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IDENTIFICATION OF BACTERIA FOUND IN *HOLOTHURIA TUBULOSA* AND DETECTION OF THEIR ANTIBACTERIAL ACTIVITY

BY

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A thesis submitted in fulfilment of the requirement for the degree of Masters of Science (Biotechnology)

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ABSTRACT

Sea cucumbers have been shown to produce antibacterial agents. This research tested the hypothesis that the antibacterial agents were produced by bacteria indigenous to the sea cucumber, as part of a symbiotic relationship. Research was done using 2 specimens of *Holothuria tubulosa* and a total of 23 bacterial strains were isolated from 5 zones of the sea cucumber and also from the sea-water as a control. All strains were grown using brain-heart agar and broth prepared with sterile sea-water. From the 23 strains, seven strains were selected for further analysis. These 7 were identified as J1:2 = *K. pneumoniae*, J2:2 = *B. subtillis*, J2:3 = *B. licheniformis*, J2:6 = *B. licheniformis*, J2:7 = *K. pneumoniae*, J2:10 = *B. subtillis* and J1:12 = *K. pneumoniae*, based on PCR 16S rRNA gene specific forward and reverse primers, and subsequent sequencing of the approximately 700bp products. Selection was based on antibacterial well diffusion testing results, with the 7 selected strains having higher and more consistent antibacterial activity in comparison to the other isolated strains from *H. tubulosa*. In turbidometric analysis, all 7 strains' extracts delayed the exponential growth phase of test pathogens *K. pneumoniae* and *S. typhimurium* compared to the negative control (negative control = approx. 120 minutes, test samples = approx. 200 minutes). Size exclusion chromatography using Sephadex G-25 (1-5kDa separation) was carried out on all 7 strains. A hundred 1ml fractions were collected for each strain and absorbance readings were taken at 600nm. Two major peaks were seen in the chromatograms for all 7 strains, the first between fractions 20 to 40 (5kDa range) and the second at fractions 50 to 70 (1kDa range). SDS-PAGE analysis on fractions 27-33 of bacterial strain J2:2 (*B. subtilis* strain) showed 3 peptide bands, X (MW = 20413), Y (MW = 18742) and Z (MW = 5792). The results from this research showed that is possible that antibacterial agents found in sea cucumbers are made by microflora indigenous to the sea cucumber.

ملخص البحث

خيار بحر شُوّفَ لإنْجاب الوكلاءِ المضادّ للجرائيم. إختبرَ هذا البحثِ الفرضية التي الوكلاءَ المضادّ للجرائيمَ أنجبوا بالبكتيريا الأصليينِ إلى خيار البحرَ، كجزء مِنْ a **علاقة تعايشية. البحث عُمِلَ إستعمال 2 مِنْ نماذج** *Holothuria tubulosa* **وما مجموعه 23 إجهاد جرثومي عُزلا مِنْ 5 مِنْ مناطق خيار البحرَ وأيضاً مِنْ ماءِ البحر كـ**a **سيطرة. كُلّ الإجهاد نُمِى إستعمال المادّة المثخّنةِ ومرق قلبِ الدماغ إستعدّا بماءِ البحر المعقّم. مِنْ الـ23 الإجهادِ، سبعة إجهادِ إختَرَ للتحليلِ الآخر. هذه الـ7 مُيّزَ J1:2 =** *K. pneumoniae*، **J2:2 =** *B. subtillis*، **J2:3 =** *B. licheniformis*، **J2:6 =** *B. licheniformis*، **J2:7 =** *K. pneumoniae*، **J2:10 =** *B. subtillis* **وJ1:12 =** *K. pneumoniae*، **مستند على بي سي آر 16 إس مبادىء قراءة جين** rRNA **المعيّنة الأمامية والعكسية، وتسلسل لاحق تقريباً 700 مُنتَجاتِ** bp**. الإختيار كَانَ مستند على نَتائِج إختبار الإنتشار الجيّدةِ المضادّة للجرائيم، بالـ7 الإجهادِ المختار سَيكونُ عِنْدَهُ نشاطُ مضادّ للجرائيمُ ثابتُ أعلى وأكثرُ بالمقارنة مع الإجهاد المَعْزُول الآخر مِنْ إتش .** tubulosa**. في تحليلِ** turbidometric**، كُلّ 7 إجهادِ ' مقتطفات أخّرتْ مرحلة نمو بمعدل متزايدَ أسباب الإختبار المرضية** *K. pneumoniae* **و** *S. typhimurium* **قارنا إلى السيطرةِ السلبيةِ (سيطرة سلبية = تقريباً. 120 دقيقة، عينات إختبار = تقريباً. 200 دقيقة). إستثناء حجم** chromatography **إستعمال** Sephadex G-25 (1-5 **إفتراق** kDa**) نُقِلَ على كُلّ 7 إجهادِ. أي مائة 1 مليلتر كسور جُمِعَ لكُلّ إجهاد وقراءات** absorbance **أخِذا في 600** nm**. قمتان رئيسيتان رَأتَا في** chromatograms **لكُلّ 7 إجهادِ، الأول بين كسور 20 إلى 40 (5 مدى** kDa**) والثانية في كسور 50 إلى 70 (1 مدى** kDa**). تحليل إس دي إس الصفحة على الكسور 27-33 مِنْ الإجهادِ الجرثومي J2:2 (***B. subtilis***) شوّفَ 3 فِرقَ** peptide**، إكس (إم دبليو = 20413)، واي (إم دبليو = 18742) وزد (إم دبليو = 5792). إنّ النَتائِجَ مِنْ هذا البحثِ شوّفَ ذلك محتملُ ذلك الوكلاءِ المضادّ للجرائيم وَجدوا في خيار البحر يَجْعلونَ بالبكتيريا الأصليينِ إلى خيار البحرَ.**

