

CHAPTER THREE

MATERIALS AND METHODS

3.1 *Streptomyces* spp. cultivation

Based on *Streptomyces*-like growth, forty four putative *Streptomyces* strains from the culture collection of Mycology and Plant Pathology Lab, Institute of Postgraduate Studies, University of Malaya, were selected at random and subcultured onto ISP 4 and ISP 2 media (Appendix A) to obtain single colony. These strains were isolated from sponge of Tioman Island. After seven days of incubation at $28 \pm 2^{\circ}\text{C}$, all the cultures were then lawned on their optimum growth media and incubated in same manner for another seven days. After that, they were cut into small blocks of agar (1cm x 1cm width) and were placed in 30% (v/v) glycerol solution (Appendix A) and then stored at -20°C .

3.2 Primary screening for antifungal activity against *Candida albicans* and *Schizosaccharomyces pombe*

All strains were tested for their antifungal activities against two yeast strains - *Candida albicans* and *Schizosaccharomyces pombe*, obtained from Microbiology Division, University of Malaya. The actinomycetes isolates were lawned on their respective optimum growth media (ISP 4/ISP 2) and incubated for seven days at $28 \pm 2^{\circ}\text{C}$. Separate lawn cultures of both yeasts were set up in triplicates. *C. albicans* was grown on sabouraud dextrose agar (SDA) (Appendix A) while *S. pombe* was grown on yeast-peptone-glucose (YPG) agar (Appendix A). Five 6-mm agar plugs of 7-day-old actinomycetes strains were placed on each SDA and YPG plate lawned with one of the test fungus each. The agar plugs were placed as shown in Figure 3.1 to allow the

diffusion of bioactive substances into the lawned test fungi during incubation (Tan *et al.*, 2004). 2.5µg nystatin at a concentration of 50µg/ml loaded on sterile 6-mm Whatman paper disc, was used as positive control. Test plates were incubated at $37 \pm 2^\circ\text{C}$ for *C. albicans* plates and $25 \pm 2^\circ\text{C}$ for *S. pombe* plates for 48-72 hours. Diameter of clear zones around the agar plugs were recorded and compared with clear zone diameters obtained from the positive control plates. Only potential strains with strong activities against any of the fungi tested were further characterized.

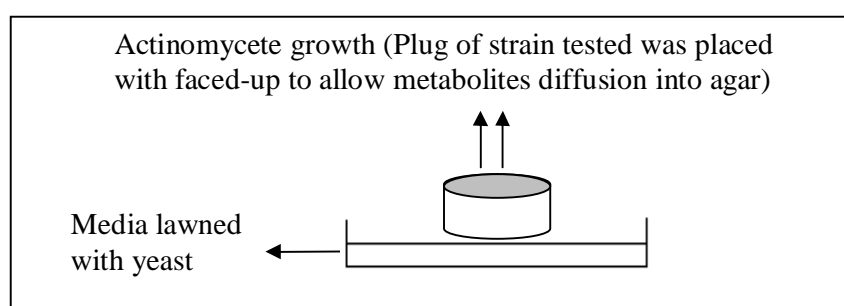


Figure 3.1: Position of test strain agar plug on media lawned with yeast.

3.3 Cultural characterisation of tested *Streptomyces* spp.

Streptomyces spp. strains were lawned on yeast extract-malt extract agar (ISP 2), oatmeal agar (ISP 3), inorganic salts-starch agar (ISP 4), glycerol asparagine agar (ISP 5), peptone-yeast extract-iron agar (ISP 6) and tyrosine agar (ISP 7) (Appendix A). Each strain was lawned in triplicates for each media before being incubated for 14 to 21 days at $28 \pm 2^\circ\text{C}$. The colour of pigments, aerial and substrate mycelium were recorded according to the ICI Dulux Colour Inspirations (2008).

3.4 Morphological studies –scanning electron microscopy (SEM)

Sterile 12mm round coverslips were inserted at an angle into lawned optimal growth media and incubated at $28 \pm 2^\circ\text{C}$ for ten days; before they were withdrawn and fixed with 2% (w/v) osmium tetroxide vapor, mounted on 0.5-inch aluminum specimen stubs

by using conductive carbon cement (Neubauer Chemikalien), followed by gold-coating and viewed under scanning electron microscope (Leica Stereoscan 440) (Appendix B).

