Cultivation of Marine Microbes for Discovering Bioactive Compounds

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ABSTRACT

The historical paradigm of the ocean being a biological desert has evolved. The increasing isolation of novel microorganisms from the ocean reveals a biodiversity as rich as a rainforest [1]. Despite covering approximately 70% of Earth's surface, the marine environments is still unexplored and under-explored [2]. With a rise in health problems caused by drug- resistant pathogens, there is an urgent need for new effective antimicrobial agents [3]. Synthetic drugs have been poor substitutes for natural products, making microbe-synthesized bioactive compounds of keen interest since they function as basic structures, which may be manipulated into drugs [4]. In this study, we explored *in situ* cultivation that allows microbes to grow inside an incubation device while utilizing compounds from the environment. Through this, a total of 42 strains were recovered. Isolated strains were tested for activity against four multi-drug resistant clinical isolates: Methicillin-resistant *Staphylococcus aureus, Klebsiella pneumoniae, Escherichia coli* and *Pseudomonas aeruginosa.* Strains that showed activity against these clinical isolates had their DNA extracted and sequenced. Interestingly, MI 3 was the only strain with activity against *K. pneumonia.* 16s rDNA identification revealed that MI 3 has only 95.02 % similarity with *Azoarcus sp.* NSC3(T), holding high potential as a novel strain.

Key words: Marine environment, novel strains, bioactive compounds

1 INTRODUCTION

The Great Plate Count Anomaly is arguably the oldest and most well-known unresolved phenomenon in microbiology. It was first discovered in 1898, when an Austrian microbiologist Heinrich Winterberg observed that the number of microbial cells in his samples was not the same as the number of colonies formed on nutrient media [5]. This was due to the fact that the overwhelming majority of microbial species are unable to grow on synthetic media *in vitro* and hence remain unexplored [6]. It is estimated that approximately 99% of all species in external environments are uncultured (do not grow under laboratory conditions), making them a source of new antibiotics worth pursuing [7]. Although marine plants and invertebrates have been widely studied as a source for natural-product discovery, their microbiological counterparts have received significantly less attention [8]. In recent times, combinatorial chemistry has been employed to produce new and effective drugs to deal with the growing number of antibiotic resistant pathogenic microorganisms [9]. However, synthetic approaches to produce antibiotics have been weak alternatives to natural products [7] and as such, natural products remain the most promising source for discovering novel antibiotics [9]. Despite this, natural-product research efforts have lost the support of many major pharmaceutical companies and, in some cases, even been replaced completely by combinatorial chemistry. This loss of interest can be attributed to the enormous effort and resources that is required to isolate active natural products [2], which may eventually result in the rediscovery of known molecules. In spite of staggering odds, technical advances in cultivation methodologies have successfully recovered a diverse set of ecologically useful species [6]. Motivated by the high rediscovery rates from traditional cultivation methods, we explored *in situ* cultivation in attempts to cultivate rare or previously unculturable microbes. *In situ* cultivation has garnered attention in recent years due to the recent drug discovery of Teixobactin that kills multi-drug resistant pathogens without developing resistance [7]. Teixobactin was produced by previously uncultured bacteria *Elftheria terrae*. Seeing how the recovery of microbes through *in situ* cultivation approaches 50 % as compared to the 1 % of cells from the soil that will grow on a regular nutrient petri dish [6] we chose *in situ* cultivation as this would increase the success rate of discovering novel strains and their accompanying bioactive compounds.

2 AIMS/ OBJECTIVES

This study aims to isolate novel strains from the waters of Sungei Buloh Wetland Reserve (mangrove, Singapore) using the strategies and concepts of *in situ* cultivation. The isolated strains were tested for potential activity against four multi-drug resistant clinical isolates (Methicillinresistant *Staphylococcus aureus (MRSA) from blood, Klebsiella pneumoniae (K. pneumoniae), Escherichia coli (E. coli)* and *Pseudomonas aeruginosa (P. aeruginosa))* which were used as test organisms for our antimicrobial assays. Strains that showed activity against these test organisms would have their DNA extracted and sequenced. Through this, we aim to discover novel strains and their accompanying unique bioactive compounds.

