ISOLATION AND CHARACTERISATION OF ANTIMICROBIAL COMPOUNDS SYNTHESISED BY *MICROCYSTIS* SP.

by

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A Thesis submitted for the degree of

Doctor of Philosophy

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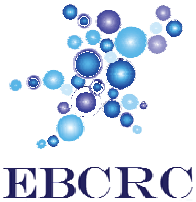


APPENDIX A STANDARD OPERATING PROCEDURES

A.I Freeze Drying

SCOPE: This standard operating procedure (S.O.P.) describes the procedure for efficient and safe operation of the Freeze Drying experiment, located in Laboratory 4.27, Molecular and Life Sciences Building, University of Adelaide.

PURPOSE; The purpose of this SOP is to deocument a procedure for safe and efficient set-up and operation (including start up and shut down) of the Freeze Dry equipment and also provides safety information considered important to the operation of the apparatus.

RESPONSIBILITIES; Kyleigh Victory and Stephen Pahl are responsible for the efficient and safe operation of the Freeze Dry experiment.

CONTENT/DESCRIPTION

Part One – Start-up Procedure

This section describes concisely and in a step by step manner exactly how to safely start the equipment, apparatus or process. Enter your name, the date and time and the total sample size to be freeze dried in the book before starting the machine

- Switch on vacuum pump and refrigeration unit at the wall.
- Position Manifold over cold trap to produce even seal when refrigeration is started.
- Ensure the vacuum pump shut off valve is open. Close the drain valve until finger tight and turn on refrigeration
- When the temperature has dropped to approximately -35°C, close the pump shut off valve and turn on the vacuum pump to warm up

When the temperature has dropped to -45°C, the machine is ready for use.

Part Two - Preparation of Samples

This section describes concisely and in a step by step manner exactly how to prepare samples to be freeze dried

- Cell cultures should be centrifuged prior to freeze drying to remove majority of liquid.
- Cell pellets must be snap-frozen in a slurry of dry ice and ethanol before freeze drying
- Remove caps from tubes and place tubes standing in a plastic container inside manifold
- Place lid on manifold
- Open pump shut off valve
- Leave to freeze dry overnight

Part Three – Shut-down Procedure

This section describes concisely and in a step by step manner exactly how to safely shutdown the equipment, apparatus or process.

- After drying (22 hours is usual), close the pump shut off valve and release the drain valve (leave pump on)
- Remove lid from manifold and take out samples. Replace caps on tubes and store on ice or in freezer until required
- Run the vacuum pump on light ballast for approximately 30 minutes to remove any water from the oil in the pump
- Open pump shut off valve and turn off pump
- Wipe off moisture and clean up cultures spills inside manifold and cold trap
- Complete log book – minimum temperature, maximum pressure achieved, time finished and any relevant comments

Part Four – Requirements

This section describes concisely and in a step by step manner exactly what requirements, equipment or materials, including personal protection are required for this apparatus or process.

Specific

- Dynavac Engineering FD3 Freeze Dryer
- Javac Double Stage High Vacuum Pump

General

- Centrifuge tubes (10ml or 50ml, depending on sample size)
- Plastic beaker/centrifuge tube rack to fit inside freeze dry manifold
- Freezer at -20°C or ice
- Reagents
- 70% v/v Ethanol
- Dry ice

Part Five – Safety and points to note

This section describes concisely and exactly what hazards are present during any phase of operation of this apparatus or process and any personal protection that may be required.

- -50°C cold trap is all that prevents the evaporating solvent from reaching the pump and possibly causing damage. Approval from Tony Richardson (Safety Equipment Officer, School of Molecular and Biomedical Science) or Chris Cursaro (Research Technician, School of Molecular and Biomedical Science) is required before any solvent other than water is used with this apparatus

- Most vacuum pumps have a ballast system that can be used to allow some air to pass through the oil in the pump. This evaporates any solvent from the oil. The ballast adjusting knob is usually beside the suction input. Ballasting is usually only required for drying larger samples

- Total volume that can be dried in 1000mls. The sample volume in any one flask must be one-fifth of the volume of the flask or smaller. Samples must be pre-frozen in a mixture of ethanol and dry ice to form a “shell” around the flask.

- Ballasting procedure: when samples are first placed in manifold, a small amount of ballast should be set and held until the samples are almost dry. When the samples are about 90% dry, shut off the ballast. After the run is complete close the pump shut off valve and set the pump to a medium ballast; run the pump for 30 min/100mls of sample dried then shut off ballast, open pump shut off valve and switch off pump.

REFERED DOCUMENTS

- Operation manual for Dynavac Engineering FD3 Freeze Dryer
- Instruction sheet provided by School of Molecular and Biomedical Sciences, University of Adelaide

A.II Cell enumeration

SCOPE: This standard operating procedure (S.O.P.) describes the procedure for efficient and safe operation of cell enumeration experiments.

PURPOSE: The purpose of this S.O.P. is to document a procedure for safe and efficient set-up, operation (including start-up and shutdown) also and provides safety information considered important to the operation of the apparatus.

RESPONSIBILITIES: Kyleigh Victory and Stephen Pahl are responsible for the efficient and safe operation of the cell enumeration procedure.

