# Evaluation of antibiofilm efficacy of actinomycetes isolated from BRIS soil of Terengganu against *Corynebacterium pseudotuberculosis*

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**Abstract.** This study was performed to identify bioactive compounds in ethanolic extracts of actinomycetes (Saccharopolyspora sp - S5, Amycolatopsis sp - S7, Actinoplanes sp - AA11, and Micromonospora sp - AA141) isolated from the Beach Ridges Interspersed with Swales (BRIS) soil of Terengganu, Malaysia and to evaluate the antibiofilm efficacy of those actinomycete extracts against *Corynebacterium pseudotuberculosis*. GCMS analysis demonstrated that all actinomycete extracts produced known antibacterial bioactive compounds. FESEM analysis verified the ability of *C. pseudotuberculosis* to form biofilm in the present study while resazurin assay revealed acceptable antibiofilm activity of actinomycete extracts at 24 h.

**Keywords:** Actinomycetes; biofilm; BRIS soil*; Corynebacterium pseudotuberculosis.*

## 1 Introduction

Caseous lymphadenitis (CLA) is a highly infectious disease that affects small ruminants such as goats and sheep. It is caused by the bacterium *Corynebacterium pseudotuberculosis*. CLA is characterized by the formation of abscesses primarily in the lymph nodes, which contain a thick, cheesy material. The formation and development of abscesses can take several months, and they contain a thick, purulent material known as caseous necrotic material. These abscesses are often painful and can cause swelling and discomfort in the infected animal. The economic impact of CL includes high costs associated with treatment, decreased milk or wool production, and potential culling of infected animals to prevent the spread of the disease [1].

*Corynebacterium pseudotuberculosis* is a Gram-positive, rod-shaped bacterium that can survive in the environment for long periods. It is highly contagious and can be transmitted through direct contact with infected animals or through contaminated objects such as feeders, water troughs, and fences. Upon entry into the host's body, the


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bacterium is phagocytosed by macrophages, which are immune cells responsible for engulfing and killing foreign particles. However, *C. pseudotuberculosis* can evade the host's immune response and survive within the macrophages, leading to the development of abscesses. Biofilms are intricate communities of microorganisms, primarily bacteria, encased within a self-produced matrix of extracellular polymeric substances (EPS) matrix [2]. Controlling biofilm formation and dispersal is essential for mitigating their harmful effects, and this can be achieved through various compounds that interfere with EPS production, disrupt quorum sensing, or promote biofilm dispersal [3]. Biofilm formation by *C. pseudotuberculosis* has previously been demonstrated [4, 5]. It has been established that actinomycete extracts offer a diverse range of potential uses in inhibiting pathogens.

Actinomycetes are a group of Gram-positive bacteria known for their ability to form filamentous structures resembling fungi. They are widely distributed in various environments, including soil, water, and plants. For instance, mangrove forests have been established as highly dynamic ecosystems that cover and protect approximately 75% of the world's tropical and subtropical coastal areas and harbor a diverse range of marine, freshwater, and terrestrial flora and fauna. The mangrove forests are unique environments that favor the production of unusual metabolites among the residing microorganisms due to the large fluctuation of salinity and tidal gradients. Actinomycetes found in various mangrove habitats such as sediments, mangrove plant rhizosphere soil, and mangrove endophytes are classified into 25 genera, 11 families, and 8 suborders. One particular area where actinomycetes have been extensively studied is in the soil of Terengganu, Malaysia. Terengganu is a state located on the east coast of Peninsular Malaysia. It is known for its diverse ecosystem, including tropical rainforests, mangroves, and coastal areas. The Beach Ridges Interspersed with Swales (BRIS) soil in Terengganu is rich in organic matter and nutrients, making it an ideal habitat for various microorganisms, including a diverse range of actinomycetes belonging to various genera such as Streptomyces, Nocardiopsis, Micromonospora, and Actinomadura.

