’s staining</td>
<td>+ ve</td>
</tr>
<tr>
<td>Acid-fast staining</td>
<td>- ve</td>
</tr>
<tr>
<td>Starch hydrolysis</td>
<td>+</td>
</tr>
<tr>
<td>Gelatin liquefaction</td>
<td>-</td>
</tr>
<tr>
<td>Casein hydrolysis</td>
<td>+</td>
</tr>
</tbody>
</table>

Optimization of media and cultural conditions

The potent strain ABTRI 12 was incubated in the broth culture for its production of antibiotics at different incubation period of 24, 48, 72, 96, 120, 144, 168 and 192 hrs. The broth was then extracted in ethyl acetate and tested against the test organisms Staphylococcus aureus, Bacillus cereus, Klebsiella pneumoniae, Pseudomonas aeruginosa. The results are shown in Figure 1. For all the cultures, it was observed that
Table 1: Occurrence and distribution of marine actinomycetes genera in different marine samples

<table>
<thead>
<tr>
<th>Location</th>
<th>Actinomycetes isolated</th>
<th>Streptomycetes</th>
<th>Micromonospora</th>
<th>Nocardia</th>
<th>Saccharopolyspora</th>
<th>Streptoverticillium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>18</td>
<td>13</td>
<td>1</td>
<td>-</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Soil sediments</td>
<td>47</td>
<td>34</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Rhizosphere soil</td>
<td>19</td>
<td>14</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>84</td>
<td>61</td>
<td>8</td>
<td>5</td>
<td>4</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 3: Antibacterial activities of fermentation products of the selected putative *Streptomyces* isolates

<table>
<thead>
<tr>
<th>Test Bacteria</th>
<th>ABTRI 10</th>
<th>ABTRI 12</th>
<th>ABTRI 27</th>
<th>ABTRI 46</th>
<th>ABTRI 49</th>
<th>ABTRI 68</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zone of inhibition (mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Gram positive</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>18</td>
<td>19</td>
<td>20</td>
<td>20</td>
<td>-</td>
<td>18</td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>21</td>
<td>24</td>
<td>-</td>
<td>17</td>
<td>19</td>
<td>20</td>
</tr>
<tr>
<td><em>Micrococcus luteus</em></td>
<td>-</td>
<td>21</td>
<td>16</td>
<td>19</td>
<td>-</td>
<td>22</td>
</tr>
<tr>
<td><strong>Gram negative</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>16</td>
<td>23</td>
<td>-</td>
<td>19</td>
<td>19</td>
<td>20</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>18</td>
<td>26</td>
<td>22</td>
<td>-</td>
<td>21</td>
<td>-</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>19</td>
<td>24</td>
<td>21</td>
<td>20</td>
<td>22</td>
<td>20</td>
</tr>
</tbody>
</table>

inhibition zone was increased with the increase in incubation period in the medium and a maximum inhibition was achieved for the culture incubated at 144 hrs. However, further increase in incubation period (at 168 and 192 hrs) showed a declining trend in inhibition zone.

![Figure 1: Influence of incubation period on antibacterial activity of marine *Streptomyces* sp. ABTRI12](image1.png)

Of the various growth medium, viz. Starch Casein broth, ISP – 5 (Glycerol asparagine agar), Glucose asparagine broth, Kuster’s broth, Maltose yeast extract broth, the starch casein broth media was highly utilized by this *Streptomyces* sp. ABTRI12 is shown in Figure 2.

![Figure 2: Influence of various growth media on antibacterial activity of marine *Streptomyces* sp. ABTRI12](image2.png)

**Antioxidant Activity**

*Estimation of Radical Scavenging Activity (RSA) Using DPPH Assay*

The DPPH free radical scavenging activity (%) of different concentrations of ethyl acetate extracts of both *Streptomyces* species and ascorbic acid is shown in Figure 3. The degree of stable DPPH* decolorization to DPPHH (reduced form of DPPH) yellow indicated the scavenging efficiency of the extract. The radical scavenging ability of different concentrations, namely 0.02, 0.04, 0.06, 0.08, 0.1, 0.12, 0.14, 0.16, 0.18, 0.2 mg/ml, of extracts of isolate was found to be 29, 35, 48, 51, 58, 62, 65, 74, 75, and 83% respectively. Ascorbic acid exhibited marked antioxidant activity of 84% in 0.2 mg/ml as compared to ethyl acetate extracts. Both ethyl acetate extracts and the standard exhibited dose dependent activity.

![Figure 3: Estimation of Radical Scavenging Activity (RSA) Using DPPH Assay](image3.png)
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Figure 3: Antioxidant activity of solvent extracts and ascorbic acid by DPPH assay

**Thin Layer Chromatographic Analysis of Compounds**

The screenings of confirmed the production of bioactive substances from the culture extracts by TLC analysis, the Rf values were ranged from 0.21 to 0.96 are shown in Table 5.