CONTENT/DESCRIPTION

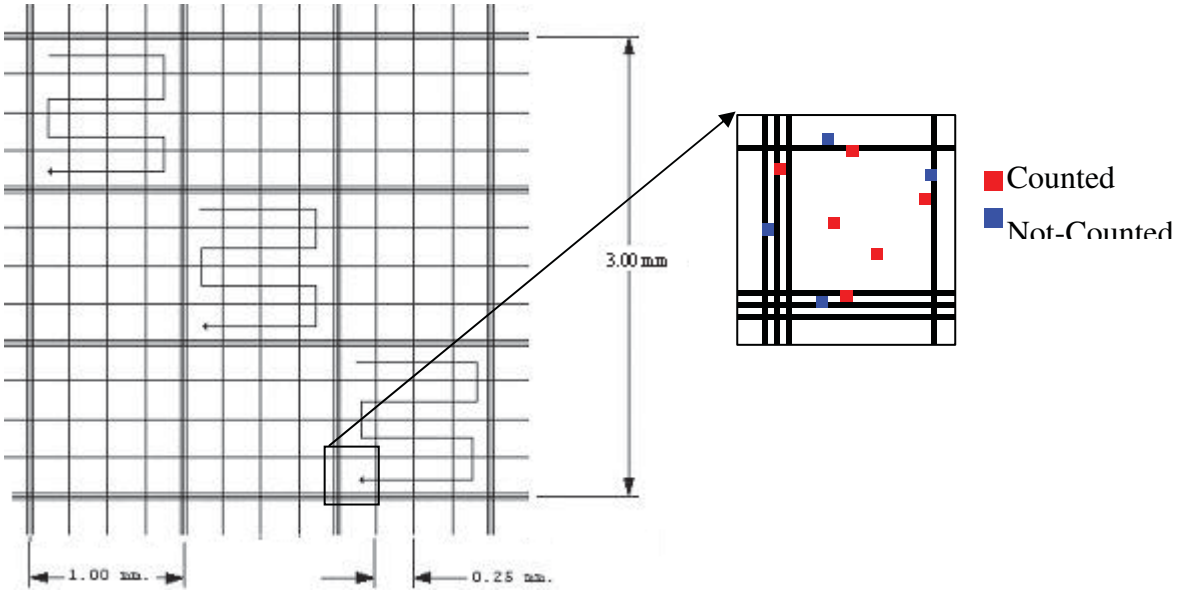
Part One – Start-up Procedure

This section describes concisely and in a step by step manner exactly how to safely start the equipment, apparatus or process.

- Using aseptic techniques take a small sample from the culture vessel and transfer into a small centrifuge tube.
- Add 50μL of Evans Blue stock solution (0.1% w/v) per 100μL of sample (final concentration 0.05% w/v) and allow to stain for 5 minutes. Evans Blue will penetrate and stain dead cell, but will be excluded from the semi-permeable membrane of living cells.
- Centrifuge stained cells (8,000 rev/min for 5 minutes), draw off and discard 100 μL of supernatant and replace with fresh media.
- Count the number of live (unstained) and dead (stained) cells as soon as possible, though the stained sample can be stored at ambient temperature for some time.
- The stained sample is loaded onto the Mod. Fuchs-Rosenthal haemocytometer by the following method:
    - Clean heamocytometer and coverslip with 70% ethanol, followed by MilliQ water
    - The raised polish surfaces are slightly moisten and the coverslip placed over the counting chamber and pressed down firmly so that Newton's rings are formed.

- With a Pasteur pipette, obtain the sample to be counted and carefully load the haemocytometer by capillary action. Do not over-fill the counting chamber.
- Allow sufficient time for the cells to settle to the bottom of the chamber.

- Remove the dust cover over the Olympus IX50 microscope (located in N210). Place the loaded heamocytometer on the stage and turn on the microscope by switching on the TH3 converter.
- Count the cells at 40X magnification according to standard procedures, shown graphically in **Error! Reference source not found.**. Cells that are within, or that touch, the left or top boundary are counted, while those that touch, or are outside, the lower or right boundary are not counted.



Mod. Fuchs-Rosenthal Haemocytometer

Part Two – Shut-down Procedure

This section describes concisely and in a step by step manner exactly how to safely shutdown the equipment, apparatus or process.

- Turn off the microscope and allow to cool down before replacing dust cover.
- Wash the haemocytometer and coverslip with 70% ethanol and rinse with MilliQ water. Carefully dry with Kimwipes and store in a safe place.

Part Three – Requirements

This section describes concisely and in a step by step manner exactly what requirements, equipment or materials, including personal protection are required for this apparatus or process.

Specific

- Microalgae culture
- Mod. Fuchs-Rosenthal haemocytometer & coverslip
- IEC Micromax Microcentrfuge SM2498 & microcentrifuge tubes
- Olympus IX50 microscope

General

- Pipette & pipette tips (sterile and non-sterile)
- Pasteur pipette
- Kimwipes
- Safety glasses, gloves

Reagents

- 70% ethanol solution
- MilliQ water
- Evans Blue solution (0.1% w/v in distilled water)

Part Four – Safety and points to note

This section describes concisely and exactly what hazards are present during any phase of operation of this apparatus or process and any personal protection that may be required.

- Evans Blue is used to stain any non-viable microalgae cells. It may cause cancer, may cause harm to an unborn child and is harmful by inhalation, in contact with skin and if swallowed. Avoid exposure, wear suitable protective clothing, gloves and eye/face protection. In case of accident or feeling unwell seek medical advice immediately and show label if possible.
- Follow operational procedure for the IEC MicroMax Microcentrifuge SM2498 and only operate once inducted.
- The Modified Fuchs-Rosenthal haemocytometer contaists two counting chambers. Each chamber consists of 1 large square, subdivided into 9 smaller

squares, each of which is subdivided into 16 smaller squares. The surfce area of each of the smallest squares is $1/16^{th}$ mm², thus the intermediate squares have a surface area of 1 mm² and the large square 9 mm². The depth of each chamber is 0.2 mm, hence the total volume of each chamber is 1.8 mm³ or 1.8µL.

REFERED DOCUMENTS

- Operational Manual for IEC MicroMax Microcentrifuge SM2498
- Operational Manual for Olympus IX50 microscope

A.III Culture absorbance measurement

SCOPE: This standard operating procedure (S.O.P.) describes the procedure for efficient and safe operation of cell absorbance measurement experiments.

