

MARINE *Streptomyces* SP. UKMCC_PT15 PRODUCING UNDECYLPRODIGIOSIN WITH ALGICIDAL ACTIVITY

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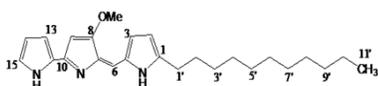
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Graphical abstract



Abstract

Marine actinomycetes are now in demand as they are capable of producing unique and novel compounds with wide biological activities. Marine *Streptomyces* sp. UKMCC_PT15 previously isolated from seawater collected in Pulau Tinggi, Johor was used in this study. Molecular identification showed high similarity of *Streptomyces* sp. UKMCC_PT15 with *Streptomyces fradiae* and *S. diastaticus* subsp. *ardesiacus* based on partial sequence of 16S rRNA gene. Further characterisations of this bacterium include spore morphology using SEM, growth on various media, salt tolerance test and carbon utilisation profile. This bacterium had straight spore chain with smooth-surfaced spores and was able to tolerate up to 11% NaCl with capabilities of utilising >40 carbon sources. Undecylprodigiosin (C₂₅H₃₅N₃O) was successfully purified through succession of column chromatography and finally using HPLC. Structure elucidation was confirmed through NMR spectroscopy, MS and comparison with established data. This compound demonstrated strong antibacterial activities against *S. aureus*, *B. subtilis* and *C. albicans* but weak antibacterial activities against *E. coli*, *P. aeruginosa* and Methicillin-resistant *S. aureus* (MRSA). Interestingly, undecylprodigiosin also demonstrated algicidal activity when tested against toxic dinoflagellates, *A. minutum* and *P. bahamense*, both of which are responsible for harmful algal blooms (HABs). Undecylprodigiosin with concentration of 10-100 µg/ml gave ~100% algicidal activity against both dinoflagellates. Further testing with undecylprodigiosin concentration < 10 µg/ml showed that undecylprodigiosin was capable of killing significantly high numbers of both dinoflagellates, giving a high algicidal activity. Findings from this study suggested the potential use of undecylprodigiosin as algicidal agent which could be used for the mitigation of HABs.

Keywords: Marine *Streptomyces*, undecylprodigiosin, antibacterial, algicidal

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1.0 INTRODUCTION

Bacteria within the order *Actinomycetales* (actinomycetes) belonging to the class Actinobacteria, are Gram positive bacteria with high

G+C content. They are filamentous with some having the ability to produce spores. Actinomycetes are common soil inhabitants and they generally have a complex life cycle, filamentous morphology, and are renowned for the production of structurally diverse

and biologically active small organic molecules commonly referred to as secondary metabolites [1]. On the premise of finding novel and unique secondary metabolites, researchers are exploring new locations due to re-discovery of similar compounds from soil actinomycetes. New and underexplored locations such as the ocean, mangrove, desert and dunes are considered as new resources of actinomycetes.

The genus *Streptomyces* represents the most important genus in the production of medically and industrially valuable molecules. Approximately over 500 species belong to this genus *Streptomyces*, the largest number of species in nature. About 75% of antibiotics in the market currently are produced from this genus [2]. *Streptomyces* has the ability to produce a wide range of bioactive molecules which includes antibiotics, immunosuppressant agents, anticancer agents, antimalarial agents, enzyme inhibitors and others [3]. Owing to this unique capability of producing different bioactive molecules makes this genus remained unchallenged without a potential competitor from other microbial group.