3 METHODOLOGY

3.1 ISOLATION CHIP

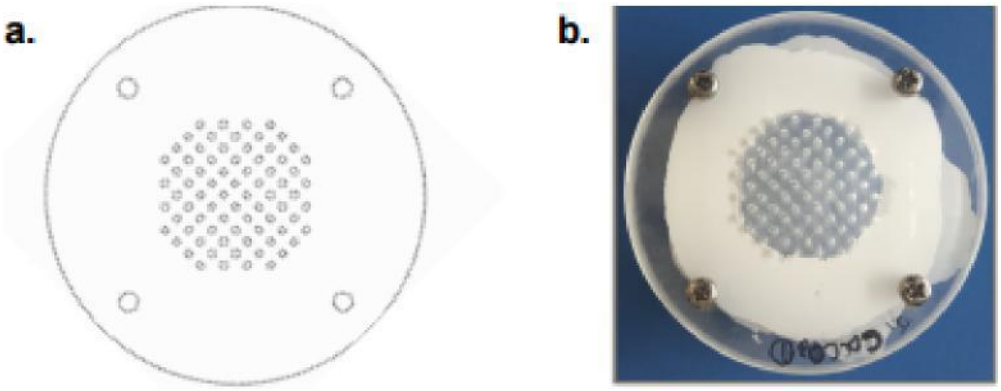


Figure 1: *In situ* incubation device a) schematic drawing and b) assembled device.

The *in situ* incubation device, which will be referred to as isolation chip in the remainder of this paper, was used for *in situ* cultivation of environmental microorganisms. The isolation chip functions very much like a diffusion chamber. Diffusion would provide cells inside the chamber with compounds from its natural environment thereby supporting the growth of cells inside the diffusion chambers [6].

Figure 1 shows the schematic drawing and the assembled device of an isolation chip that we had designed. The membranes were secured and protected with silicone glue and two additional discs before the screws were used to complete the set up. Modified SMS medium was used as the isolation media. Each 1 L preparation contains: 0.125 g casein; 0.1 g potato starch; 1.0g casamino acid; 20 g agar and 1 L autoclaved seawater. At the end, pH was adjusted to 7.0. The central plate was dipped into molten SMS medium containing appropriately diluted soil suspension. Excess agar was removed and the device was assembled with three discs tightened together with matching holes. The central plate consists of 76 agar wells with microbes loaded at 10^{-3} dilution factor. The central plate was then covered with a 0.03 µm polycarbonate

membranes to allow growth factors or other signalling molecules from nature to diffuse into the microbes loaded agar to support the growth of these unculturable bacteria.

3.2 SOIL SAMPLING

For isolation and cultivation of microbes, samples were collected from three different locations in Sungei Buloh Wetland Reserve. The coordinates of the sampling locations are as follows.

Location 1: N01 °26.686' E 103°43.602' (mangrove sediment). Location 2: N01 °26.641' E103°43.507' (mangrove sediments near the aerial roots of *Rhizophora* species). Location 3: N01 °26.641' E 103°43.507' (mangrove sediment). The environmental temperature measured was 30ºC, sediment pH ~7; slightly acidic and the seawater salinity was 16 part per trillion.

3.3 PRE-TREATMENT METHODS

A total of three pre-treatment methods were used- air dry, microwave irradiation and

calcium carbonate ($CaCO_3$). Pretreatment of soil samples, by both drying and heating, served to stimulate the isolation of spores of rare actinomycetes [10]. All sample were air dried in different desiccators based on their location. After 10 days, each sample was divided into three portions for different methods of pretreatment. One portion was air-dried and served as a control with no pre-treatment utilised while the other two received microwave treatment and $CaCO_3$ treatment respectively. $CaCO_3$treatment involved air dried soil samples with equal volumes of $CaCO_3$ for additional 11 days at 28ºC under humid conditions [11]. At the same time, samples undergoing microwave treatment were air dried for 11 days before receiving proper microwave irradiation [12].