PURPOSE: The purpose of this S.O.P. is to document a procedure for safe and efficient set-up, operation (including start-up and shutdown) also and provides safety information considered important to the operation of the apparatus.

RESPONSIBILITIES: Kyleigh Victory is responsible for the efficient and safe operation of the cell absorbance measurement procedure.

CONTENT/DESCRIPTION

Part One – Start-up Procedure

This section describes concisely and in a step by step manner exactly how to safely start the equipment, apparatus or process.

- Switch on the Shimadzu UV-1601 UV Visible Spectrophotometer and PC. Allow both to run initialisation procedures, and open spectrophotometer software on PC. Select an appropriate wavelength for measuring cell absorbance, in this case 680 nm.
- "Zero" the spectrophotometer by filling the 1 mL cuvette with fresh medium. Place the cuvette into the holder, ensuring the cuvette is aligned correctly.
- Close the lid, and select "zero" on the computer screen.
- Discard the medium. Using aseptic techniques transfer 1 mL of culture by pipette into the cuvette.
- Align the cuvette in the spectrophotometer, and close the lid. Select "Read" from the available menu options. Cell absorbance will be displayed on the screen and on the spectrophotometer.
- Repeat for each culture to be measured, rinsing the cuvette with fresh medium or distilled water between samples.

Part Two – Shut-down Procedure

This section describes concisely and in a step by step manner exactly how to safely shutdown the equipment, apparatus or process.

- After the final sample, rinse the cuvette with 70 % v/v ethanol followed by distilled water, and invert onto a Kimwipe tissue to dry.
- Save the cell absorbance to a file, then shut down PC and spectrophotometer.

Part Three – Requirements

This section describes concisely and in a step by step manner exactly what requirements, equipment or materials, including personal protection are required for this apparatus or process.

Specific

- Microalgae culture
- 1 mL cuvette
- Shimadzu UV-1601 UV-Vis Spectrophotometer

General

- Pipette & pipette tips (sterile and non-sterile)
- Kimwipes
- Safety glasses, gloves

Reagents

- 70% ethanol solution
- MilliQ water
- Fresh medium

Part Four – Safety and points to note

This section describes concisely and exactly what hazards are present during any phase of operation of this apparatus or process and any personal protection that may be required.

- Follow operational procedure for the Shimadzu UV-1601 UV-Vis Spectrophotometer and only operate once inducted.

REFERED DOCUMENTS

- Operation manual for the Shimadzu UV-1601 UV-Vis Spectrophotometer

A.IV Bioactivity assay – Microtitre Plate

SCOPE; This standard operating procedure (S.O.P.) describes the procedure for an efficient and safe procedure for Microtitre plate assays.

PURPOSE; The purpose of this SOP is to document a procedure for safe and carrying out of a bioactive test assay using a Microtitre plate and also provides safety information considered important to the operation of the apparatus.

RESPONSIBILITIES; Kyleigh Victory and Gemma Large are responsible for the efficient and safe operation of Microtitre plate assays.

CONTENT/DESCRIPTION

Part One – Preparation of samples

This section describes concisely and in a step by step manner exactly how to safely start the equipment, apparatus or process.

- Samples are freeze-dried using the procedure stated in the Freeze dry SOP
- Samples are then extracted using the procedures stated in the Extraction SOP

Part Two – Test assay protocol

This section describes concisely and in a step by step manner exactly how to prepare samples to be freeze dried

- Grow bacteria/fungi to be used as test organisms at 37°C on a shaker table at 180 rpm for approximately 5 hours in LB (Miller) broth (can be grown overnight).
- Remove an aliquot (approximately 1 in 10000 dilution) and transfer to the required volume of LB broth for screening (195 µl inoculated broth per well in a microtitre plate).
- Transfer the newly inoculated medium into a sterile multichannel pipette reagent reservoir.
- Aliquot 195 µl into each well of a sterile microtitre plate.
- Add 5 µl of a filter seterilised 10 mg/ml amplicillin to each well in column 12.

- Add 5 µl of DMSO into each well in column 11.

- Add 5 µl of test extract according to plate format.
- Incubate at room temperature, at 100 rpm overnight on shaker incubator.
- Measure absorbance at 600 nm the following day.

Part Three – Requirements

This section describes concisely and in a step by step manner exactly what requirements, equipment or materials, including personal protection are required for this apparatus or process.

Specific

- Sterile microtitre plate
- UV spectrophotometer plate reader
- General
- Shaker table
- 37°C Incubator
- eppendorf tubes
- pipettes and tips
- sterile conical flasks
- autoclave

Reagents

- LB (Miller) broth
- Ampicillin
- Dimethyl sulphoxide (DMSO)
- Bacterial cultures

Part Four – Safety and points to note

This section describes concisely and exactly what hazards are present during any phase of operation of this apparatus or process and any personal protection that may be required.

Ensure that basic laboratory personal protective equipment is worn and ensure that protective gloves are worn when handling algae samples.

DMSO is an irritant, therefore personal protective equipment must be used when handling. Use in a well ventilated area. Keep container securely sealed when not in use. Keep ignition sources away from DMSO. Clean up spills immediately and avoid breathing vapours and contact with skin and eyes. Contain and absorb spill with sand, earth, inert material or vermiculite.

REFERED DOCUMENTS

- Freeze dry SOP
- Extraction SOP

A.V Extractions – Hydrophilic and Lipophilic

SCOPE: This standard operating procedure (S.O.P.) describes the procedure for efficient and safe operation of the extraction of freeze-dried algae samples.

PURPOSE; The purpose of this SOP is to deocument a procedure for safe and efficient set-up and operation of various extraction techniques and also provides safety information considered important to the extraction.