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and in quality, as a thesis for the degree of Masters in Science (Biotechnology).

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DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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To Mama, Papa, Dee-Dee, Dr. Haathi, Moisey the American and Prince Jols – I love you all.

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CHAPTER ONE

INTRODUCTION

1.1 RESEARCH OBJECTIVES

The main objective of this research was to culture bacteria from Malaysian sea cucumbers and to obtain antibacterial agents from these bacteria. This was based on a hypothesis that a symbiotic relationship existed between the bacteria and the sea cucumber; the sea cucumber acted as a host for the bacteria and the bacteria provided protection for the sea cucumber by producing antibacterial agents to kill other bacteria that may be pathogenic to the sea cucumber.

1.2 SELECTION OF SEA CUCUMBERS AND ISOLATION OF BACTERIA

There are several locations in Malaysia where sea cucumbers are commonly found. Among these sampling sites is Pangkor Island, a small island off the coast of the north-western state of Perak in peninsular Malaysia. It was from here that the sea cucumbers used in the proceeding research were obtained.

Since culturing bacteria from a marine organism is desired, any growth media will have to contain salt. Two methods were considered and they were either to collect sea-water from where the sea cucumbers were gathered and use this in preparation of culture media or to make artificial sea water. Several websites such as The German Collection of Microorganisms and Cell Cultures (DSMZ) contain formulae for making artificial sea-water.

Luria-Bertani (LB) agar and broth, brain heart agar and broth and several customized media were used. A higher concentration i.e. a 2x culture media was also

used. This was achieved either by doubling the quantity of the media powder or by reducing the amount of water used to prepare the agar or broth.

1.3 MICROSCOPY

In this research, only bright field optical microscopy was used, which is a relatively basic microscopy method. Due to this, this method suffers from several limitations. A very low contrast of observed material is noted. The resolution of this method is also quite bad. These limitations can be countered however by simple remedies such as reducing the amount of light used to illuminate the sample or to carry out staining techniques such as the Gram stain to improve the resolution.

The Gram stain is a simple but powerful staining method that separates bacteria into two groups. Depending on the quantity of peptidoglycan present in the cell wall, bacteria can either be Gram positive or Gram negative.

Peptidoglycan is a polymer made up of sugars and amino acids, arranged in a crystal lattice structure. It forms a homogenous layer outside the plasma membrane of bacteria, giving structural rigidity to the cells. In Gram positive bacteria, the peptidoglycan layer is significantly thicker than Gram negative bacteria. This is what causes Gram positive bacteria to have a dark purple color. On the contrary, Gram negative bacteria do not stain purple.