Marine *Streptomyces* species are widely distributed in marine organisms such as in the fishes, molluscs, sponges, seaweeds, besides sediments and seawater as they can produce abundant spores that are readily dispersed. Furthermore, their unique capabilities of producing metabolites of commercial interest make them desirable to be pursued in search of novel metabolites despite the fact that there is still perception that marine *Streptomyces* will produce the same compounds as their terrestrial counterpart. Marine actinomycetes are prolific secondary metabolite producers, and majority of the compounds discovered are produced from the single genus *Streptomyces*. Many studies have shown that besides producing metabolites that are known from the terrestrial, marine *Streptomyces* are also capable to produce new metabolites [4],[5]. The past decades have witnessed the increasing number of novel metabolites possessing potent bioactivity isolated from marine-derived *Streptomyces* [6]. Marine *Streptomyces* are also capable of producing a variety of bioactive metabolites that possess a wide range of activities such as antibacterial, antifungal, antitumor, immunosuppressant agent, and many more. Examples of metabolites produced by marine *Streptomyces* include peptides, quinones, terpenes, macrolides, polyketides and enzyme inhibitor [7],[8].

Although many compounds have been described for actinomycetes, the algicidal potential of actinomycetes is yet to be explored. To date, few studies described the algicidal activity of the actinomycetes themselves or the compounds derived from them against microalgae particularly in mitigating harmful algal blooms (HABs) [9], [10], [11]. Harmful algal blooms (HABs) commonly known as red tides are caused by rapid growth and accumulation of microalgae in the ocean. HABs pose significant adverse impact on the marine environments, aquaculture activities and the public health.

Alexandrium minutum and *Pyrodinium bahamense* are among the important member of paralytic shellfish toxin (PST)-producing marine dinoflagellates especially in tropical waters. *Pyrodinium bahamense* produces saxitoxin and other toxin derivatives while *Alexandrium minutum* produces gonyautoxins (GTX), all of which cause Paralytic Shellfish Poisoning (PSP) when human ingest shellfish especially filter-feeding bivalves because they accumulate toxins as they fed this organism [12]. In the context of harnessing marine actinomycetes full potential, this study aimed at assessing the potential of compound produced by this bacterium as antimicrobial and algicidal agents beside identification and characterisation of marine *Streptomyces* sp. UKMCC_PT15.

2.0 METHODOLOGY

2.1 Molecular Identification of and Characterisation of Marine *Streptomyces* UKMCC_PT15

Marine *Streptomyces* UKMCC-PT15 was previously isolated from seawater collected in Pulau Tinggi, Johor. Genomic DNA *Streptomyces* UKMCC_PT15 was extracted using Wizard® Genomic DNA Purification Kit (Promega) following the manufacturer's protocol. PCR amplification of 16S rRNA gene was performed on the extracted DNA using the following set of primers: 27F (5'-AGAGTTTGATCCTGGCTCTCAG-3') and 1492R (5'-GGTACCTGTTACGACTT-3'). The PCR reaction was carried out using under the following conditions: initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 55°C for 60 s and 72°C for 4 min; and extension step at 72°C for 10 min. The amplification products were confirmed using 1% agarose gel and sent to 1st Base Laboratory, Malaysia for sequencing.

Several media were used for growth and cultural characterisation of *Streptomyces* UKMCC_PT15 namely, SYE (Starch-Yeast Extract-Seawater), Marine Agar (MA), ISP2, ISP3, ISP4, Czapek's agar, Gause Synthetic Agar, Nutrient agar (NA) and Potato dextrose agar (PDA). All plates were incubated at 30°C for 2-3 weeks. The colour of aerial and substrate mycelia, the presence/absent of diffusible pigment and growth of *Streptomyces* UKMCC_PT15 were observed after the incubation period.

For salt tolerance test, the medium used for this purpose was SYE without the seawater but with the addition of different amount of NaCl in the range of 1% - 12%. The plates were incubated at 30°C for 2-3 weeks.

Scanning electron microscopy (SEM) was performed on 7-day old culture grown on SYE agar to assess the morphological characteristics. Colonies on plate was cut into 1 cm x 1 cm and fixed in 0.1% glutaraldehyde for overnight. Serial dehydration from 30% to 100% was performed followed by critical point drying (CPD). Before viewing, samples were sputtered with gold and viewed with SEM Philips XL 300.