RESPONSIBILITIES; Kyleigh Victory and Gemma Large are responsible for the efficient and safe operation of these extraction methods.

CONTENT/DESCRIPTION

Part One - Preparation of Samples

This section describes concisely and in a step by step manner exactly how to prepare samples ready for extraction

Freeze dry samples prior to extraction by following the procedure outlined in the Freeze-dry SOP

Part Two – Extraction Methods

This section describes concisely and in a step by step manner exactly how to carry out the various extraction techniques

Part 2.1 – Aqueous Extraction

Part 2.1.1 – Distilled water (Ostensvik *et al*. 1998)

- Resuspend 50mg of freeze dried cyanobacterial material in 3ml distilled water
- Carefully mix and keep the sample was kept at room temperature for 30min
- Centrifuge at 2500g for 6 min
- Withdraw the supernatant fluid with a pipette, and transfer 100µl of the aqueous extract to each well in the agar diffusion test.

Part 2.1.2 – Distilled water and Sonication (Ördög *et al*. 2004)

- Resuspend the freeze dried algae in distilled water (10mg ml-1)
- Ultra-sonicate for 2min

Part 2.2 – Solvent Extraction

- *Part 2.2.1 - Three time methanol extraction* (Oudra *et al*. 1998)

- Add 100% methanol to freeze-dried cells (50-75mg dry cells per ml of methanol)
- Centrifuge at 4000g for 10min (4°C)
- Retain the supernatant
- Re-extract the pellet by repeating the above method twice
- Dilute the three methanolic extracts with Milli-Q® ultra pure water (final methanol concentration of 20% v/v)

Part 2.2.2 - Methanol/Sonication on ice (Ohta *et al*. 1998)

- Adjust algal concentration by adding methanol to the pellet in the quantity of 0.1g fresh weight ml-1
- Sonicate on ice
- Centrifuge
- Filter the supernatant through a 0.22µm Millipore (or equivalent) filter

- *Part 2.2.3 - Chloroform: methanol* (Robles-Centeno *et al*. 1996)

- Extract 0.7g freeze dried material with chloroform: methanol (2:1 v/v) over low heat (32°C) for 30min
- Partition extract with water and discard aqueous phase
- Remove chloroform by flash evaporation

Part Three – Requirements

This section describes concisely and in a step by step manner exactly what requirements, equipment or materials, including personal protection are required for this apparatus or process.

Specific

- Branson Digital Sonicator
- Speed Vac (Rotary Vacuum)

General

- Centrifuge tubes (10ml or 50ml, depending on sample size)
- Centrifuge
- 0.22µm Millipore (or equivalent) filter
- eppendorf tubes

Reagents

- Distilled water
- Milli-Q® ultra pure water
- 100% v/v Methanol
- Chloroform
- Ice

Part Four – Safety and points to note

This section describes concisely and exactly what hazards are present during any phase of operation of this apparatus or process and any personal protection that may be required.

- Ensure that personal protective equipment is worn and that protective gloves are worn when handling algae samples.

- Methanol is a flammable liquid – follow instructions in MSDS for safe and appropriate use,

- Chloroform is a human carcinogen – follow instructions in MSDS for safe and appropriate use

REFERED DOCUMENTS

Freeze dry SOP

A.VI Digital Sonification

SCOPE: This standard operating procedure (S.O.P.) describes the procedure for efficient and safe operation of the project 'Analysis and Classification of antimicrobial compounds produced by microalgae and cyanobacteria'.

PURPOSE: The purpose of this S.O.P. is to document a procedure for safe and efficient set-up, operation and shutdown) of the Branson Digital Sonifier located in A311, and also provides safety information considered important to the operation of the apparatus.

RESPONSIBILITIES: Kyleigh Victory is responsible for the efficient and safe operation of the project 'Analysis and Classification of antimicrobial compounds produced by microalgae and cyanobacteria'.

CONTENT/DESCRIPTION

Part One – Set-up Procedure

This section describes concisely and in a step by step manner exactly how to safely set up the equipment, apparatus or process.

- Clean the contacting surfaces of the converter and horn, and remove any foreign matter from the threaded stud and threaded hole
- Coat one of the mating surfaces completely with a very thin film of silicone grease (without additives), using an amount about the size of a paper match head. Excessive grease may diminish performance
- Thread the horn stud into the converter and tighten, using spanner wrenches provided. The recommended torque is 24.85 Nm
- To attach the Tip to the Horn, clean contacting surfaces of the horn and tip, and remove any foreign matter from the threaded stud and hole
- Hand assemble the tip to the horn. Using the spanner wrench on the horn and an open end wrench on the tip, tighten the tip.
- Once assembled, mount the converter/horn/tip assembly in a laboratory retort stand with suitable support.
- Set the on/off switch on the control unit to OFF

- Plug the line cord into the unit, and then into an appropriate power outlet, ensuring the power supply is grounded

Part Two – Operation

This section describes concisely and in a step by step manner exactly how to safely operate the equipment, apparatus or process

- Turn on the Branson Digital Sonifier using the switch below the control panel on the Digital Sonifier Unit
- The Digital Sonifier will execute a pre-programmed series of calibrations – DO NOT attempt to use the unit until these calibrations are complete (approx 1 minute)
- Ensure the horn is connected to the converter, and attach the microtip if required using the spanner wrench provided. Ensure all fittings are tight and flush before use.
- Using keys on Control Panel, set time required, and pulse mode if desired. Illuminated LEDs next to the keys indicate which parameters are available for manipulation. Press ENTER to store values.
- Set the Amplitude using the dial on the right of the Digital Sonifier (displayed on Control Panel screen). If using the microtip, DO NOT exceed 70% Amplitude
- Place sample in suitable vessel (beaker or tube). The vibrating horn or microtip must not be placed against solid objects - can lead to breakage
- As heat is generated during operation, an ice bath may be required to maintain temperature of the sample.
- Place vessel in clamp below horn assembly. Gently lower assembly into sample, ensuring tip is not touching base or sides of vessel, but is submerged in sample.
- Press START key on control panel when experiment ready. Operation may be paused or stopped at any time during experiment using START/PAUSE or STOP keys.
- To reduce contamination of samples, tip should be cleaned between samples by vibrating tip in 70% Ethanol (5-10secs sufficient).