3.5 Physiological characterisation of tested *Streptomyces* spp.

3.5.1 Temperature range and optimum temperature for growth

All strains were lawned on ISP 4 media which was the optimum growth media and incubated in different temperatures: 10°C, 15°C, 25°C, 35°C, 45°C and 50°C. The growth was observed after seven days of incubation.

3.5.2 Formation of melanoid pigment

All strains were plated on peptone-yeast extract-iron agar and tyrosine agar and incubated at $28 \pm 2^\circ\text{C}$ for 4 days. The cultures were observed for melanoid formation (Shirling & Gottlieb, 1966).

3.5.3 Liquefaction of gelatin

Difco gelatin (12%) was dissolved in distilled water and dispensed into 20ml Mc Cartney bottles prior to autoclaving. Each bottle was inoculated with a 6mm plug of 7-day-old *Streptomyces* spp. culture (triplicates for each strain tested). Inoculated gelatin was incubated at $28 \pm 2^\circ\text{C}$ for 14 days. On days 7 and 14, triplicate tubes were placed at $3-5^\circ\text{C}$ for 30 minutes to one hour and the degree of liquefaction noted. The colour of soluble pigment was also recorded (Shirling & Gottlieb, 1966).

3.5.4 Hydrolysis of starch

All strains were cultured on ISP 4 agar plate at $28 \pm 2^\circ\text{C}$ for a week. Then, 3ml of iodine was dropped onto the medium. If the tested strains produced amylase, a transparent zone would appear around the colony as starch was degraded. Negative

control was set up by using only ISP 4 media; the same manner was done and compared with the tested cultures for hydrolysis of starch test.

3.5.5 Hydrogen sulfide (H₂S) production

Difco peptone-iron agar (Appendix A) was supplemented with 0.1 % Difco yeast extract. The medium was dissolved, dispensed into flasks, autoclaved at 121°C for 15 min, and allowed to solidify after pouring into Petri dishes. The inoculated media was incubated at 28 ± 2°C and the cultures were observed at 6 and 18 hours for the production of hydrogen sulfide which was indicated by the presence of a pronounced blue-black discoloration of the medium surrounding the colonies (Gottlieb, 1960).

3.5.6 Sodium chloride (NaCl) tolerance

Sodium chloride tolerance was investigated by lawning all strains on the respective optimum growth media. The media was added with 2,4,6,8 and 10% (w/v) of sodium chloride. Every strain was set up in triplicates for each percentage of sodium chloride tested. The growth were observed after one to two weeks of incubation and recorded.

3.5.7 Carbon sources utilization

Basal medium and salt solution (Appendix A) were prepared and dissolved prior to sterilization at 121°C for 15 minutes. The final pH of all media was adjusted to 6.8 to 7.0. Filter sterilized sucrose, D-xylose, L-arabinose, rhamnose, glucose, D-fructose, lactose, raffinose, inositol, maltose, D-mannitol and D-sorbitol (1% - w/v) were added individually to the molten carbohydrate-free medium, mixed properly and 15ml was poured into Petri dishes. Then, six of one cm diameter sterile plastic straws for each plate were placed on the surface of the media. Second layer of 20ml media was then poured on the first layer with the straws on it, making six wells after solidified. All the

straws were taken out. Then, spores were harvested from 7-day-old cultures. Using sterile hockey stick, all mycelium and spores on the surface of the media were harvested with 5ml of sterile distilled water and pipetted out to a 5ml sterile test tube. The mixture then vortexed for one to two minutes, and was ready to be used as spore suspension. 250µl spore suspension of each potential strain was loaded in each well for each test strain. Each carbon source utilization test was set up in triplicates. Glucose was used as positive control and media without carbon sources as negative control. After loading, test plates were incubated at $28 \pm 2^\circ\text{C}$ for 10 days. The growth of each strain on carbon source media was observed and compared to positive and negative control before recorded.

