Detection, enumeration and isolation of antibiotic producing actinomycetes from d...

Detection, enumeration & Isolation of Antibiotic Producing Actinomycetes from Different Soil Samples of the Sunshine Coast, Australia

Abstract

The aim of the present study was to isolate and evaluate the antimicrobial potential of actinomycetes in differing soils of the Sunshine Coast, Australia. Secondary metabolites of actinomycetes are prominent source of antibiotics.

A total of 8 soil plugs, eachwere screened for antimicrobial activity and antibiotic content.

The ethyl acetate extract of fermented broth an actinomycete strain, identified as Streptomyces pratensis exhibited significant antimicrobial activity against Staphylococcus aureus ATCC 29213 with MIC 0. 25 µg/ml and Mycobacterium tuberculosis Strain H37Rv with MIC 0. 062 µg/ml. The strain S. pratensis IIIM06 was grown on large scale and their broth was extracted with ethyl acetate. The extract was subjected to various chromatography techniques which led to the isolation of four compounds whose structures were established as actinomycin C1, actinomycin C2, actinomycin C3 and actiphenol on the basis of spectral data analysis. Actinomycin C1, C2 and C3 exhibited potent antimicrobial activity against S. aureus as well as M. tuberculosis. The isolated indigenous actinomycetes exhibited good antibacterial activity and the study reveals that IIIM06 is a promising strain and could be of great potential for industrial applications.

Biotech – new areas – extremophiles

Undiscovered (Actinomycetes)

order *Actinomycetales*

genus *Streptomyces*

family *Actinosynnemataceae*

Introduction

Diverse microorganisms thrive in heterogeneous soil habitats, generally in much higher numbers than freshwater and marine habitats, at typically, 10^6 – 10^9 bacteria per gram. Organic matter concentrations are high in soils, favouring the growth of heterotrophic microbes, that play a critical role in decomposition and recycling. Availability of fixed forms of nitrogen for microbial survival is a limiting factor in soils. Soils are composed of many microhabitats. Autochthonous microbes fill functional roles called niches in their habitats. In surface soils, indigenous microbes feed on high concentrations of organic nutrients. The abiotic limitations of certain soils restrict the development of microbial populations (Atlas & Bartha 1998).

Soil moisture is linearly to microbial respiration. With low water content, most bacteria, become inactive, however many yeasts and filamentous fungi can thrive and out-compete other microbes for nutrients (Cook & Orchard 2008). *Actinomyces* are indigenous to soils, but usually in low quantities, although they are resistant to drying and can survive in dry soils, preferring alkaline or neutral pH and low acidity. *Actinomycetes* may constitute 10 – 33% of soil bacteria, with the genera *Nocardia* and *Streptomyces* most

copious. They are capable of decomposing complex humic substances (Atlas & Bartha 1998) being mycelial, they undergo elaborate morphological divergence (Barka et al. 2014).

Actinomycetes , of the order *Actinomycetales* , are a group of filamentous, Gram-positive bacteria that are an abundant source of secondary metabolites, often produced in large amounts, highlighting their importance in industry (Kurtböke 2012). Many of *Actinobacteria* are soil bacteria, but some are also pathogenic or saprophytic organisms. The Class *Actinobacteria* comprises 6 classes, 6 orders, 14 suborders, and 56 families (Wink, Mohammadipanah & Hamedi (eds.) 2017).

Streptomycetes is dominant among *Actinomycetes* . They are comprised of a sizable bacterial phylum, and are located in both aquatic and terrestrial ecosystems (Dhananjeyan, Selvan & Dhanapal 2010). The non-*Streptomyces's* are called rare *Actinomycetes,* composing approximately 100 genera, which live in marine environment (Dhananjeyan, Selvan & Dhanapal 2010). Genomically, the richness of secondary metabolites in *Streptomyces* and other mycelial *Actinomycetes* is second to none in the microbial arena (Barka et al. 2014). Consequently, these bacteria are of major importance for biotechnology, medicine, and agriculture (Kurtböke 2012). Members of the genus *Streptomyces* produce many commercially significant compounds, especially antibiotics (Kurtböke 2012) producing approximately two-thirds of all naturally derived antibiotics in current clinical use, as well as many anticancer, anthelmintic, and antifungal compounds (Barka et al. 2014).

German Nobel laureate Paul Ehrlich coined the term ‘ magic bullet’ in 1900 and developed the concept of chemotherapy to treat microbial diseases regarding the development of chemotherapeutic agents, that inhibit or kill pathogens without harming the host. The first antibiotic, penicillin, was discovered by Alexander Fleming in 1928 and clinical trials were done in 1940 (Totora, Funke & Case 2016). Since the 1940s, the history of antibiotic discovery and development is associated with *Actinobacteria* and largely to members of the genus *Streptomyces* (Wink, Mohammadipanah & Hamedi (eds.) 2017).