- Following final sample tip should be cleaned with 70% Ethanol to ensure all solid particles are removed

Part Three – Shut-down and Maintenance Procedure

This section describes concisely and in a step by step manner exactly how to safely shutdown the equipment, apparatus or process.

- Ensure tip/horn has been cleaned with ethanol
- Switch off Control Unit
- The horn and tip should be periodically observed for erosion (a side effect of the cavitation process) and must be cleaned regularly to minimise erosion
- Remove horn and tip, and clean all surfaces, including threads with alcohol. Ensure both are clean and completely dry before reassembling.

Part Four – Requirements

This section describes concisely and in a step by step manner exactly what requirements, equipment or materials, including personal protection are required for this apparatus or process.

Specific

- Branson Digital Sonifier 250
- Power cordset
- Converter
- Horn and Horn Tips (eg Microtip)
- Spanner wrench and Open-ended wrenches

General

- Beakers/centrifuge tubes
- Laboratory retord stand and clamps
- Ice Bath (optional)
- Safety glasses, gloves, ear plugs

Reagents

- 70% Ethanol
- Ice

Part Five – Safety and points to note

This section describes concisely and exactly what hazards are present during any phase of operation of this apparatus or process and any personal protection that may be required.

- Do not operate the equipment with the cover removed. High voltage is present within the equipment when connected to the plant wiring

- Do not turned on the ultrasonics without the converter and horn attached

- Do not touch the horn or tip when ultrasonics are active. When handling, removing or attaching a horn or tip, be sure that the ON/OFF switch is set to OFF. Touching the horn or tip while the unit is on can result in serious personal injury

- The tip must be installed clean and dry, or the power supply may not tune and operate correctly

- To remove a horn, use spanner wrenches shipped with the system. Never attempt to remove a horn by holding the converter housing in a vice. If necessary, secure the largest portion of the horn in a soft-jawed vice

- Do not exceed 70% Amplitude when operating with the Microtip. The microtip is recommended for volumes from 3-20ml

- Do not allow the horn or microtip to contact lab stands, beakers, test tubes or similar objects. Microtip failure may result. Breakage of glassware may result in the loss of a specimen

- An ice bath may be necessary to minimise temperature rise of specimens. Consideration should also be made for the type of vessel used – those with low heat conductivity should be used

- Plastic vessels are not recommended unless the sample being processed will be unaffected by heat or unless ultrasonic treatment is pulsed

REFERED DOCUMENTS

- Operational manual for Branson Digital Sonifier Models 250 & 450

A.VII Agar Disc Diffusion

SCOPE: This standard operating procedure (S.O.P.) describes the procedure for efficient and safe operation of the bioactivity assay of algal extracts by agar disc diffusion.

PURPOSE; The purpose of this SOP is to document a procedure for safe and efficient set-up and operation of bioactivity assays for algal extracts (lipophilic and hydrophilic) by the agar disc diffusion method and also provides safety information considered important to the assay.

RESPONSIBILITIES; Kyleigh Victory and Gemma Large are responsible for the efficient and safe operation of these extraction methods.

CONTENT/DESCRIPTION

Part One - Preparation of Samples

This section describes concisely and in a step by step manner exactly how to prepare samples ready for extraction

- Freeze dry samples prior to extraction by following the procedure outlined in the Freeze-dry SOP
- Perform extractions (solvent extraction or aqueous extraction) by following the procedure outlined in the Extraction SOP

Part Two – Bioactivity Assays

This section describes concisely and in a step by step manner exactly how to carry out the bioactivity assay by agar disc diffusion

Part 2.1 – Preparation of Sterile Discs

- Punch discs (6-8mm) from a piece of Whatman filter paper and collect the discs in a 10ml McCartney bottle
- Autoclave the McCartney bottle containing the filter discs to sterilise.

Part 2.2 – Preparation of Agar Plates

- Prepare sufficient Luria Bertani agar to pour agar plates for all bioactivity assays required.
- pour approximately 15ml of agar into each 90mm petri dish in laminar flow hood and flame surface using Bunsen burner to remove air bubbles and sterilise
- Replace lids following flaming and leave to set for 20 min.
- Plates may be stored at 4°C until required.

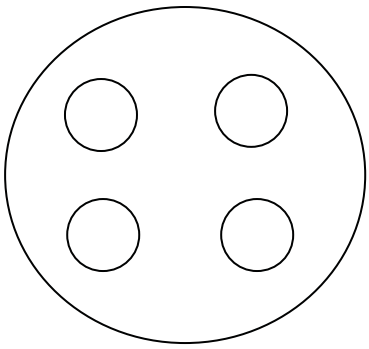
Part 2.3 – Preparation of Test Organisms

- Obtain bacterial cultures to be used as test organisms from School of Molecular and Biomedicacl Sciences at least 2 days before required.
- Obtain fungal cultures from Mycology Department, Women's and Children's Hospital
- Sterilise 10ml of Luria Bertand Broth per test organism by pouring broth into 20ml McCartney bottles and autoclaving.
- Inoculate aseptically (using a Bunsen burner and inoculating loop) each broth sample with a different organism.
- Agitate samples overnight on an orbital shaker (100 rpm and 25°C)

Part 2.4 - Bioactivity Assay

- Inoculate agar plates by placing a 100µl of inoculated culture in the centre of the plate and spreading aseptically to ensure an even distribution.
- Incubate plates for 1 hour at final test temperature.
- Impregnate sterile filter paper discs with 20-30µl of the respective microalgal extracts and dry in an oven at 50°C
- Sterile filter discs should also be impregnated with samples of the extract solvents as controls, and with an known bactericidal/antifungal (eg an antibiotic). Incubate 1 plate of each organism with control-impregnated discs.
- Place sterile discs impregnated with extracts on inoculated agar plates (4 discs per plate, equally spaced) using sterile tweezers. Perform all tests in triplicate. (See)
- Incubate bacterial plates at 30°C for 48 hours, and the fungal plates at 25°C for 72 hours.