Carbon utilisation was determined using The Biolog GP2 MicroPlate following the manufacturer's instructions. The spores were collected and resuspended in a GP Inoculating Fluid and the turbidity was adjusted to 20%. Thioglycollate solution was then added to the solution to prevent the cells from clumping together. About 150 µl of the cells suspension was pipetted into each wells of GP2 MicroPlate. The MicroPlate was incubated at 30°C for 2 days. The MicroPlate was read using Biolog MicroStation™ and compared to the GP Database.

2.2 Purification and Structure Elucidation of Undecylprodigiosin

Streptomyces sp. UKMCC_PT15 was cultured in liquid medium of SYE and incubated at 30°C for 7 days with shaking at 150 rpm. The cells were harvested and extracted with methanol overnight, followed by evaporation to dryness using a rotary evaporator. Crude extract was subjected to flash column chromatography and sephadex LH-20 column chromatography and finally, undecylprodigiosin was purified using HPLC with diode array detector L-7455 (Hitachi) and a BioSil® C₁₈ column (10 x 250 mm, 5µ) (Bio-Rad) with mobile phase comprised of 95% methanol and 5% of 0.1% acetic acid and flowrate of 3 ml/min at a wavelength range of 200-800 nm. Structure elucidation of compound undecylprodigiosin was conducted through NMR spectroscopy (¹H, ¹³C, HSQC, HMBC and COSY), FTIR and ESI-MS.

2.3 Evaluation of Antimicrobial Activities

Disc diffusion assay was carried out following the method as described by Kirby-Bauer et al. [13] with modification. Cell suspension of the test microorganism was prepared following a 0.5 McFarland turbidity standard. Test microorganisms used were as follows: *E. coli* ATCC 10536, *P. aeruginosa* ATCC 27853, *P. mirabilis* ATCC 12453, *S. aureus* ATCC 11632, MRSA N12, *B. subtilis* ATCC 11774 and *C. albicans* from Institute for Medical Research, Malaysia (IMR). Mueller Hinton agar was then inoculated with the test microorganism followed by placing the 6 mm disc containing 20 µg of undecylprodigiosin as well as disc containing reference antibiotic (chloramphenicol) on the agar. The agar plate was inoculated at 37°C (bacteria) for 24 hours or 28°C (*C. albicans*) for 2 days. The test was carried out in duplicate. The antimicrobial activity was determined as a clear zone of inhibition surrounding the disc with the diameter expressed in mm.

2.4 Evaluation of Algicidal Activity

Alexandrium tamarense and *Pyrodinium bahamense* cultures used in this study were from marine microalgae culture collection at Universiti Kebangsaan Malaysia. The cultures were maintained in sterile ES-DK medium prepared with natural sea

water and incubated at 26 °C under a 14 h: 10 h light dark cycle. Both dinoflagellates in the logarithmic growth phase was diluted with ESDK medium to a final density of 2 x 10⁴ cells/ml. ESDK medium (1 ml) with various concentrations of undecylprodigiosin were added to each tested algal suspension (1 ml) in the well of 6-well plate. The final concentrations of undecylprodigiosin used were 1 to 100 µg/ml. After incubation at 26 °C for 24 h, the number of viable algal cells was determined using a hemocytometer at magnification of x100. The algicidal activity was calculated using the following formula:

$$\text{Algicidal activity (\%)} = \left(1 - \frac{N_t}{N_0} \right) \times 100$$

N_t = viable individuals of algal after the treatment
N₀ = initial individuals of algal

3.0 RESULTS AND DISCUSSION

3.1 Marine *Streptomyces* sp. UKMCC_PT15

Partial sequence of *Streptomyces* sp. UKMCC_PT15 was deposited to GenBank under accession number JF950277. Comparison with GenBank database showed *Streptomyces* sp. UKMCC_PT15 had high similarity (98%) with *S. fradiae* and *S. diastaticus subsp ardesiacus*. SEM image revealed straight spore chain with smooth-surfaced spores (Figure 1). *Streptomyces* sp. UKMCC_PT15 showed good growth on SYE, MA, PDA, ISP2 and ISP4 but poor growth on ISP3, Czapek's and Gause synthetic media. Diffusible pigment was not produced in any of the medium tested. The substrate mycelium of *Streptomyces* sp. PT15 was red in colour when growing on SYE, MA, PDA, ISP4, Czapek's and Gause's synthetic media but lost its red pigment when growing on NA, ISP2 and ISP3 media.