Researchers have conducted numerous studies on actinomycetes in the BRIS soil of Terengganu, aiming to identify and characterize their bioactive compounds. For example, reference [6] have demonstrated the antibacterial effect of Streptomyces sp. AA13, Amycolatopsis sp. AA12 and Micromonospora sp. AA141 against a panel of microorganisms consisting of *Escherichia coli* DSM 30083, *Bacillus subtilis* DSM 10, *Pseudomonas fluorescens* DSM 50090, and *Klebsiella pneumoniae* DSM 30104. Meanwhile, reference [4] have characterized the structure, biochemical composition, and antimicrobial susceptibility pattern of *C. pseudotuberculosis* biofilm. However, the potential application of actinomycetes isolated from BRIS soil of Terengganu to inhibit *C. pseudotuberculosis* biofilm remains uncertain. Therefore, this study was performed to determine the bioactive compounds of the ethanolic extract of actinomycetes isolated from BRIS soil of Terengganu and evaluate their antibiofilm efficacy against *C. pseudotuberculosis* biofilm.

# 2 Methodology

## 2.1 Preparation of Actinomycetes

Actinomycete isolates (Saccharopolyspora sp - S5, Amycolatopsis sp - S7, Actinoplanes sp - AA11, and Micromonospora sp - AA141) of BRIS soil of Terengganu were obtained from Microbiology Lab, Faculty of Science and Mathematics, Universiti Pendidikan Sultan Idris, Tanjung Malim, Perak. They were grown and isolated on yeast malt agar supplemented with 25 µg/mL cycloheximide and 25 µg/mL nalidixic acid to avoid microbial contamination. The isolates were characterized based on aerial spore mass color, reverse substrate mycelial and diffusible pigment by referring to the National Bureau of Standards (NBS) Color Name Chart. Colonies with suspected actinomycetes were picked and subcultured on ISP2 medium and incubated for 14 days at 28 °C.

## 2.2 Extraction of Actinomycete Isolates

Actinomycete isolates were extracted using ethanol as previously reported with minor modifications [7]. In brief, 1 mL of actinomycete culture was centrifuged at 14,000g for 10 min. Pellet was collected and mixed with 1 mL of ethanol and incubated overnight at room temperature. The mixture was then centrifuged at 14,000g for 10 min. Supernatant was collected and evaporated at 60 °C using heating block and stored at -20 °C. For GCMS analysis, the dried extract was reconstituted in a small volume of absolute ethanol. For antibiofilm assay, the dried extract was reconstituted in a small volume of 2% (v/v) DMSO.

## 2.3 Gas Chromatography-Mass Spectrometry

GC-MS analysis was performed using a Hewlett-Packard 6890N gas chromatography system with mass spectrometry (Hewlett-Packard 5973 inert mass selective detector). One µL of SMME was injected with a split ratio of 30:1. Helium gas was used as a carrier at 1.5 mL/min. The temperature of the HP-5MS column (length 30.0 m, internal diameter 0.25 mm, film-0.25 µm) was set at 150 °C for one min after the sample injection while the temperature was maintained at 290 °C with a 10 °C/min rate. The mass spectra generated during GC-MS analysis were interpreted using the National Institute of Standards and Technology (NIST) database.

## 2.4 Preparation of test microorganism

A clinical isolate of *C. pseudotuberculosis* was obtained from Veterinary Laboratory Service Unit (VLSU), Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM). It was cultivated in nutrient broth (Difco Laboratories, USA) at 37 °C for 48 h and adjusted to optical density (OD) of 0.7 at 600 nm before the biofilm assay.

### 2.5 FESEM

*C. pseudotuberculosis* biofilm was grown in a 6-well microplate. Overnight inoculum (4 mL) and fresh nutrient medium (1 mL) were added into the microplate wells. Glass cover slips were introduced into the biofilm assay with the surface of interest facing upward. The microplate was incubated overnight at 37 °C. Then, the glass cover slips were removed from the microplate and rinsed with phosphate-buffered saline twice. The biofilm on the glass cover slip was fixed in 4% (v/v) formaldehyde at 4°C for 3 h, rinsed with sterile distilled water thrice and dehydrated (once in 25% (v/v), 50% (v/v), 75% (v/v) and twice in 100% of ethanol each for 10 min). The glass slides were air dried overnight and observed at 5000x magnification using FESEM (Hitachi, Japan).