Antibiotics are defined as ‘ natural chemical products from any type of cell that kill or inhibit the growth of other cells’ (Waites et al. 2001). When used for therapy, antibiotics are characterised as antimicrobial drugs. The 1930s - 1960s are known as golden era of antibiotic discovery, development and production (Totora, Funke & Case 2016).

There is currently a pressing need for the discovery of new antibiotics. Since the 1970s new antibiotics have been very difficult to discover and therefore, to commercialise (Waites et al. 2001). Globally, many antibiotics are inappropriately utilised for inappropriate uses. In addition, expired, impure, and fake counterfeit antibiotics are common. Even when antibiotic use is appropriate, dose regimens are usually shorter than required for full infection eradication; a process that encourages the survival of resistant strains of bacteria (Henderson & Sutherland 2017).

The introduction of an antibiotic into the microbial environment is a selective factor that may gradually select for resistant pathogens (Totora, Funke &

Case 2016). Superinfections occur when normally resistant microbiota multiply excessively, or a pathogen develops resistance to the drug being used, commonly by acquisition of antibiotic resistance genes by transformation, conjugation, transduction, or by a mutation (Henderson & Sutherland 2017). Aminoglycosides are products of *Streptomyces* species (Waites et al. 2007). Probably the best-known aminoglycoside is Streptomycin, which was discovered in 1944. It disrupts the “ reading” of mRNA codons on bacterial ribosomes, specifically, the 30-s portion, thereby interfering with protein synthesis (Totora, Funke & Case 2016; Waites et al. 2007). Streptomycin, is a broad-spectrum antibiotic, affecting a broad range of Gram-positive or Gram-negative bacteria, although rapid development of resistance and serious toxic effects have reduced its use (Atlas & Bartha 1998).

Objectives

The objectives of the current experiment included the calculation of the moisture contents of three different Queensland soil samples and the isolation of *Actinomycetes* from the soil samples. Additionally, the antibiotic activity of *Actinomycete* s previously isolated from the different soils using the Kirby-Bauer disc diffusion technique was tested.

Methods

(Part 1a)Calculation of the moisture contents of different soil samples

To calculate the water mass (M_W) and the dry weight (M_D) for the beach sand, an empty soil tin (including lid) was placed on a balance to record its mass (M_1). The lid was then removed and inverted beneath the tin, sand was added to half full. The lid was placed back on the tin and the mass of the tin and lid and sample and weighed (M_2). The same procedure was followed for the bush soil and agricultural soil. The three soil sample tins were dried in an oven at 105 ^{o}C for 24 hours with the lids off before the isolation of *Actinomycetes* to determine their moisture content. Following drying the samples were cooled to room temperature (23 ^{o}C), the tin lids were replaced and the mass of the tins containing dry soils were recorded (M_3). The soil moisture content for the three soil samples was then calculated. The mass of water lost from the sample was calculated (M_W), followed by the dry weight of the sample (M_D). Using these values, the soil moisture content (W) was calculated and recorded. The soil moisture content was expressed as a percentage of the dry weight based on the water mass lost from the moist weight (see Table 1).

(Part 1b)Isolation of streptomycetes from different soil types

Three dilutions were created by weighing 1g of each of the three dried soil samples and adding each to a Schott bottle containing 99mL of sterile water. The Griffin shaker was used to shake the bottles for 15 minutes. Three sterile test tubes were labelled 10^{3}, 10^{4} and 10^{5}. An automatic pipette was used to add 1. 0mL of the shaken soil suspension into the 10^{3} test tube and the dilution carefully mixed. Using a sterile pipette tip for each, the procedure

was repeated for the remaining 10^4 and 10^5 dilutions. Starch-casein agar (SCA) plates were supplemented with Cycloheximide (50ppm, Sigma-Aldrich Co., Australia) and Nystatin (50ppm) to remove fungi and Nalidixic acid (25ppm, Sigma-Aldrich Co., Australia) to lower the numbers of gram negative bacteria (Kurtböke 2007). The plates were appropriately labelled before the 10^5 dilution tube was used to inoculate and evenly spread a 0. 20mL aliquot of the dilution onto three isolation plates. The process was repeated creating three replicates for each dilution (triplicates). The plates were dried around a Bunsen burner for 20 minutes, with the lid ajar, for 20 minutes prior to incubation at 28 ^{o}C for 7-10 days. Fourteen days following this experiment, the SCA plates with approximately 30-300 colonies of white-gray filamentous *Actinomycetes* bacteria were counted by marking each colony on the bottom of the Petri plate with a pen. The number of microorganisms per gram of original sample (CFU/g) were then calculated.