Streptomyces sp. UKMCC_PT15 could tolerate up to 11% NaCl demonstrating its adaption to the marine environment. This bacterium was found to show intense dark red pigmented colonies at 4% NaCl that gradually decreased as the NaCl concentration increased. At 8% of NaCl, a very faint red almost pale pink colony was observed. Concentration of NaCl higher than 8% resulted in the disappearance of the red pigment completely. This suggested the influence of NaCl on the pigment production in this bacterium. Interestingly, this bacterium was able to utilize >40 carbon sources present in the Biolog GP2 MicroPlate, amongst them were dextrin, palatinose, glycerol, gentiobiose, maltose, xylose, mannitol and mannose.

3.2 Structure Elucidation of Undecylprodigiosin

After a succession of column chromatography, undecylprodigiosin was successfully purified through HPLC using combination of 95% methanol and 5% of 0.1% acetic acid as mobile phase at retention time of 12 min (Figure 2). Undecylprodigiosin was a deep red

magenta solid with maximum UV absorption at 530 nm in methanol.



Figure 1 SEM image of *Streptomyces* sp. UKMCC_PT15

$^1\text{H-NMR}$ spectrum recorded aromatic protons at δ_{H} 6.21, 6.84, 7.00, 6.09 and 6.95 ppm, a triplet at δ_{H} 4.01 ppm corresponded to OMe and, NH protons at δ_{H} 12.85 and δ_{H} 12.69 ppm (Figure 3). $^{13}\text{C-NMR}$ spectrum recorded 13 carbons corresponded to the pyrrole rings - δ_{C} 93.0, 111.9, 112.5, 116.3, 117.8, 121.3, 122.0, 125.9, 127.5, 129.2, 148.7, 153.1 and 166.2 ppm. OMe was observed at δ_{C} 58.7 ppm and there was an overlap at δ_{C} 29.7 – 31.9 ppm. Further NMR experiments - HSQC, HMBC and COSY were carried out to elucidate the structure of this compound. ESI-MS was recorded at 394.28 m/z ($[\text{M}+\text{H}]^+$). Based on NMR spectroscopy results, ESI-MS data and by comparison of the data with the published data [14] the compound was established as undecylprodigiosin $\text{C}_{25}\text{H}_{35}\text{N}_3\text{O}$ (393.5 g/mol).

Prodiginines are red tripyrrole antibiotics containing a pyrrolylpyromethane skeleton. Members of this group include prodigiosin, undecylprodigiosin, cycloprodigiosin and cyclonoylprodigiosin. Undecylprodigiosin ($\text{C}_{25}\text{H}_{35}\text{N}_3\text{O}$) is a linear tripyrrole and highly nonpolar compound. Undecylprodigiosin has maximum UV absorption of at 535 nm in methanol, 535 nm in acidic methanol, and 470 nm in alkaline methanol [15]. Undecylprodigiosin is produced by *Streptomyces longisporus ruber*, *Streptoverticillium rubrericiculi*, *Actinomadura madurae*, *Streptomyces coelicolor* A3(2) and *Saccharopolyspora* sp. [16]. Undecylprodigiosin is the major component of a mixture of at least 3 prodiginosins produced by *S. coelicolor*. The production of this undecylprodigiosin usually occur in a growth phase-dependent manner, occurring in the transition and stationary phases of liquid-growth cultures and coincides with the onset of morphological development in surface-grown cultures [17]. Although *Streptomyces* sp. UKMCC_PT15 showed high similarity to *S. fradiae* and *S. diastaticus subsp ardesiacus*, there was no report on the production of undecylprodigiosin from these two *Streptomyces* species. Initially, it was thought that