<table>
<thead>
<tr>
<th>Rf values</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>UV (254 nm)/Absorbance</strong></td>
</tr>
<tr>
<td>0.21</td>
</tr>
<tr>
<td>0.77</td>
</tr>
<tr>
<td>0.87</td>
</tr>
<tr>
<td>0.95</td>
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</tbody>
</table>

**Characterization of crude extracts**

**Infrared spectroscopy**

The presence of some functional group as revealed by IR spectral is shown in Figure 4. The FTIR Spectral analyses of extracts ABTRI 12 show certain common absorption band at 3398 cm⁻¹ is a characteristic of hydroxyl υ (O-H) and υ (N-H) vibrational frequency which are interchangeable. A common 3 vibrational peak between 2899 and 2977 cm⁻¹ are characteristic of a υ (C-H) symmetrical vibration of saturated hydrocarbon. The vibrational frequency υ (C-O) was observed in the spectra of the extracts at 1047 and 1087 cm⁻¹. Deviation from this region to a higher wave number was observed which is indicative of a secondary amide. These peaks were sharper than the υ (O-H) peaks due to reduction in hydrogen bonds which increases with electronegativity. Vibrational peaks at 1654 cm⁻¹ in extract signify the possibility of an aromatic compound. Based on the physical state (oily) of the extracts and the characteristic features of the infrared vibrational peaks in the spectra, terpenoids, long chain fatty acids and secondary amine derivatives are possible compounds in the extracts.

**DISCUSSION**

Actinomycetes are indeed well adapted and are functional members of the aquatic microbial community (Jensen et al., 1991). The result of this study corroborates the report of Rifaat et al. (2003) that actinomycetes are widely spread in various bodies of water and attached biofilms, where they play a great part in the carbon cycle due to their ability to grow at low concentration of carbonaceous substance and to degrade recalcitrant organic matter. Actinomycetes especially *Streptomyces*, have been reported from the marine sub habitats such as marine sediments (Takizawa et al., 1993; Vijayakumar et al., 2007); marine soil (Peela et al., 2005; Vijayakumar et al., 2007) and also from almost all parts of the world. Also, the dominance of *Streptomyces* among the actinomycetes especially in soils has been reported by many workers (Jensen et al., 1991; Peela et al., 2005). Hence they have worldwide distribution, which indicate their plasticity and adaptability to extremely varied environment. Also, a higher number of isolates were recovered from the rock scrapings compared to water sample, thus suggesting that actinomycetes can adhere to natural or artificial surfaces and within sessile multicellular communities known as biofilms. Biofilms is a complex microbial community including diverse species with a variety of functions.

The selected 6 isolates showed good antifungal activity, this characteristic features not remains with antifungal alone and also added antibacterial activity too. This wide spectrum activity nature is very common in marine actinomycetes (Ramesh, 2009; Jayaprakashvel, 2012). Morphological characterization of ABTRI 12, a broad spectral antagonistic isolates developed dark grey to white coloured spore mass. Further the strain developed spiral nature spore chain in its aerial mycelium. Sivakumar et al., (2005) reported that the characters can be used as marker by which an individual strain can be recognized. The present investigation concluded that the physiological characteristics of actinomycetes varied...
depending on the available nutrients in the medium and the physical conditions. Upon the growth of the isolates on various media, starch casein agar was observed to be the best medium for maximal growth.

In recent years much attention has been devoted to natural antioxidants and their association with health benefits (Ali et al., 2008). There are several methods available to assess antioxidant activity of compounds. An easy, rapid and sensitive method for the antioxidant screening of plant extracts is free radical scavenging assay using 1,1-diphenyl-2-picrylhydrazyl (DPPH) stable radical spectrophotometrically. In presence of an antioxidant, DPPH radical obtains one more electron and the absorbance decreases (Koleva et al., 2002). In this study, the scavenging activity of ethyl acetate extracts was found to be dose dependent i.e., higher the concentration, more was the scavenging activity. Though the DPPH radical scavenging abilities of the extracts were less than that of ascorbic acid, the study showed that the extracts have the proton-donating ability and could serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants.

The cultural extracts obtained from Streptomyces isolated from sediment soil showed nine bioactive regions were detected on the TLC plate with Rf values from 0.21 to 0.96 (Ilic et al., 2005). Similar result was obtained from the cultural extracts of marine sponges associated Streptomyces was reported (Dharmaraj and Sumantha, 2009). IR spectra of crude extract showed some different vibrational peaks of these functional groups in the extract depicts that the diverse activity they exhibited against test organisms during the susceptibility screening. The distribution of the antibiotic inhibition phenotype of Streptomyces with great antibacterial and antifungal activity which gave a similar spectra profile has also been reported (Ilic et al., 2007). Nevertheless, further investigation is needed in order to purify and determine the structure of the active components in the extracts.

CONCLUSION
The results of this study indicated that Streptomyces sp. ABTRI 12 possess significant DPPH free radical scavenging activity and antimicrobial activity too. Based on the results it can be concluded that the isolate produces extracellular secondary metabolite capable of scavenging DPPH free radicals so it can be an antioxidants. However further studies are needed to identify the chemical nature of the secondary metabolite produced by Streptomyces sp. ABTRI 12.

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REFERENCES
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