- After incubation observe plates for zones of organism growth inhibition and measure diameters of zones.



Figure_Apx A-A-1: Schematic Diagram of agar plate with sterile discs placed at equally spaced intervals

Part Three – Requirements

This section describes concisely and in a step by step manner exactly what requirements, equipment or materials, including personal protection are required for this apparatus or process.

Specific

- 90mm Petri Dishes
- 6-8mm filter discs
- inoculating loop
- spreader
- General
- 20ml McCartney Bottles
- Bunsen burner

Reagents

- Distilled water
- 100% v/v Methanol
- Chloroform
- ethanol
- LB Agar

- LB broth
- Bacterial and fungal cultures

Part Four – Safety and points to note

This section describes concisely and exactly what hazards are present during any phase of operation of this apparatus or process and any personal protection that may be required.

- Ensure that personal protective equipment is worn and that protective gloves are worn when handling algae samples and bacterial/fungal cultures

- Perform all bacterial and fungal culture work in Laminar flow hood.

- Methanol is a flammable liquid – follow instructions in MSDS for safe and appropriate use,

- Chloroform is a human carcinogen – follow instructions in MSDS for safe and appropriate use

- Ethanol is a flammable liquid – follow instructions in MSDS for safe and appropriate use,

- Take care when using Bunsen burner, and turn off gas when not in use

REFERED DOCUMENTS

- Freeze dry SOP

- Extraction SOP

A.VIII Growth of axenic cultures

SCOPE: This standard operating procedure (S.O.P.) describes the procedure for efficient and safe operation of the growth of axenic *Microcystis* cultures.

PURPOSE: The purpose of this SOP is to document a procedure for safe and efficient set-up and operation of axenic culturing techniques, and also provides safety information considered important to the methods.

RESPONSIBILITIES; Kyleigh Victory is responsible for the efficient and safe operation of the axenic culture method.

CONTENT/DESCRIPTION

Part One - Preparation of Media

Refer to Appendix C for instructions to prepare B-12 media.

Part Two – Culturing

This section describes concisely and in a step by step manner exactly how to carry out the culturing techniques:

- Add 5 ml of B-12 liquid medium to a test-tube and add cells to be cultured.
- Culture under 2,000-1x illumination at 30°C for 7 – 10 days.
- Vigorously shake using a vortex mixer for 2 min.
- Serially dilute 10-fold in sterilised, deionised, distilled water.
- Vigorously stir each dilution for 30 to 60 s.
- Follow the method described in Part 2.1 to prepare pour plates or Part 2.2 for preparation of spread plates.

Part 2.1 – Pour Plates

- Pipette 100 µL of 100-fold diluted culture onto a sterile agar plate.
- Add approximately 10 mL molten (40 °C) B-12 medium mixed with agar. [The medium contains various concentrations of agar, agar-noble, agarose and low-melting agarose]. Keep medium molten at 40 °C in a water bath.
- Allow plates to solidify at room temperature.

- Incubate plates under 2,000-lx illumination in an inverted position at 30°C

Part 2.2 – Spread Plates

- Pour molten B-12 medium mixed with agar media into a sterile Petri dishes.
- Allow plates to solidify at room temperature.
- Pipette 100 µL of diluted culture onto the surface of the solid media and spread aseptically.
- Seal plates with Parafilm and incubate under 2,000-lx illumination in an inverted position at 30°C.

Part Three – Isolation of axenic species

- When colony formation is observed, transfer cells in the colony using a sterile toothpick, to fresh agarose medium and incubate again to produce a second culture.
- Take 5-6 cells from fully propagated colonies in the second culture and inoculate in CB medium (5 ml per tube).
- Culture for 1 to 2 weeks.
- Examine cells using a phase-contrast microscope to determine if any bacterial contamination has occurred.
- Repeat Step 1 if contamination has occurred, until the culture is free of contamination.
- Inoculate non-contaminated cells into 100 ml of CB medium in a 200 ml Erlenmeyer flask and culture at 30 °C under 2000 lx illumination.
- Heavily inoculate the resulting cultured cells into the following 3 test media to observe for any microbial contamination (1 mL of culture per 10 mL of medium): diluted nutrient agar (0.5 g of meat extract, 1 g of peptone, 0.5 g of NaCl [pH 7.0] per litre); thioglycolate (TGC) medium; and potato dextrose agar.
- Culture at 30°C for 3 weeks.
- Repeat the procedures if contaminating microbes are observed.

Part Four - Requirements

This section describes concisely and in a step by step manner exactly what requirements, equipment or materials, including personal protection are required for this apparatus or process.

Specific

- Parafilm
- Illumination at 2,000-1x
- 200 ml Erlenmeyer flask
- vortex mixer
- General
- petri dishes
- agar plates

Reagents

- thioglycolate (TGC) medium
- agar
- agar-noble
- agarose
- low-melting agarose
- meat extract
- peptone
- NaCl (pH 7.0)
- Distilled, deionised water
- potato dextrose agar

Part Five – Safety and points to note

This section describes concisely and exactly what hazards are present during any phase of operation of this apparatus or process and any personal protection that may be required.

- Refer to the relevant MSDS document for more information.