3.5.8 Nitrate reduction test

Synthetic nitrate broth was made by preparing two solutions of media and salt solution (Appendix A) to test for reduction of nitrate. Solution I and II (Appendix A) were mixed, dispensed into test tubes and sterilized at 121°C for 15 minutes. Tubes were inoculated by transferring a loop of the strains prior to incubation at $28 \pm 2^\circ\text{C}$ for every 7, 14 and 21 days. A red colour, indicating the presence of nitrites, developed after 1ml of sulfanilic acid reagent (8g of sulfanilic acid per 1L of 5N acetic acid) and 1ml of dimethyl- α -naphthylamine reagent (6ml dimethyl- α -naphthylamine per 1L of 5N acetic acid) were added and shaken after each addition. If no red colour formed after addition of the test reagents, a pinch of zinc dust was added. If nitrate was still present, it was reduced to nitrite and the red colour appeared. If no colour developed after the addition of zinc, the nitrate had been used completely or reduced to products beyond nitrite (Gottlieb, 1960).

3.5.9 pH sensitivity

Optimum growth media was prepared and adjusted to pH 4.5, 5.5, 6.5, 7.5, 8.5 and 9.5. Every strain then subcultured on each adjusted pH media (set up in triplicates) prior to be incubated at $28 \pm 2^\circ\text{C}$ for seven to ten days. The growth of the strains was observed and recorded as well.

3.6 Identification based on 16S rRNA gene sequence analysis

For molecular part, all test strains were cultured at $28 \pm 2^\circ\text{C}$ on non-sporulating media (Appendix A) for three days or before dense sporulation occurred, as the presence of spores was known to interfere with DNA extraction (Tan, 2007). Only selected strains based on primary HPLC result (see 4.6.1) in chemical profiling part were identified (which expected to have interesting compounds).

3.6.1 DNA extraction of selected *Streptomyces* spp.

A single colony was picked with a sterile loop and transferred directly into a sterile 1 ml vial containing 150 μl of TE buffer and glass beads. 2.5 μl of lysozyme (50mg/ml) and 5 μl of proteinase K (20mg/ml) were then added. In order to mix the suspension properly, it was vortexed for 30 seconds before being incubated at 37°C for two hours prior to be centrifuged at 13500 rpm for 10 minutes. Lastly, the supernatant was transferred to a new tube and incubated at 75°C for 15 minutes (Tan, 2007). The DNA extracts were checked using electrophoresis gel (see 3.6.3). Only DNA extracts exhibiting clear bands will be used as template for PCR amplification.

3.6.2 Amplification of 16S rRNA gene using PCR

DNA extracts of pure *Streptomyces* spp. culture were used as the source of DNA template for PCR amplification of 16S rRNA genes. PCR kit available from Fermentas

(MBI Fermentas) was used and the reaction mixture was prepared in 0.5ml PCR tubes. The volume of reaction mixture used was 50µl, which consisted of 1X PCR buffer, 0.2mM dNTPs, 1.0µl each of forward and reverse primers, 1.5mM MgCl₂ and 1U *Taq* DNA polymerase. Sterile distilled water was added to adjust the final reaction volume to 50µl. Universal primers (1st Base), as shown in Table 3.1 were used for the amplification 16S rRNA (Khamna *et al.*, 2009). The expected size of product is 1500 bp. DNA extract (1µl) was added lastly into the reaction mixture. Single reaction mixture without the DNA extract was used as negative control.

Table 3.1: Universal primers used in the amplification of 16S rRNA gene

Primers	Nucleotide sequence	Reference
27f	5'-AGAGTTTGATCCTGGCTCAG-3'	Whitehouse <i>et al.</i> (2010)
1525r	5'-AAG GAG GTG ATC CAG CC-3'	Khamna <i>et al.</i> (2009)

PCR amplification was performed with a Swift Maxi thermal cycler (ESCO, Singapore). The reaction mixture was subjected to heating at 95°C for 2 minutes as initial DNA denaturation step, followed by denaturation at 95°C for 30 seconds, then 30 seconds of primer annealing at 55°C and 1 min extension at 72°C; for a total of 30 cycles. The PCR process ended with a final extension step at 72°C for 10 min. After PCR, the product was either immediately analyzed by gel electrophoresis or stored at -20°C until use.