(Part 1c)Testing antibiotic activity of actinomycete isolates previously isolated from the three different soil types

The Kirby-Bauer antibiotic sensitivity test procedure was used to determine the sensitivity of *S. epidermis* and *E. coli* bacteria to several different antibacterial agents (Bauer, Kirby & Sherris 1966). The experimental procedure was adapted from Microbiology: A Laboratory Manual (Cappuccino and Sherman, 2005). Triptone-soy agar (TSA) plates were inoculated the test organisms *S. epidermis* and *E. coli* (0. 2 mL per organism). Plates were dried around the Bunsen burner for 10 min. The top of the plates was then divided into four compartments using a marker pen. Pure bacterial cultures that

were isolated in part 1C of the experiment had small ‘ plugs’ cut from the agar lawns on which the organisms were grown by the laboratory technicians. A sterile needle was used to lift the plugs, which were placed equidistantly in an inverted position onto the TSA plate compartments, previously inoculated with *E. coli* and *S. epidermis* bacteria. This procedure was performed in the biological safety cabinet. The plates were carefully handled to avoid dislodging the plugs and they were not inverted. They were then incubated for 48 hours at 37 ^{o} C by the laboratory technicians.

The antimicrobial activity was recorded during the next laboratory class (fourteen days later) by measuring to the nearest whole mm with a ruler, the clearing zones around the colonies. The zone sizes were then compared to a Clinical and Laboratory Standards Institute standardised chart (Cappuccino & Sherman 2005).

Results

(Part 1a)Calculation of moisture content (%) of differing soil type samples

Soil moisture calculations were measured, and are displayed in table 1. The moisture content for the organic rich agricultural field soil was the highest at 42%, followed by the bush-land soil at 11%. The moisture content for the beach sand was by far the lowest at 0. 3%.

Table 1: Soil types and the moisture content of soil samples.

Calculation	Beach sand (g)	Bush soil (g)	Agricultural soil (g)
M_1	31. 9	31. 4	31. 9
M_2	91. 6	66. 7	53. 2
M_3	91. 4	63. 1	46. 9
M_W	0. 2	3. 6	6. 3
M_D	59. 5	31. 7	15. 0
W	0. 34%	11. 36%	42. 0%

M_1 = empty soil tin mass; M_2 = soil tin containing moist soil sample; M_3 = soil tin containing dried soil sample; M_W = mass water in soil; M_D = dry mass of soil; W = Moisture content.

(Part 1b) Isolation of *streptomycetes* from different soil types

As shown in table 2, the agricultural soil had the highest microbial content at …. CFU/g, followed by the bushland soil with …. CFU/g. The beach sand had a considerably lower microbial content at …. CFU/g.

As the results were visually indistinct, it was decided not to photograph the plate counts.

Table 2: Colony forming units (CFU)/g dry weight of soil samples

Dilution	Soil moisture (%)	Aliquot volume (mL)	Number of colonies /plate	CFU/g Moist soil	CFU/g Dry soil	CFU/g Sample
10-4 mean			1	5×10^4	6.5×10^3	
10-5 mean						
Bush Soil						
10-4 mean						
10-5 mean						
Agricultural Soil						
10-4 mean						

10-5

mean

(1c) Testing antibiotic activity of actinomycete isolates previously isolated from the three different soil types

Based on the figures shown in Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Disk Susceptibility Tests* , Tenth Edition, 2008, the *E. coli* and *S. epidermis* bacteria were determined to be resistant, intermediate or susceptible to the antibiotics (Cappuccino & Sherman 2005). The largest zone of growth inhibition, indicated by the size of the clear zone of 14mm surrounding sample plug number 03 on, displaying intermediate resistance. The next largest zone of growth inhibition, indicated by the size of the clear zone of 11mm surrounding sample plug number 29b, displaying low intermediate resistance.

The remaining three sample plugs, numbered 33, 28 and 01 showed resistance to antimicrobial activity. The remaining sample plugs, 33, 12 and 76, showed resistance.

Plug sample number	*E. coli* Inhibition zone size	Susceptibility	Plug sample number	*S. Epidermis* Inhibition zone	Susceptibility

			size	
# 03	14mm	Intermediate?	# 29b	11mm
# 33	0mm		# 33	0mm
#28	0mm		# 12	0mm
# 01	0mm		# 76	0mm

Table 2: Origins of different *streptomyces* species isolated from soil and the strength of their antibiotic activity

Isolate number	Soil type	Bacterial host	
		S. epidermis	*E. coli*
1	Agricultural soil	++	+
3	Bushland soil	+	+
12	Beach sand	–	–
28	Agricultural soil	++	–
29b	Bushland soil	++	–

33	Beach sand	–	–
76	Beach sand	–	+

Discussion

(Part 1a)Calculation of moisture content (%) of differing soil type samples

The agricultural soil had the highest moisture content as *Actinomycetes* dominated in wet soils and the moisture content affected their growth.