3.4 IN SITU CULTIVATION

Three isolation chips were buried in Sungei Buloh at each of the locations stated in section 3.2 for *in situ* incubation. Using mangrove sediments and seawater collected from each of the three locations in Sungei Buloh, three isolation chips were buried in them for simulated in-lab incubation. These were then placed on a belly dancer (Stovall Life Science Incorporated) set to orbital shaking to simulate tidal action, which the strains would be subject to in their natural environment. The isolation chips were incubated for a total of three weeks.

3.5 PRODUCTION MEDIA

Vaatanen nine-salt solution (VNSS) medium was prepared as follows for 1L preparation [13]: peptone 1.0 g; yeast extract 0.5 g; glucose 0.5 g; starch (soluble) 0.5 g; $FeSO_4.7H_2O$ 0.01 g; Na_2HPO_4 0.01 g; NaCl 17.6 g; Na_2SO_4 1.47 g; $NaHCO_3$ 0.08 g; KCl 0.25 g; KBr 0.04 g; MgCl2.6H2O 1.87 g; $CaCl_2$. $2H_2O$ 0.41 g; $SrCl_2$. $6H_2O$ 0.01 g; H_3BO_3 0.01 g; double-distilled water 1,000 mL. Solidified media contained 1.5% (w/v) agar.

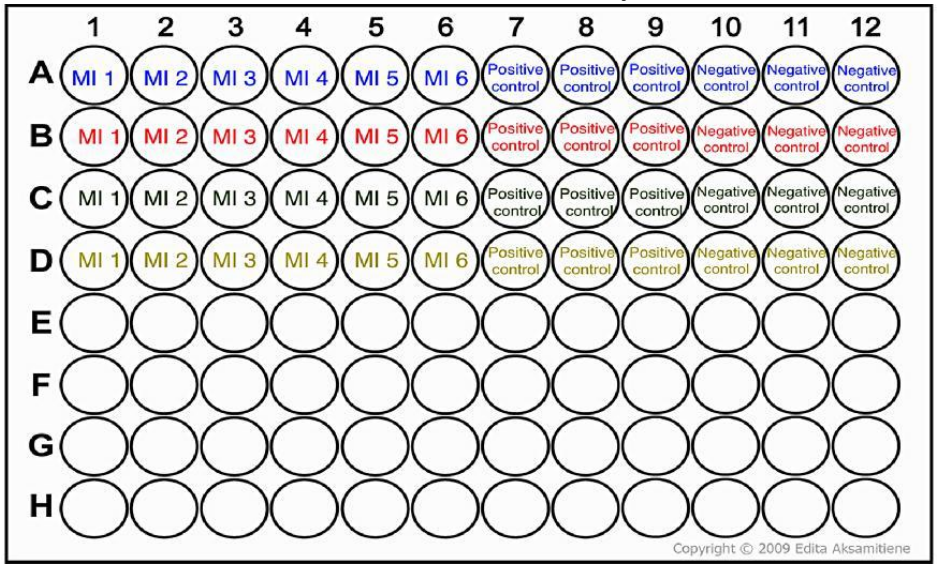
3.6 CHARACTERIZATION OF ISOLATES

Isolated strains were characterised based on their morphological features followed by sub culturing in Luria Bertani (LB) agar.

3.7 ANTIMICROBIAL ASSAY

All 42 strains were streaked from glycerol stock onto VNSS media inside a 6 well plate with one strain per well. The agar inside the 6 well plate was left to dry into a thin film before extraction with 950 mL of dimethyl sulfoxide (DMSO). 2% of DMSO was added to each well to extract secondary metabolites produced by each strain. 4 □□ of extract, 4 □□ of antibiotics

(Rifampicin for MRSA and tetracycline for *K. pneumoniae, E. coli, P. aeruginosa*) in positive control and 4 iL of DMSO in negative control was added to each well of the 96 well plate based on the layout (Figure 2). To ensure a constant optical density (OD600) before any of the strains were added, an



OD600 value of 0.00255 [14] was chosen. A spectrophotometer (UV-1650PC) was used to measure OD600 of the clinical isolates.