3.6.3 Agarose-gel electrophoresis

A 4-µl aliquot of DNA or PCR product was mixed with 4µl of 6X gel-loading buffer (Fermentas) and analyzed by electrophoresis through a 0.4% (w/v) agarose gel in 0.5X TBE buffer at 120V for approximately 20 min, with 4 µl of GeneRuler 100-bp DNA

ladder (Fermentas, MBI Fermentas) as the marker. A 5X stock TBE solution was prepared by mixing 54.0g Tris base (445mM), 27.5g boric acid (445mM) and 20ml of 0.5M EDTA (10mM) solution at pH 8.0, in 1.0L sterile distilled water. The working solution of 0.5X TBE buffer was obtained by diluting the stock solution (50ml) with sterile distilled water (450ml). After electrophoresis, the gel was stained with 0.5 µl/ml ethidium bromide for 5-10 min and destained for 15-20 min in distilled water. DNA bands were visualized by UV transillumination at 312nm and photographed (Tan, 2007).

3.6.4 Purification of 16S rRNA gene

Prior to sequencing, PCR products exhibiting single bands with the expected size were purified using Qiagen DNA purification kit according to manufacturer's instructions. The purified products were then checked using gel electrophoresis again to confirm the quality of the DNA.

3.6.5 Sequencing of 16S rRNA gene

Purified PCR products were sent to 1st Base Company (Selangor, Malaysia) to be sequenced using 27f forward and 1525r reverse primers (Table 3.1) using ABI system.

3.6.6 DNA sequence analyses

Chromatograms of the reverse and forward sequences obtained from 1st Base Laboratory were viewed using Chromas Lite software (<http://www.softpedia.com/get/Science-CAD/Chromas-Lite.shtml>). Firstly, by using the horizontal and vertical scroll bars, the sequences were examined from the beginning to the end. Any ambiguity found at various sites along the DNA sequence or recorded as

“N” instead of A, C, G or T were corrected (if possible) and the whole sequence were proofread to determine the sequence quality.

In order to get an almost full sequence of each studied *Streptomyces* spp. strain, both forward and reverse sequences files were opened in MEGA 4 (Kumar *et al.*, 2008). The reverse sequence was set as reversed complement in both Chromas Lite (after proofread) and MEGA 4. By using clustal W alignment application in MEGA 4, both the forward and reversed complement sequences were contiguously aligned, resulting of high-lighted bases indicating identical and different bases between the two overlapped sequences. After that, the overlapped sequences were copied and saved as consensus sequence, resulting three sequences in one MEGA 4 file; forward sequence, consensus sequence and reverse complement sequence. Bases which were overlapped were supposed to be identical to ensure the full sequence to be constructed was correct. Therefore, different overlapped bases between the forward and reverse complement sequences were checked and edited manually by comparing the best base peak in chromatogram of forward and reverse complement in Chromas Lite. In the same manner, the messy sequences, in front of forward sequence and at the back of reverse complement sequence in Chromas Lite chromatogram, were deleted in MEGA 4 file. Now, an almost full sequence was created with the combination of the forward and reverse complement sequences. This sequence was then saved in the FASTA format before being analyzed in BLAST, EzTaxon and Ribosomal Database Project websites to get the species with highest percentage of similarity and identity.

BLAST program are available online at <http://blast.ncbi.nlm.nih.gov/Blast.cgi>. Nucleotide blast was applied before uploading the consensus sequence (in FASTA format) of the studied strain in the space provided. Then, the ‘choose search’ was set to ‘others database’; ‘nucleotide collection’. A click of BLAST tab will ‘search’ the

nucleotide database collection (nr/nt) using Megablast (Optimize for highly similar sequences).

The almost full sequences were uploaded in two web-based tools; EzTaxon (<http://147.47.212.35:8080/>) and RDP (<http://rdp.cme.msu.edu/>). The search results will show a list of the most similar strains to the query sequences.

The highest percentage of similarity strains from EzTaxon and RDP were then checked at <http://www.bacterio.cict.fr/> for validation and references on the description of the species.