(Part 1b) Isolation of streptomycetes from different soil types

The agricultural soil had the highest moisture content. There was a correlation between the soil type and the microbial content, as the number of microorganisms was dependent both on the moisture content of the soil and the nutritional matter consisting of organic compounds.

(1c) Testing antibiotic activity of actinomycete isolates previously isolated from the three different soil types

There was no correlation between microbial numbers and *antagonistic* activities of the soil bacteria as the effect of antagonists on pathogens is a continuum, from stimulation to no inhibition of one or several pathogens (Broadbent, Baker & Waterworth 1971). The soils bioactivity was dependent on the soil type, due to the number of organic compounds contained within it.

Streptomyces strains isolated from soil are able to degrade organophosphate insecticide and can grow on this compound as the only source of carbon and energy (Atlas & Bartha 1998)

Limitations

Possible careless handling may have affected the interpretation of the differing soil results (Carter & Gregorich (eds.) 2008).

Future directions

New areas opening up in the field of biotechnology promise discovery of further antibiotics from extremophiles, as not all metabolites from the order *Actinomycetales* have been exhausted. Over 150, 000 bioactive metabolites are still undiscovered from the genus *Streptomyces* alone. Additionally, more than 50 rare actinomycete taxa are reported to be the producers of 2500 bioactive compounds. The failure to discover new bioactive compounds does not represent a lack of new compounds yet to be discovered, but rather a challenge for new research and screening breakthroughs (Kurtböke 2012).

References

- Atlas, RM & Bartha, R 1998. *Microbial ecology: fundamentals and applications.* Benjamin, Cummings Publishing Company, Menlo Park.
- Bauer, AW, Kirby, WMM, Sherris, JC, Truck, M 1966. Antibiotic susceptibility testing by a standardized single disk method. *American Journal of Clinical Pathology.* 45: 4.
- Barka, EA, Vatsa, P, Sanchez, L, Gaveau-Vaillant, N, Jacquard, C, Meier-Kolthoff, JP, Klenk, HP, Clément, C, Ouhdouch, Y & van Wezel, P 2016.

Taxonomy, physiology, and natural products of *Actinobacteria* , [published correction appears in *Microbiology & Molecular Biology Reviews* . 9; 80(4): iii]. *Microbiology and Molecular Biology Reviews* . 2015; 80(1): 1–43. doi: 10. 1128/MMBR. 00019-15

- Broadbent, P, Baker, KF & Waterworth, Y 1971. Bacteria and *Actinomycetes* antagonistic to fungal root pathogens in Australian soils. *Australian Journal of Biological Sciences* 24. doi: https://doi. org/10. 1071/BI9710925
- Carter, MR & Gregorich, EG 2008. *Soil sampling and methods of analysis* , 7th edn, Canadian Society of Soil Science, Taylor & Francis Group, LLC, Boca Raton.
- Clinical and Laboratory Standards Institute 2008. *Performance Standards for Antimicrobial Disk Susceptibility Tests* , 10th Edition.
- Cook, FJ & Orchard, VA 2008. Relationships between soil respiration and soil moisture. *Soil Biology and Biochemistry* . 40.
- Henderson, NM & Sutherland, RK 2017. Nocardia and Actinomyces. *Medicine,* 45. doi: https://doi. org/10. 1016/j. mpmed. 2017. 09. 012
- Kurtböke, DI 2012. Biodiscovery from rare actinomycetes: an eco-taxonomical perspective, *Applied Microbiology and Biotechnology* , 93: 5. doi: 10. 1007/s00253-012-3898-2
- Kurtböke, DI, Neller, RJ & Bellgard, SE 2007. Mesophilic *Actinomycetes* in the Natural and Reconstructed Sand Dune Vegetation Zones of Fraser Island, Australia, *Microbial Ecology* , 54: 332. doi: https://doi. org/10. 1007/s00248-007-9207-4

- Tortora, G J, Funke, BR & Case, CL 2016. *Microbiology: an introduction* . Pearson, Boston.
- Dhananjeyan, V, Selvan, N & Dhanapal, K 2010. Isolation, characterization, screening and antibiotic sensitivity of *Actinomycetes* from locally (near MCAS) collected soil samples. *Journal of Biological Sciences,* 10. doi: 10. 3923/jbs. 2010. 514. 519
- Waites, MJ, Morgan, NL, Rockey, JS & Higton, G 2001. *Industrial microbiology: an introduction* . Blackwell Publishing, Victoria.
- Wink, J, Mohammadipanah, F & Hamedi, J (eds) 2017. *Biology and biotechnology of Actinobacteria.* ebook, Springer International Publishing AG 2017, Cham, viewed 2 July 2019, https://link. springer. com/book/10. 1007%2F978-3-319-60339-1#editorsandaffiliations