The purple color comes from crystal violet, which is the primary stain used in Gram staining. When crystal violet is used to stain bacterial cells, it forms complexes in the inner and outer layers of the cell membrane. When a solvent like acetone is used to decolorize the sample, bacteria with a thin peptidoglycan layer lose their outer membrane and thus, the purple stain is washed away. In cells with thicker peptidoglycan layers, dehydration occurs and the stain is trapped in between the

complex folds of the peptidoglycan lattice. These bacteria are thus known as Gram positive bacteria. For the Gram negative variety, a counter-stain like safranin is used to confer the reddish or pink hue typical of this group.

1.4 BIOCHEMICAL TESTS

Biochemical tests are used in microbiological work as another method of elucidating the identity of bacteria. There are numerous types of tests that can be carried out, some using highly specific components while some employ simple reagents. Examples of biochemical tests are the oxidase, catalase and indole test.

1.4.1 Oxidase test

This test determines if a bacterial species has the enzyme cytochrome oxidase present. To test for this, an oxidase disc, which is impregnated with a redox agent, is used. If the enzyme is present, the redox agent is oxidized and turns purple or dark blue.

1.4.2 Catalase test

This test determines if a bacterial species has the enzyme catalase. To test for this, hydrogen peroxide (H_2O_2) is added to a suspension of the bacterial cells. If the enzyme is present, oxygen is liberated, resulting in a very noticeable bubbling.

1.4.3 Indole test

Indole is a component of the amino acid tryptophan. Some bacteria break down tryptophan for nutritional needs using the enzyme tryptophanase. The presence of indole can be detected through the use of Kovac's reagent. Kovac's reagent, which is yellow, reacts with indole and turns red.

1.5 ANTIBIOTIC DIFFUSION TEST

Antibiotic diffusion is one method that is used to test the efficacy of an antibacterial agent against a bacterial species. The principle is that a liquid will take a certain amount of time to diffuse through a solid medium like agar. The diffusion time depends on several factors such as temperature, the viscosity of the liquid and the porous properties of the agar. Two methods are generally used and they are the disc diffusion method and the well diffusion method.

In the disc diffusion method, small discs made from filter paper are soaked in the antibacterial agent and then are placed on an inoculated agar plate. The antibacterial agent diffuses out of the discs into the agar, where it inhibits the growth of the test bacteria. The well diffusion method is different in that the inoculated agar plates have holes or wells punched into them and the test agent is pipetted directly into these wells. The principle of diffusion through the agar remains the same.

The disc diffusion method is only efficient if the test agent is known to be an efficient antibacterial agent. Otherwise, the small volume of the test agent may not be sufficient to yield a proper result, which would then give rise to a false negative result. The well diffusion method allows a much greater amount of the test agent, up to 100µl to be used, which in turn would give much better results. The exact quantity of the test agent being used is known, which is essential in determining the minimum inhibitory concentration (MIC) based on dosage.

1.5.1 Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration of an antibacterial agent is the lowest concentration of that agent that effectively inhibits the growth of a bacterial species. The MIC is not constant for an agent; it depends on several factors such as the type or

test organism used, inoculum size, type of culturing media used, pH, inoculation temperature and the availability or lack of oxygen. If these parameters are standardized however, it is then possible to compare the activity of different antibacterial agents against a single test pathogen or to compare a single antibacterial agent's efficacy against several test pathogens.

The simplest way of determining the MIC of an antibacterial agent would be to use the tube dilution method. Here, several tubes are prepared, each containing a preset amount of test inoculum, culturing media and varying concentrations of the antibacterial agent. After incubation, the first tube that does not have any bacterial growth would be the tube with the MIC concentration of the antibacterial agent. The limitation of this method is that it doesn't allow the identification of the type of antibacterial agent used i.e. if it is a bacteriocidal agent (kills the cells) or if it is a bacteriostatic agent (inhibits cell growth).

The second method used in determining the MIC value of an antibacterial agent is the diffusion method. In either of the methods, the antibacterial agent diffuses into the agar and due to this, its concentration lessens. Eventually, the concentration of the agent is insufficient to prevent bacterial growth. An inhibition zone thus exists, defined by a clear "ring" around the disc or well. The outer circle of this ring is the MIC of the antibacterial agent. Measuring the zones of inhibition and plotting them against the concentration of the antibacterial agent using a logarithmic scale, gives a standard curve, from which the concentration of an unknown test agent can be determined by measuring the inhibition zone.