3.7 Chemical profiling, isolation and identification of active compounds in extracts of potential strains

From this level onwards, all methods and analysis were done under Associate Professor Dr Charles Santharaju Vairappan supervision in Institute of Tropical Biology and Conservation, University Malaysia Sabah (UMS). To depict the method in a simple way, flow chart procedure of chemical profiling and compound isolation is shown in Appendix H.

3.7.1 Fermentation of *Streptomyces* spp.

600ml broth of ISP 2 (Appendix A) was prepared in 2Liter of Schott bottle and sterilized. A 5-day-old culture of strain X34 and X42 from agar plate were subjected to spore count (10^6 spores/ml). 60ml (10% - v/v) of inoculum were used to inoculate the broth media of ISP 2. Inoculated broth was then incubated at room temperature in a rotary shaker at 100-150 rpm for five to seven days prior to extraction.

3.7.2 Extraction of bioactive components

Five to seven days old broth cultures were filtered using filter paper before extraction. Then the filtered broth was added with ethyl acetate (1:1) and left overnight. Extraction was done using ethyl acetate in ratio of 3:1 (filtrate broth: ethyl acetate) and partitioned in a separating funnel to separate the ethyl acetate layer from the water fraction. The principle of this partitioning process is to separate compounds of different polarity from the filtrate broth. In this case, water is much polar compared to ethyl acetate (Houghton & Raman, 1998).

The partitioning process was repeated three times after which the organic phase (ethyl acetate) collected into conical flask. In order to remove traces of water from the organic solution, an inert drying agent, sodium sulphate anhydrous was added and left for two hours. Then the solution was filtered and evaporated to dryness using rotary evaporator. The crude extract obtained was subjected to TLC and tested against *S. pombe* for antifungal bioassay.

3.7.3 High performance liquid chromatography (HPLC) for interesting compounds screening

Extract of potential strains (based on primary screening of antifungal activity) were profiled using HPLC to screen for interesting compounds. Only extract of strains which was expected to have interesting compounds were further profiled and isolated.

About 5mg of the extract was dissolved in 1ml of acetonitrile. The sample then injected automatically into HPLC column following conditions as shown in Table 3.2 below.

Table 3.2: Conditions of high performance liquid chromatography (HPLC) used to screen *Streptomyces* spp. strains for interesting compounds.

Item	Condition
Solvent system	Gradient mode (30% MeCN: 0-5min, 30-100% MeCN: 5-55 min, 100% MeCN: 55-60 min)
Column	X Terra™ RP18 5µm (ID 3.0x250 mm)
Flow	1.0 ml/ml
Detector	UV; 220 nm, 254 nm
Column temperature	35°C
Injection volume	10 µl in MeCN

3.7.4 Thin layer chromatography (TLC)

SiO₂ gel TLC plate (20x20cm) was cut into width of 10cm x 6.5cm (for six samples). A line was drawn 1cm from bottom of the plate. Crude extract was dissolved in ethyl acetate and spotted on the line (Figure 3.2). Crude extract solutions were applied to the plate surface using capillary tube. Three solvent systems were used to develop the plates; hexane: ethyl acetate (3:1); chloroform and toluene. Once developed, the plates were dried using a hair-dryer. To detect double bonds compounds, the plates were observed under 254 and 364nm UV light and any spots or separated compounds detected were circled. The plates were then fully sprayed with a mist of molybdo (IV) phosphoric acid n-hydrate and heated over hot plate at 100°C until coloured spots were visible (Houghton & Raman, 1998).

3.7.5 Column chromatography

A 100ml size column was packed using a simple dry-pack method (Houghton & Raman, 1998). A small amount of cotton, just enough to prevent the silica from leaking out, was clamped down lightly to plug the column. In the meantime, the column was clamped to a ring stand using a 3-pronged clamp. About 200g of dry silica gel adsorbent were soaked in 350ml hexane in a 500ml beaker with a spatula inside and sonicated for

30 minutes. The sonicated silica gel were then poured carefully into the column to fill the column to just below the indent on the column, which left a space of 4 to 5cm on top of the adsorbent. The silica gel was then packed by flushing hexane about seven to eight times repeatedly and a rubber pump was used to speed up the process. The packed silica gel was then left for one hour before loading sample.