### 2.6 Microplate Biofilm Assay

Ethanolic extracts of actinomycetes were tested in the range between 10 µg/mL - 50 µg/mL. Overnight inoculum (100 µL) and actinomycete extracts (100 µL) were added into a 96-well microplate. Equal volume of intellectual property (IP)-protected antibiofilm cocktail and fresh broth were also added as positive and negative controls, respectively. After overnight incubation at 37 °C, the medium was discarded, and the microplate wells were washed with distilled water twice and heat-fixed at 60 °C for 30 min.

### 2.7 Resazurin Assay

Resazurin solution (0.02%, w/v) was prepared and stored at 4 °C in the dark until further use. To suspend the biofilm fractions, 220 µL of phosphate-buffered saline and 30 µL of 0.02% (w/v) resazurin were loaded into the microplate wells. The microplate was incubated at 37 °C for at least 3 h and microplate reader (ThermoFisher Scientific, USA) was used to measure absorbance values at 570 nm. The absorbance values from the microplate assay were used to calculate the percentage inhibition of biofilm according to the following equation:

$$Percentage\ (\%)\ Inhibition\ = \frac{(OD\ Negative\ Control - OD\ Experimental)}{(OD\ Negative\ Control)} \times 100 \quad (1)$$

### 2.8 Data Analysis

The experimental results were expressed as the mean ± standard error of the mean of five triplicates. The differences between the samples and control were determined using independent T-test and the result was considered significant if $p<0.05$. The half-maximum biofilm inhibitory concentration (BIC50) values for the biofilm inhibition were calculated by using GraphPad Prism software version 8.0 [8].

# 3 Results

## 3.1 Bioactive Compounds

Table 1 shows a list of bioactive compounds produced by ethanolic extracts of actinomycete isolates. A total of six, four, three and four bioactive compounds were identified in isolates AA11, AA141, S5 and S7, respectively. Cyclopentasiloxane was identified in both isolate AA11 and AA141. Cyclodecasiloxane was identified in both isolate AA141 and S7. Diisooctyl phthalate was identified in both isolate AA11 and S7. Meanwhile, 3,4-dihydroxyphenylglycol was identified in all isolate AA11, AA141 and S7. The major bioactive compounds identified in isolates AA11, AA141, S5 and S7 were found to be dimethyl trisulfide, cycloheptasiloxane, thiazolo [3,2-a] pyrimidine and diisooctyl phthalate, respectively (Figure 1).

**Table 1.** Bioactive compounds identified in ethanolic extracts of actinomycetes isolated from BRIS soil of Terengganu, Malaysia. Those with known antibacterial activity are indicated by *

| | Retention time (Peak area) | | | |
|---|---|---|---|---|
| | AA11 | AA141 | S5 | S7 |
| Cyclopentasiloxane* | 13.7 (2.89%) | 10.67 (1.33%) | | |
| Cycloheptasiloxane* | | 16.48 (17.49%) | | |
| Cyclodecasiloxane* | | 23.08 (13.38%) | | 23.08 (3.76%) |
| 2,4-dihydroxybenzoic acid* | | | 18.91 (1.88%) | |
| 3,4-dihydroxyphenylglycol* | 18.99 (1.48%) | 22.82 (2.68%) | | 18.87 (4.11%) |
| Cholestane* | | | 21.06 (2.83%) | |
| Dimethyl trisulfide* | 21.22 (20.44%) | | | |
| Succinic acid* | 22.46 (14.4%) | | | |
| Diisooctyl phthalate* | 22.68 (13.01%) | | | 22.48 (19.92%) |
| Octadecamethylcyclononasiloxane* | 22.83 (16.94%) | | | |
| Benzoic acid* | | | | 21.54 (2.23%) |
| Thiazolo [3,2-a] pyrimidine* | | | 24.06 (9.29%) | |