Figure 2: Layout of 96 well plate for antimicrobial assay (blue represents MRSA, red- *K. pneumoniae*, green- *E. coli*, yellow- *P. aeruginosa*)

The amount of clinical isolate to be added into each well was determined based on their optical density, which was calculated using theformula: (Initial OD600) □□□□□□ □□ □□ □□□□□ =□i□□□ $_{OD600}$ (14□□) with the final OD600 = 0.00255. The initial $_{(OD600)}$ before incubation was measured using Infinite M200 Pro. The 96 well plate was then placed into an incubator at temperature 37ºC. After 19 hours of incubation, OD600 was measured again using the same machine.

3.8 IDENTIFICATION

DNA isolation, followed by 16S rDNA PCR amplification was carried out using primers 27F (5' AGAGTTTGATCMTGGCTCAG 3') and 1525R (5'AAGGAGGTGWTCCARCC 3'). 16S rDNA PCR amplification was performed using iCycler (Biorad) as follows: 2.5 □□ 10X Thermopol Reaction Buffer; 0.5 □□ 10 mM dNTPs; 0.5 □□ 10 □M Forward Primer; 0.5 □□ 10□M Reverse Primer; 100 ng Template DNA; 0.125 □L Taq DNA Polymerase and Nuclease-free water to 25 □L. The PCR conditions were an initial denaturing time of 30 seconds at 95.0 ºC; followed by 30 cycles of 30 seconds at 95.0ºC, 15 seconds at 45.0ºC and 2 minutes at 68.0ºC; with a final extension time of 5 minutes at 68.0ºC and hold at 10.0ºC. Amplified products were first analyzed using gel electrophoresis (1% agarose). DNA was subsequently extracted from the agarose gel and sent for sequencing for identification of various species isolated.

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4 RESULTS AND DISCUSSION

4.1 RESULTS

4.1.1 ISOLATION FROM INCUBATION DEVICE

Table 1: Results obtained from *in situ* incubation device

Type of Cultivation	Pre-Treatment	Location	Number of Bacteria	Number of Wells
Sungei Buloh	Air Dry	2	6	54
Sungei Buloh	Calcium Carbonate	2	4	44
Sungei Buloh	Microwave	2	8	40
Sungei Buloh	Air Dry	3	0	64
Sungei Buloh	Calcium Carbonate	2	15	37
Sungei Buloh	Microwave	3	14	24
Lab	Air Dry	1	16	32
Lab	Air Dry	2	57 (2 actinomycetes)	66
Lab	Air Dry	3	46	46
Lab	Microwave	1	50	57
Lab	Microwave	2	21	30
Lab	Microwave	3	42	56
Lab	Calcium Carbonate	1	2	61
Lab	Calcium Carbonate	2	0	19
Lab	Calcium Carbonate	3	9	66

Through *in situ* incubation in Sungei Buloh and simulated in-lab incubation, we were able to recover a total of 42 strains. Among them, only two were actinomycetes. Isolates recovered from the isolation chip were denoted as MI followed by their number in recovery order. This naming system will be used for the rest of the paper to refer to the strains isolated.