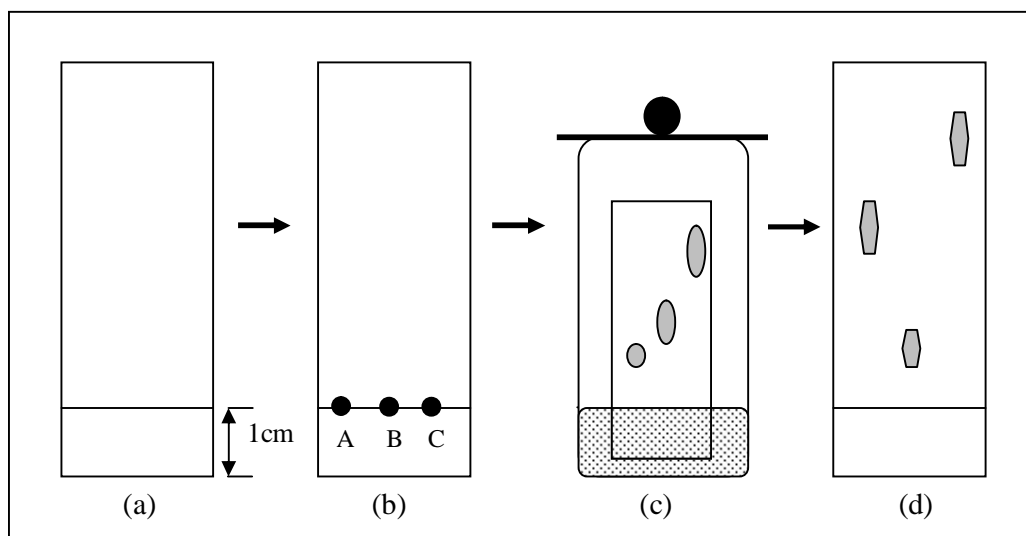


Figure 3.2: Thin layer chromatography (TLC); a) marked TLC plate, b) TLC plate dotted with crude extract of test strains, c) TLC plate developed in chamber and d) developed TLC plate.

500mg of sample to be introduced was diluted in 0.5ml of hexane and sonicated for a few seconds. It was then transferred carefully into the column using Pasteur pipette by distributing over the whole surface of the hexane which covered the silica gel, as not to disturb the packing. The tap at the end of the column was opened carefully to let the mobile phase run through until the level of the supernatant at the top of the column was just above (1cm) the top surface of the stationary phase. The tap then closed to prevent the bed drying out. At this time, the first aliquot of mobile phase eluant was introduced to the top of the column.

In this study, the gradient solvent system used was labeled as F1 to F6 (Table 3.3). The ratio of the mobile phase eluant (increasing polarity) was used to purify (or separate into components) the sample. Each of the eluant was set up to 250ml. All these eluants were introduced onto the column one by one after which each eluant finished and collected separately using a 500ml of round bottom flask. The collected eluants were evaporated dryness by rotary evaporator, transferred into vials, and kept at 4°C prior to use.

Table 3.3: Gradient solvent system of column chromatography for active *Streptomyces* spp. crude extract against *Schizosaccharomyces pombe*.

Fraction	Solvent	Ratio	In 250ml
F1	hexane: ethyl acetate	9:1	225 : 25
F2		8:2	200 : 50
F3		7:3	175 : 75
F4		6:4	150 : 100
F5		1:1	125 : 125
F6	ChCl ₃ : MeOH: dH ₂ O	65:25:4	172.9 : 66.5 : 10.6

ChCl₃: chloroform, MeOH: methanol, dH₂O: distilled water

3.7.6 Preparative thin layer chromatography (PTLC)

About 40mg crude extract of active fraction diluted in 1ml of ethyl acetate and applied in the form of a band on a preparative TLC plate. The plate was then developed in (suitable solvent system) hexane: ethyl acetate (2:3) to separate the components. Basically, PTLC works with the same mechanism as the TLC. Visualization was done by examination under UV light and all the bands detected were marked as well. A narrow strip at the edge of the plate was cut and sprayed with molybdo (IV) phosphoric acid n-hydrate before heating and comparing to the original plate. The position of the

bands marked in the narrow strip was then used to estimate the position of the bands in the original plate. The silica gel of the marked bands then scraped off carefully and mixed with an excess volume of ethyl acetate, sieved by cotton and evaporated to dryness. The fractions obtained were then subjected again to TLC and ¹H-NMR.