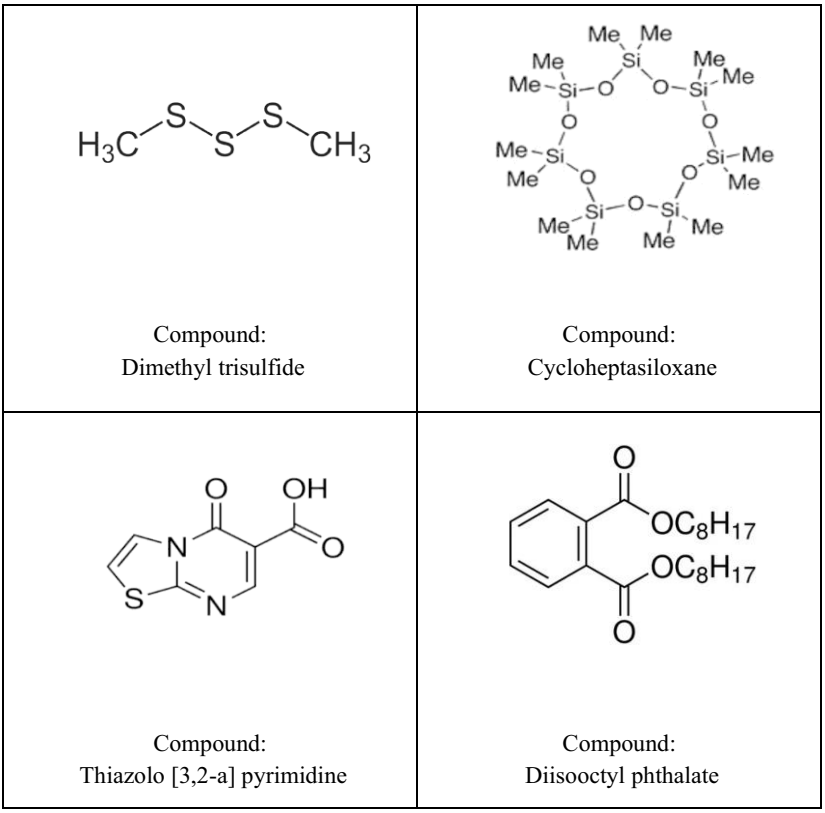


**Fig. 1.** The major bioactive compounds in isolate AA11 (Dimethyl trisulfide), AA141 (Cycloheptasiloxane), S5 (Thiazolo [3,2-a] pyrimidine) and S7 (Diisooctyl phthalate)

### 3.2 Biofilm Morphology

Figure 2 shows biofilm formation by *C. pseudotuberculosis* at 24 h. Rod-shaped cells of *C. pseudotuberculosis* were attached to surface and encapsulated in fibrous extracellular matrix. This result verified the ability of *C. pseudotuberculosis* to form biofilm in the present experiment.

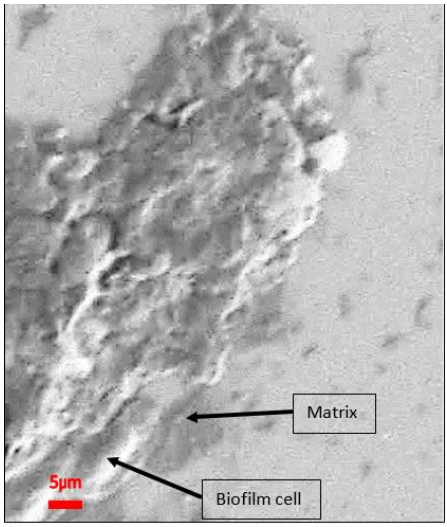


**Fig. 2.** FESEM image of *C. pseudotuberculosis* biofilm at 5000x magnification.

### 3.3 Viability of *C. pseudotuberculosis* Biofilm

Figure 3 shows viability of *C. pseudotuberculosis* biofilm in the presence of actinomycete isolates. All actinomycete isolates were tested against *C. pseudotuberculosis* at different stages of biofilm life cycle namely 6 h (initial attachment), 12 h (microcolony formation), 18 h (immature biofilm), and 24 h (mature biofilm). Overall, *C. pseudotuberculosis* biofilm showed lower viability in the presence of different concentrations of ethanolic extracts at all stages.


**Fig. 3.** Heatmap for viability of *C. pseudotuberculosis* biofilm in the presence of ethanolic extracts of actinomycete isolated from BRIS soil of Terengganu, Malaysia.