Streptomyces sp. UKMCC_PT15 to be similar with *S. fradiae* which is also a red pigmented bacterium, however, phenotypic and genotypic characteristics showed otherwise. *S. fradiae* is known to produce a variety of compounds such as actinomycin Z, tylosine, frenolicin and hybrimycin but no undecylprodigiosin was reported being produced by *S. fradiae*. Whereas *S. diastaticus subsp ardesiacus* is a yellow/beige pigmented bacterium and was reported to produce polyene macrolide antibiotics, rimocidin and CE-108 and antifungal, oligomycins A and C. Thus, this may indicate the possibility of *Streptomyces* sp. UKMCC_PT15 to be a new species of *Streptomyces*.

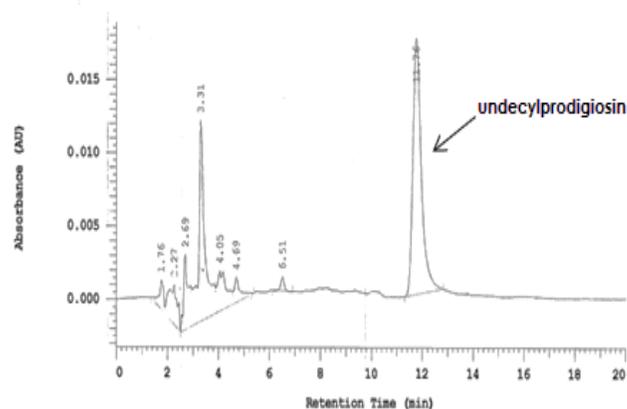


Figure 2 HPLC profile of undecylprodigiosin

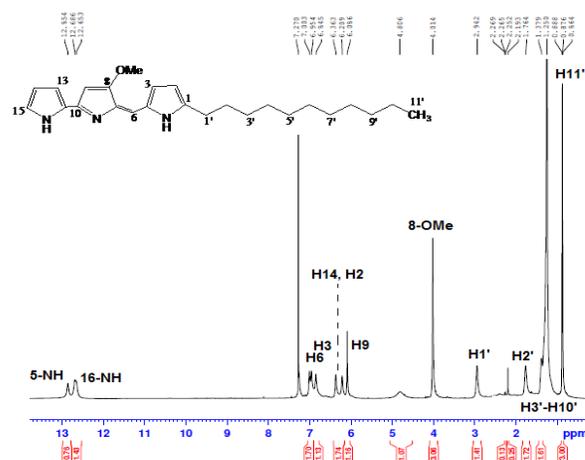


Figure 3 $^1\text{H-NMR}$ spectrum of undecylprodigiosin in CDCl_3

3.3 Antimicrobial Activity of Undecylprodigiosin

Undecylprodigiosin demonstrated strong activity against *S. aureus*, *B. subtilis* and *C. albicans* but weak activity against *E. coli*, *P. aeruginosa* and MRSA (Table 1). Stankovic *et al.* [18] also reported similar findings for antimicrobial activity of undecylprodigiosin from *Streptomyces* sp. JS520 against *B. subtilis* and *C. albicans*. Undecylprodigiosin, however, had no effect

on *P. mirabilis* which indicated the resistance of *P. mirabilis* to undecylprodigiosin. Difference in susceptibility between Gram-negative and gram positive bacteria towards undecylprodigiosin might be related to the cell wall morphologies. Gram-positive bacteria lacks an outer polysaccharide membrane bearing the structural lipopolysaccharide components, making the cell wall more permeable to lipophilic solutes [19]. Furthermore, the presence of porins at the outer membranes of Gram-negative bacteria which serve as membrane channels and can be closed to prevent the absorption of antimicrobial substance upon contact with low molecular weight compounds help to increase the resistance of Gram-negative bacteria [20]. Nevertheless, undecylprodigiosin holds the prospective to be developed further for antimicrobial agent.