3.7.7 Bioassay – antibiotic assay (AA) disc diffusion method

Isolation of active compounds was only done for active components of tested *Streptomyces* spp. extract against *Schizosaccharomyces pombe* which means *Candida albicans* was not included in this part onwards. This is due to be more focused only to one test yeast. Bioassay guided isolation was done by disc diffusion method to detect any compounds or fractions with antifungal activity.

A yeast-peptone-agar (YPG) broth (Appendix A) was prepared in duplicate test tubes before sterilization. The sterilized broth was then inoculated with a loopful of *S. pombe* culture and incubated at 30°C overnight. Using spectrophotometer, the culture turbidity was adjusted to an optical density (OD) McFarland 0.105 (±0.005). Then, 0.1ml of the *S. pombe* adjusted culture was used to seed yeast-peptone-agar (YPG) agar plates (Appendix A). 10mg of each crude extract was dissolved in 0.5ml methanol as samples to be tested, which 25µl of the sample was loaded onto a 6mm paper disc. The loaded paper discs were then put on the seeded ISP 2 agar plates and incubated at 30°C for two to three days (Vairappan *et al.*, 2001). The diameter of any inhibitory zone were measured and recorded.

3.7.8 Preparative thin layer chromatography (PTLC) bioassay guided

PTLC plate was cut into four pieces of 5 x 3.5cm size (for three samples). These small plates then labeled, dotted with diluted crude extract and developed as well in 3:1 hexane to ethyl acetate ratio in the same manner as TLC method (3.7.4). Only one plate

was further sprayed with molybdo (IV) phosphoric acid n-hydrate, which was used as a reference or control for this bioassay. Another three plates were then put in Petri dishes separately and pre-sterilized under UV light in a closed laminar flow for ten minutes. In the meantime, sterilized 100ml of yeast-peptone-glucose (YPG) agar (Appendix A) was prepared. A loopful of two day old *S. pombe* culture was dissolved in the fresh YPG agar and slowly poured into the Petri dishes with developed PTLC plate inside. The YPG agar was just enough to cover the surface of the PTLC plate. After solidification, the Petri dishes were then incubated at 30°C for two to three days. Any clear zone after the incubation period was recorded.

3.7.9 High performance liquid chromatography (HPLC)

About 5mg of active fraction (obtained after column chromatography) was dissolved in 1ml of acetonitrile. The sample was then injected automatically into HPLC column following conditions as shown in Table 3.4.

3.7.10 Structure identification of active compounds

Structure identification was done using nuclear magnetic resonance (NMR) and fourier transformer infrared spectroscopy (FTIR). The samples of active compounds were dissolved in deuterated benzene and (C_6D_6) analyzed using NMR (JEOL), 600MHz for 1H -NMR, ^{13}C -NMR, DEPT, HSQC, HMBC, COSY, NOESY, and TOCSY under these parameters; acquisition scans – 16, relaxation delay – 5sec.

Table 3.4: Conditions of high performance liquid chromatography (HPLC) used in this study to profile active fraction of *Streptomyces* spp. strain with antifungal properties against *S. pombe*.

Item	Condition 1	Condition 2	Condition 3	Condition 4
Column	Luna phenyl-Hexyl	Luna phenyl-Hexyl	C18 (2)	C18 (2)
Solvent system	75% of 70% MeCN	90% of 70% MeCN	75% of 70% MeCN	50% of 50% MeCN
Flow rate	2.0ml/min	2.0ml/min	2.0ml/min	2.0ml/min
Detector	210nm	210nm	254 nm	210 nm
Injection volume	100µl	100µl	20µl	100µl
Sample concentration	5mg/ml MeCN	5mg/ml MeCN	5mg/ml MeCN	5mg/ml MeCN
Sensitivity of plotter	0.050 AU	0.050 AU	0.050 AU	0.050 AU