### 3.4 Biofilm inhibition

Figure 4 shows biofilm inhibition by ethanolic extracts of actinomycete isolates at 24 h (mature biofilm). The highest percentage inhibition by isolates S5, S7, AA141, and A11 were found to be 12.65%, 13.40%, 20.08%, and 16.96%, respectively. All actinomycete isolates showed concentration-dependent biofilm inhibition, except isolate AA141. Isolate S5 and S7 insignificantly ($p>0.05$) inhibited biofilm at all test concentrations. Isolate AA141 significantly ($p<0.05$) inhibited biofilm at all test concentrations. Isolate AA11 significantly ($P<0.05$) inhibited biofilm at the test concentrations of 30 µg/mL – 50 µg/mL.

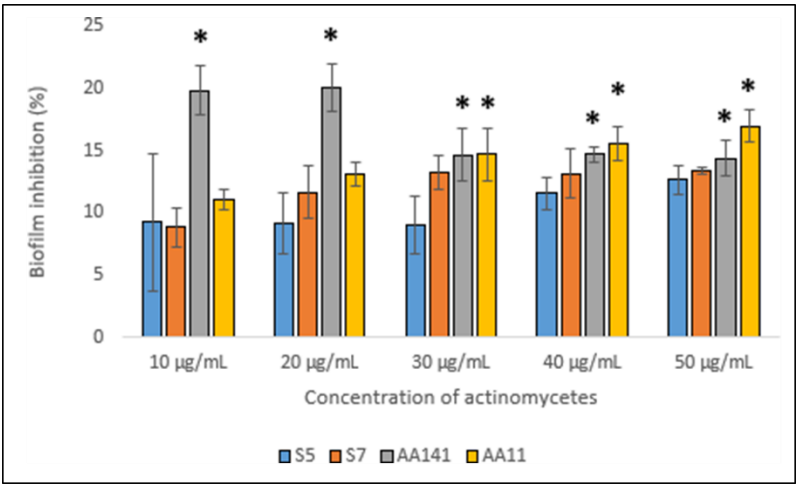


**Fig. 4.** Inhibition of *C. pseudotuberculosis* biofilm (24 h) by ethanolic extracts of actinomycete isolated from BRIS soil of Terengganu, Malaysia, n = 5. Significant difference between control and test concentration is shown by *.

### 3.5 Biofilm inhibitory concentration (BIC50) values

The BIC50 value was computed to ascertain the concentration necessary for inhibiting 50% of biofilm. A lower BIC50 value suggests stronger antibiofilm activity. The BIC50 values showed that isolate AA141 (11.72 µg/mL) was most effective against *C. pseudotuberculosis* biofilm, followed by isolates AA11 (12.05 µg/mL), S7 (14.34 µg/mL), and S5 (16.76 µg/mL).

## 4 Discussion

One of the challenges in the treatment of CLA is the ineffectiveness of conventional antibiotics against biofilm-associated bacteria. Biofilm-embedded bacteria exhibit increased resistance to antimicrobial agents compared to their planktonic counterparts [9]. Antibiofilm study allows for the discovery and development of alternative

strategies that can disrupt biofilm architecture and enhance the susceptibility of bacteria to antibiotics. This can involve the use of compounds or approaches that target the biofilm matrix, enzymes that degrade the matrix, or agents that interfere with bacterial communication pathways necessary for biofilm formation [10]. The present study used resazurin assay to explore the potential of actinomycetes isolated from BRIS soil of Terengganu for the control of *C. pseudotuberculosis* that causes CLA. In this assay, resazurin, a blue, non-fluorescent dye, is reduced to resorufin, a pink, fluorescent compound, by metabolically active cells. The same experimental approach has been widely used in other antibiofilm studies [2, 4].