4.1.2 16s rDNA SEQUENCING RESULTS

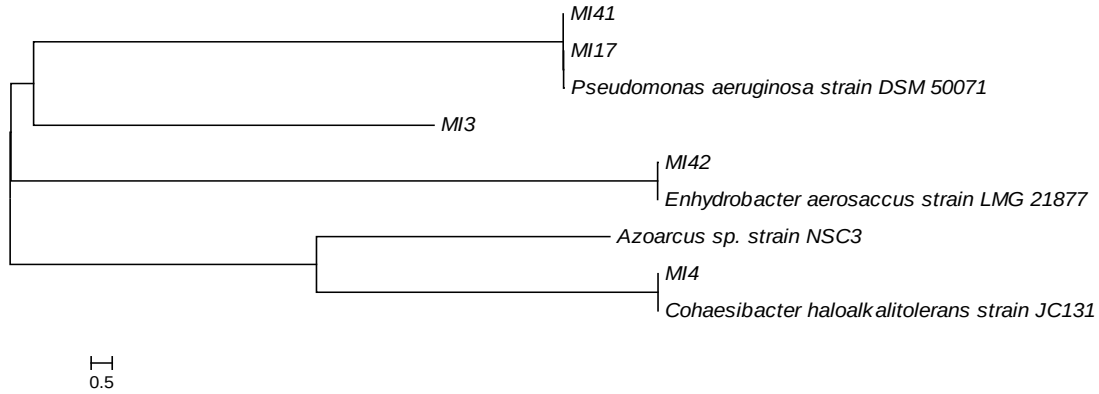

Figure 3: Phylogenetic tree constructed from 16s rDNA partial sequencing

Of the 42 strains isolated, 5 strains (MI 3, 4, 17, 41 and 42) were selected to have their DNA extracted and sequenced. Sequencing results show that 2 of them fall under the genus *Pseudomonas*, one of them *Enhydrobacter* and another *Cohaesibacter*.

4.1.3 ANTIMICROBIAL ASSAY

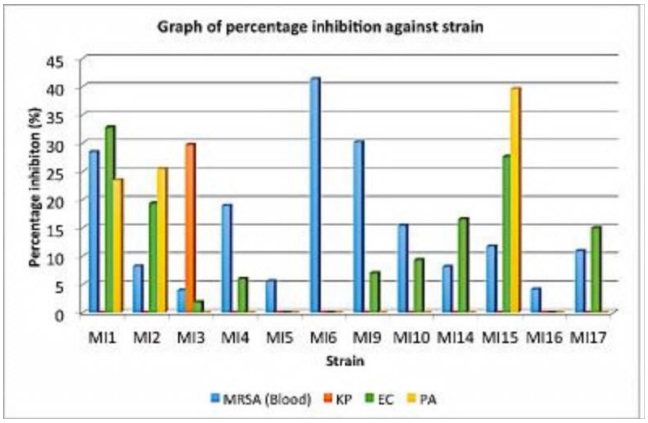

Figure 4: Results obtained from antimicrobial assay

A total of 12 isolates were tested for potential activity against four multi-drug resistant clinical isolates (MRSA, *K. pneumonia, E. coli, P. aeruginosa)*. All 12 isolates show activity against at least one test organism. Percentage inhibition was used to assess antibiotic activity. Of the 12 isolates, MI 1, MI 3 and MI 15 showed strong activity against multiple test organisms both gram-positive and gram-negative.

In addition, all 12 isolates tested had activity against MRSA (gram-positive). Notably, only MI 3 had activity against KP (gram-negative).

4.2 DISCUSSION

4.2.1 ISOLATION USING INCUBATION DEVICE

From our results, we can conclude that the number of isolates recovered from simulated in-lab incubation is significantly more than those recovered from *in situ* cultivation in Sungei Buloh.

Furthermore, we found that the pre-treatment method that recovered the most isolates came from microwave irradiation followed by air dry and lastly $CaCO_3$. This might be because microwave irradiation increases the specific activities of several key enzyme systems from cell lysates and walls such as thermonuclease and higher levels of enzymatic activity are linked with higher cell numbers [15] as discovered in a study by Dreyfuss and Chipley.

Lastly, isolation chips buried in soil samples at location 2, near the aerial roots of *Rhizophora* species recovered the most isolates. This is most likely due to endophytic actinomycetes being able to establish an endosymbiotic relationship with root nodules of the plant.

4.2.2 ANTIMICROBIAL ASSAY

MI 1, MI 3 and MI 15 showed activity against multiple test organisms both gram positive and gram negative. Of the 12 isolates tested, only MI 3 displayed activity against K. pneumoniae.

The antimicrobial assay method we employed in our study may not have been suitable for the strains we isolated as the method we utilized was meant to screen the antibiotic activity of actinomycetes. However, of the 42 strains we isolated, only 2 were actinomycetes, and as such the percentage inhibition of all strains fell below 50%. In order to improve our antimicrobial assay method, we could have utilized the modified resazurin assay [16] proposed by Sarker.