- Personal protective equipment must be worn at all times when handling chemicals.

REFERENCES

The axenic culture method was described by (Shirai M. 1989).

A.IX Isolation of Single Cells

SCOPE: This standard operating procedure (S.O.P.) describes the procedure for efficient and safe operation of the isolation of single cells of microalgae and cyanobacteria.

PURPOSE: The purpose of this SOP is to document a procedure for safe and efficient set-up and operation of isolating single-cells of microalgae and cyanobacteria and also provides safety information considered important to the methods.

RESPONSIBILITIES; Kyleigh Victory is responsible for the efficient and safe operation of the preparation and isolation method.

CONTENT/DESCRIPTION

Part One - Preparation of micropipette from a Pasteur pipette

This section describes concisely and in a step by step manner exactly how to carry out single-cell isolation using a micropipette.

- Hold the Pasteur pipette in both hands so the middle of the pipette is held over the flame of a Bunsen burner (or similar flame source).
- Rotate the pipette slowly, allowing the pipette to warm to melting point.
- When the pipette becomes pliable, remove it from the flame and gently pull both ends to produce a thin tube and eventually separates into 2. .
- Allow to cool for a few seconds.
- Using a pair of tweezers, carefully snap the uneven ends from each micropipette to create smooth, even tips
- If a jagged edge results, discard micropipettes and repeat the process.

Part Two – Isolation

This section describes concisely and in a step by step manner exactly how to carry out the isolation techniques.

- Place 4 drops of pre sterilised medium or distilled, deionised water equally spaced on a microscope slide. Rest the slide on a glass Petri dish and place under a microscope. (A dissecting microscope is recommended)

- .Attach one end of a flexible latex/rubber tube (preferably diameter just larger than that of the micropipette). Insert a pipette tip (1000 µL) into the other end to act as a mouthpiece
- Draw a small amount of water into the micropipette to act as a cushion.
- Place a finger over the open end of the mouthpiece and place the tip of the micropipette near the target cell. (in a Petri dish or similar, pour a small amount of the sample containing the cells of interest). Remove the finger to gently allow capillary action to draw 1 – 2 cells into the micropipette.
- Remove the tip from the sample and immerse in the first droplet of culture medium/water.
- Gently blow into the mouthpiece, discharging the captured cell(s).
- Clean the tubing/capillary tube between isolations by drawing up fresh media or water and discarding into a waste receptacle.
- Viewing the droplets through the microscope, capture a cell from the first droplet by capillary action (steps 4-6). Carefully discharge the cell into the second droplet.
- Repeat transfer to reach the last droplet – should have isolated a single cell at this stage.
- Capture the single cell from the final drop and discharge into a culture vessel (tube or McCartney bottle containing 10 mL culture medium).

Part Two – Technique advice

- Cells must be able to survive in the culture medium chosen, preferably without stress.
- When capturing and discharging cells from the micropipette, drawing and expelling pressure must be slight. Excessive pressure or rapid movement can damage the cell.
- Unnecessary additional isolation should be avoided as it often leads to cell damage.
- The diameter of the micropipette opening should be at least twice that of the cell in order to avoid cell damage.
- When isolating filaments, chains of cells or long single cells, the micropipette should be directed to one end of the filament or chain. The micropipette

should be held at an angle so that the filament/cell slides up the micropipette tip without severe bending.

- Time between capture and discharge of cells should be short to void adherence to the insides of the micropipette

Part Three - Requirements

This section describes concisely and in a step by step manner exactly what requirements, equipment or materials, including personal protection are required for this apparatus or process.

Specific

- 100 µL Pasteur pipette (Pyrex disposable)

General

- Flame source (Bunsen burner, blow torch)
- Microscope (preferably dissecting)
- Microscope slides
- Petri dish
- Fine-bore rubber tubing
- Forceps/tweezers

Reagents

- Culture medium
- Distilled water

Part Four – Safety and points to note

This section describes concisely and exactly what hazards are present during any phase of operation of this apparatus or process and any personal protection that may be required.

- Glass ends removed from micropipettes and used pipettes should be discarded carefully as they are extremely sharp.

REFERENCES

Single cell isolation procedure described by (Andersen and Kawachi 2005)

A.X High Pressure Liquid Chromatography

SCOPE: This standard operating procedure (S.O.P.) describes the procedure for efficient and safe operation of separation of cyanobacterial extracts by high pressure liquid chromatography.

PURPOSE: The purpose of this SOP is to document a procedure for safe and efficient set-up and operation of high pressure liquid chromatography of cyanobacterial extracts and also provides safety information considered important to the methods.

RESPONSIBILITIES; Kyleigh Victory is responsible for the efficient and safe operation of the high pressure liquid chromatography method.

CONTENT/DESCRIPTION

Part One - Preparation of micropipette from a Pasteur pipette

This section describes concisely and in a step by step manner exactly how to carry out single-cell isolation using a micropipette.

Start up Procedure:

- Switch on all machines
- Pump A= Solvent A = CH_3CN/TFA
- Pump B = Solvent B = CH_3CN/H_2O/TFA
- Place appropriate solvent line into solution and cover top of solvent bottle (Schott bottle) with aluminium foil
- Beginning with Pump A, open purge valve and purge lines for approximately 2 min to remove air bubbles. Close purge valve
- Repeat purging of lines for Pump B.
- To purge the system, rinse the lines with Solvent B for 5 min, then Solvent A, collecting any waste in a suitable container.
- Check flow rates of both pumps. To check flow of Pump A, set flow rate to 1 mL/min, at 100% Solvent A, and 0% Solvent B. To check flow of Pump B, set flow rate to 1 mL/min, at 0% Solvent A, and 100% Solvent B.