Actinomycetes are a group of filamentous, Gram-positive bacteria known for their ability to produce a wide range of bioactive compounds, including volatile bioactive compounds. These compounds have ecological significance in microbial communities, potential applications in agriculture and medicine, and are the subject of ongoing research to harness their production for various purposes. Antibacterial bioactive compounds produced by actinomycetes such as dimethyl trisulfide has previously been reported [11]. Dimethyl trisulfide is a volatile sulfur compound and is a major aroma constituent in cooked vegetables. It is also a bacterial and human decomposition product. The present study identified dimethyl trisulfide as a major bioactive compound in isolate AA11. The dimethyl trisulfide is known to be one of major phytochemical constituents in essential oil of *Allium tenuissimum* flower [12]. Antibacterial potential of this compound has been shown to cause damage to the surface structure and cytomembrane of *E. coli* leading to the leakage of cellular nucleic acids [13]. Cycloheptasiloxane is a silicon-based compound. It is also a hydrophobic compound, meaning it repels water. The present study identified cycloheptasiloxane as a major bioactive compound in isolate AA141. It is also known to be one of the major bioactive compounds in sea cucumber extracts [14]. Reference [15] has reported antibacterial, immunomodulatory, antitumor, antifungal and antifouling properties of cycloheptasiloxane.

In the present study, isolates S5 and S7 insignificantly inhibited *C. pseudotuberculosis* biofilm. This inhibitory effect is most probably due to the presence of major antibacterial bioactive compounds, thiazolo [3,2-a] pyrimidine and diisooctyl phthalate, respectively. This finding generally corroborates the fact that actinomycetes have inhibitory potential against fungal species [16] and foodborne pathogens [17]. To date, the information pertaining to the antibiofilm mode of action of bioactive compounds identified herein has not yet been reported elsewhere. Nevertheless, some of them have been demonstrated to exhibit common antibacterial mode of action. For instance, reference [18] has demonstrated that thiazolo [3,2-a] pyrimidine inhibits bacteria by binding to receptor methionyl-tRNA synthetase which is involved in protein biosynthesis. Thus, it is possible that isolate S5 containing thiazolo [3,2-a] pyrimidine may inhibit *C. pseudotuberculosis* biofilm using the same mode of action as the protein biosynthesis also takes place in biofilm stage [19, 20]. In the present study, isolates S5 and S7 displayed relatively lower biofilm inhibition. This phenomenon is not understood; however, it may be due to the failure of those compounds to completely penetrate the extracellular matrix that protects the biofilm cells. Our FESEM image revealed the extracellular matrix that surrounds and protects the biofilm cells.

A multidimensional approach that combines different strategies such as antibiotics, antifungals, plant extracts, disinfectants, nanoparticles and actinomycete extracts is necessary to effectively control biofilm infections [8, 21, 22, 23]. By integrating these diverse approaches, a comprehensive strategy can be developed to tackle biofilm infections effectively, addressing both microbial growth and biofilm matrix stability. The potential application of marine actinomycetes in inhibiting *C. pseudotuberculosis* biofilm was presented herein. The related literature information remains scarce, however, the potential application of plant extracts to control *C. pseudotuberculosis* infection has previously been reported [24]. They demonstrated the antibacterial efficacy *of Ocimum sanctum*, *Moringa oleifera* and *Murraya koenigii* leaves against *C. pseudotuberculosis* isolated from camel.

## 5 Conclusion

We demonstrate that the ethanolic extract of actinomycetes isolated from BRIS soil of Terengganu, Malaysia produces various antibacterial bioactive compounds. The major bioactive compounds in isolates S5, S7, AA11, and AA141 were found to be thiazolo [3,2-a] pyrimidine, diisooctyl phthalate, dimethyl trisulfide, and cycloheptasiloxane, respectively. The present study also provides preliminary information about the antibiofilm potential of all actinomycete isolates against *C. pseudotuberculosis*, however, further experimental works are necessary to confirm the findings reported herein.

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**Paper Contribution to Related Field of Study.** Marine actinomycetes have garnered immense interest in drug discovery due to their ability to produce a vast array of bioactive compounds which often possess potent biological activities. Many well-known antibiotics, such as streptomycin and tetracycline, are derived from terrestrial actinomycetes. Actinomycetes-derived compounds have demonstrated antimicrobial activities against multidrug-resistant bacteria, making them valuable in the fight against the global antibiotic resistance crisis. By exploring the vast marine biodiversity and isolating new strains of actinomycetes, scientists could discover novel compounds that can be developed into effective therapeutics. The work presented in this article may be useful in assisting the development of therapeutic strategy to control CLA disease in small ruminants and sustain livestock industry.

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