Table 1 Antimicrobial activity of undecylprodigiosin from *Streptomyces* sp. PT15

Test microorganisms	Diameter of inhibition zone (mm)
<i>E. coli</i>	9
<i>P.mirabilis</i>	-
<i>P. aeruginosa</i>	10
<i>S. aureus</i>	15
MRSA	10
<i>B. subtilis</i>	11
<i>C. albicans</i>	13

3.4 Algicidal Activity of Undecylprodigiosin

Concentrations of undecylprodigiosin in the range of 10 to 100 µg/ml gave almost 100% algicidal activity for *A. minutum* and *P. bahamense*. Undecylprodigiosin at concentrations 5 to 9 µg/ml gave >95% and >90% algicidal activity for *A. minutum* and *P. bahamense* respectively. At the lowest concentration of undecylprodigiosin (1 µg/ml), algicidal activity was detected at 88.3% for *A. minutum* and 80.6% for *P. bahamense* (Table 2). Treatment with undecylprodigiosin caused the algal to shrink and eventually lead to cell death. Based on the results obtained, it could be implied that undecylprodigiosin was capable of killing significantly high numbers of both dinoflagellates even at low concentrations.

Numerous activities including anticancer, immunosuppressive properties and antiprotozoal had been described for undecylprodigiosin [21],[22], [23]. However, there was no report yet on the application of undecylprodigiosin as algicidal agent. In terms of algicidal potential, another member of the prodiginines named prodigiosin is more prominent compared to undecylprodigiosin as demonstrated by several studies. Prodigiosin produced by γ -Proteobacterium MS-02-063 was found to be able to kill *Gymnodinium impudicum*, *Alexandrium tamarense*, *Cochlodinium polykrikoides*, *Heterosigma akashiwo* and *Heterocapsa circularisquama* [24], and prodigiosin from *Serratia marcescens* was a potent algicidal agent against *Nitzschia closterium*,

Skeletonema costatum and *Anabena flosaquae* [25]. Additionally, little is known on the effectiveness of applying bacteria or compounds derived from them in mitigating *Alexandrium* spp. and *Pyrodinium* spp. Manset et al. [26] reported the use of algicidal bacteria against *Pyrodinium bahamense* and *Alexandrium* spp. through direct and indirect interactions between the algicidal bacteria and dinoflagellates. Wang et al. [27] described algicidal protein against *A. tamarense* but so far there is no report yet on algicidal compounds derived from bacteria against *Pyrodinium* spp. Thus, this is the first report on the use of undecylprodigiosin from *Streptomyces* sp. UKMCC_PT15 as algicidal agent against *A. minutum* and *P. bahamense*. Further refinement and understanding the underlying mechanism may aid in developing undecylprodigiosin as algicidal agent in mitigating harmful algal blooms.

Table 2 Algicidal activity of undecylprodigiosin from *Streptomyces* sp. PT15 against *A. minutum* and *P. bahamense*

Undecylprodigiosin (µg/ml)	Algicidal Activity (%)	
	<i>Alexandrium minutum</i>	<i>Pyrodinium bahamense</i>
1	88.3	80.6
2	90.1	83.2
3	92.5	86.0
4	93.0	88.7
5 - 9	>95.0	>90.0
10 - 100	99.9	99.8

4.0 CONCLUSION

In conclusion, undecylprodigiosin purified from marine *Streptomyces* sp. UKMCC_PT15 holds the potential not only as antimicrobial agent but also as algicidal agent against the harmful algae *A. minutum* and *P. bahamense*. Assessment on the practical aspects may help to improve the utilisation of undecylprodigiosin in mitigating harmful algal bloom in the field.

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