In this modified resazurin assay, Resazurin is an oxidation–reduction indicator used to assess cell growth. It is a blue non-fluorescent and non-toxic dye that becomes pink and fluorescent when reduced to resorufin by oxidoreductases within viable cells. Using this, we would be able narrow down the strains that have the ability to inhibit the growth of test organisms. Following the modified resazurin assay, the antimicrobial assay utilized in this paper would be used to determine percentage inhibition.

Future works would involve optimization of experimental deign of the antimicrobial assay as employing a good microbial assay would result in the generation of high-quality data with the greatest accuracy, speed and efficiency [16].

4.2.3 IN SITU CULTIVATION

Interestingly, MI 3, the only strain with potential against *K. pneumonia*, has a less than 98.5% similarity with type strain *azoarcus sp. NSC3*. 16s rDNA identification revealed that MI 3 has 95.02 % similarity with *Azoarcus sp.* NSC3(T), holding high potential as a novel strain. Of course, future works would include carrying out another round of 16s rDNA identification for MI 3 to confirm its novelty. This makes *in situ* cultivation a promising way of discovering previously unculturable strains and the subsequent discovery of novel bioactive compounds in the future highly possible.

In addition, manipulations could be made to the production media to enhance recovery effectiveness. These manipulations include the addition of halogens, namely bromine and chlorine into the production media as the majority of halogen-containing secondary metabolites found in the marine environment have bromine present in them [17]. Seeing how our study focuses on the isolation of marine bacteria, the addition of bromine into production media would be beneficial in the synthesis of halogenated metabolites by isolated strains. Furthermore antibiotics could be incorporated into the production media. This serves to stimulate the innate self-protection method of antibiotic producers. The use of antibiotic selection during isolation helps filter out all non-resistant strains and identify resistant strains, which have the potential to synthesize antibiotics [18]. Lastly, double distilled water in production media could be replaced with seawater collected from Sungei Buloh as certain genus of marine-derived actinomycetes require seawater to grow [19].

5 CONCLUSION

The quest for new chemical entities produced by nature to treat human diseases is considered a resource-intensive and time-consuming process and thus, many big pharmaceutical companies worldwide have largely pulled out of natural product screening programs [9]. This is because the cultivable microbial species are widely considered overmined for secondary metabolites, and the probability of discovering a novel bioactive compound is low [6]. One way to facilitate the process of screening strains and their unique secondary metabolites, as Demain suggests is “to build a library of unique chemical diversity, microbial, and plant secondary metabolites” [20].

One way we could have improved our study would be to employ both the methods of direct plating as well as *in situ* incubation in the isolation of bacteria. This is because there is essentially no overlap between species isolated by the isolation chip and those isolated using traditional petri dish methods [6]. Therefore, by employing both methods, it would increase the number of strains isolated and the possibility of discovering a novel strain.

In spite of the tedious process in isolating novel strains and their accompanying bioactive compounds, soil and water are rich resources, and with time and resources devoted into exploring these environments, rewards will definitely abound in the form of novel antimicrobial agents. [21]. Apparently 99% of the diverse bacterial species are unexplored [22] making it highly possible to discover more novel metabolites by screening unusual or difficult to isolate strains belonging to the two most prolific groups of producers, the filamentous actinomycetes and the fungi [23]. With continued innovation, such as in the case of *in situ* cultivation which allows access to a vast and large variety of previously inaccessible microorganisms [6], the arrival of isolation chips will contribute to the resolution of the greatest obstacle in microbiology, the “great plate count anomaly”. *In situ* cultivation has filled the gap between the cultivable and uncultivable species and set the foundation for drug discovery from the previously uncultivated microbial majority [6]. Taking into account the vastness of the marine environment, the potential fruits of this treasure house represented by the oceans are unimaginable [9]. This is but the beginning of the discovery of new antimicrobial agents because after all nature is much better at designing antibiotics than humans [24].

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