1.6 TURBIDOMETRY

Turbidometry is a process where the amount of light of a predetermined wavelength absorbed by a solution is measured. A clear solution would have a 100% light transmission and so would not absorb any light. A completely turbid solution would have the reverse effect; light absorption would be total. Absorbance readings are normally measured with a spectrophotometer and the obtained readings are optical density (OD) units.

In microbiology, turbidity in solutions is caused by the presence of bacterial cells. It can be said that the number of cells present in a solution is directly proportional (within certain limits) to the OD. At higher cell numbers however, a problem can occur. It is important to note that spectrophotometers measure unscattered light as OD readings. When there is a high number of cells present in a liquid, light scattered by one cell may be scattered back again into its original path, giving a false reading. This however, is not a major concern as the cell density of the solution would have to be significantly high for this to occur.

Turbidometric analysis provides several useful parameters in relation to bacterial cell growth. Growth rates, lag phases, stationary phases and cell death rates can all be determined from a turbidometry plot. In the case of antibacterial testing, the minimum inhibitory concentration (MIC) of an antibacterial agent can also be determined via this method.

1.6.1 Antibacterial testing using turbidometry

As was mentioned before, turbidometry can be used in antibacterial testing. Different concentrations of the antibacterial agent are used in preparation of the culture medium and the OD readings are taken at set time intervals. A growth curve is plotted with the

OD readings and from there it is possible to determine the MIC of the antibacterial agent. There are three general categories of antibacterial agents and they are bacteriostatic agents, bacteriocidal agents and bacteriolytic agents. Bacteriostatic agents do not really kill cells. Growth is merely inhibited. This means that the total cell count remains static. The cells become viable once again as soon as the bacteriostatic agent is removed. Bacteriocidal agents kill the cells but do not cause lysis; the number of viable cells decreases but the total cell count remains the same. Finally, bacteriolytic agents kill the cells by causing cell lysis. In this case, the total cell count and the number of viable cells decrease.

If OD readings were to be taken when using these antibacterial agents, only bacteriolytic agents would cause a drop in OD readings. This is because the other 2 types of agents do not cause any changes in the total cell count of the sample medium.

1.7 CHROMATOGRAPHY

Chromatography is used to separate and identify the chemical components in a complex mixture via a process called adsorption. All types of chromatography have a stationary phase and a mobile phase. The stationary phase is a phase that is fixed in place in either a column or a flat surface, whereas the mobile phase is a phase that is either a liquid or gas that moves through the stationary phase. Separation is based on the rate at which components travel through the mobile phase (Chi-San Wu, 2003). This rate is affected by molecular size of the components, differences in hydrophobicity and ionic charge.

Chromatography consists of 2 general types which are column chromatography and planar chromatography. In column chromatography, the stationary phase is packed in a column and the mobile phase travels through the

column, either by gravitational effect or by external pressure. In planar chromatography, the stationary phase is held either on a flat plate or on paper and the mobile phase travels through it via capillary action or by gravity.

1.7.1 Gel filtration chromatography

This method employs the use of porous gel molecules to separate the compounds of a mixture based on size or weight. What happens is that compounds that have a molecular size larger than the pores of the solid phase travel straight through the column and are eluted first. Smaller molecule compounds become trapped in the pores and thus take longer to elute. Elution time is based on the ability of the smaller molecules to traverse through the pores of the solid phase.

Several types of solid phase materials are used and among them is Sephadex, a column packing material produced by Pharmacia Fine Chemicals, Inc. Several different particle sizes are available and selection is based on the molecular size ranges of the compounds of interest in a mixture. Sephadex must be allowed to swell properly in a buffer or solvent before being used. The swelled particles can then be packed in a chromatography column or other suitable columns. This inert material has a high tolerance to variations in pH

1.7.2 The rate of migration of solutes in chromatography

The efficacy of a chromatography column in separating compounds in a mixture depends on many factors. One factor is the rate at which the mixtures are eluted from the column. Four factors play a part here and they are partition coefficient, retention time, capacity factor and selectivity factor.