- Attach the column, ensuring column orientation is correct (check arrows on side of column)
- Run solvent A through column for 5 min under isocratic conditions until the UV-Vis detector stabilises (monitor the wavelength on the detector)
- Change pump operation to External Mode, and use the computer to set the required conditions

Loading samples:

- Load the file:"Male Cane Toad" – this protocol has the required conditions and solvent gradient program preset.
- Run the following gradient: 15-80% Solvent A over 30 min at 2 mL/min; 100% Solvent A for 5 min at 1 mL/min; 100% Solvent A for 1 min at 0.1 mL/min. The system will hold at 0.1 mL/min until altered by operator
- Run a "blank" sample, by turning the injector port to start the program, but without injecting a sample. The system will operate at desired conditions. Press F3 to start recording.
- Allow the program to run, until returned to initial conditions
- Inject sample: Pre filter the sample using a syringe filter. Draw up 100 µL using the HPLC injection syringe. Ensure there are no air bubbles near the needle end of the syringe
- Turn the injection port to "Inject". Ensure syringe is level and insert needle smoothly into injection port .Gently inject sample, remove needle and turn port to "Load", simultaneously pressing F3 to commence recording activity.
- Place a small waste container beneath the outlet wire to collect excess solvent and any unwanted elute
- Fraction Collection: Depending on the fraction size to be collected, use 1.5 mL microcentrifuge tubes or sterile 10 mL centrifuge tubes to collect fractions of eluted material by monitoring the wavelength. A steep, rapid climb indicates a change in material (eg other than solvent) eluted from the column.
- Use separate tubes for different fractions, and label clearly. Record the retention time at the peak wavelength as a fraction is collected
- Following each run (approximately 30 min and column wash), allow the system to return to initial conditions until the UV-Vis detector has stabilised

- Load the next sample, and repeat procedure until all samples have been completed, or shut down.

Shut down procedure:

- return the system to isocratic conditions
- Clean the column for 5 min using acetonitrile: Flow rate 1 mL/min, pump A 100 %, Pump B 0%.
- Stop pumps and remove column. Replace the plugs in the ends of the column, and wrap in foil.
- Remove solvent bottles, and replace with filtered water (place both solvent lines in same bottle), or if system is to remain dormant for several days, use isopropanol. Clean the lines for 5 min
- Replace water with methanol and repeat line cleaning for further 5 min
- Allow pressure to return to 0 kPa and switch off pumps and computer

Part Two - Requirements

This section describes concisely and in a step by step manner exactly what requirements, equipment or materials, including personal protection are required for this apparatus or process.

Specific

- ICI Instruments LC1200 UV-Vis Detector
- ICI Instruments LC1110 HPLC Solvent Pump (Solvent A)
- ICI Instruments LC1110 HPLC Solvent Pump (Solvent B)
- VYDAC Protein and peptide C18 column

General

- Sterile filters
- syringe

Reagents

- Acetonitrile (HPLC Grade)

- 0.1% Trifluoroacetic acid

Part Three – Safety and points to note

This section describes concisely and exactly what hazards are present during any phase of operation of this apparatus or process and any personal protection that may be required.

- Refer to the relevant MSDS document for more information on acetonitrile and trifluoroacetic acid.

REFERENCES

HPLC Protocol prepared by Daniel Bilusich, Discipline of Chemistry, School of Chemistry and Physics, University of Adelaide. O9iio

A.XI DNA Extraction Techniques

MO BIO UltraClean Soil DNA Isolation Kit

SCOPE; This standard operating procedure (S.O.P.) describes the procedure for efficient and safe operation DNA extraction from cyanobacteria using the MO BIO UltraClean Soil DNA Isolation Kit

PURPOSE; The purpose of this SOP is to document a procedure for safe and efficient set-up and operation of the MO BIO UltraClean Soil DNA Isolation Kit as applied to microalgae and cyanobacteria and also provides safety information considered important to the methods.

RESPONSIBILITIES; Kyleigh Victory is responsible for the efficient and safe operation of the MO BIO UltraClean Soil DNA Isolation Kit.

CONTENT/DESCRIPTION

Part One – Extraction of DNA from freeze-dried cyanobacterial cells

This section describes concisely and in a step by step manner exactly how to extract DNA from microalgae and cyanobacteria using the MO BIO UltraClean Soil DNA Isolation Kit (Alternative Protocol, for maximum yields).

- To the 2 mL Bead Solution tubes provided, add 0.12 g of freeze dried cyanobacterial cells. Gently vortex to mix
- Add 60 µL of Solution S1 and invert several times or vortex briefly
- Add 200 µL of Solution IRS (Inhibitor Removal Solution). Only required if DNA is to be used for PCR.
- Secure bead tubes horizontally using the Mo Bio Vortex Adapter tube holder for the vortex, or secure tubes horizontally on a flat-bed vortex pad with tape. Vortex at maximum speed for 10 min
- Make sure the 2 mL tubes rotate freely in your centrifuge without rubbing. Centrifuge tubes at 10, 000 ×g for 30 s. CAUTION: Be sure not to exceed 10, 000 ×g or tubes may break
- Transfer the supernatant to a clean microcentrifuge tube (provided)

- Note: depending on sample type and moisture content, expect 400-450 µL supernatant (may still contain particles)
- Add 250 µL of Solution S2 and vortex 5 s. Incubate 4 °C for 5 min
- Centrifuge the tubes for 1 min at 10000 ×g
- Avoiding the pellet, transfer entire volume of supernatant to a clean microcentrifuge tube (provided)
- Add 1.3 mL of Solution S3 to the supernatant (careful, volume touches rim of tube) and vortex for 5 s
- Load approximately 700 µL onto a spin filter and centrifuge at 10000 ×g for 1 min. Discard the flow through, add the remaining supernatant to the spin filter and repeat centrifuge at 10000 ×g for 1 min. A total of 3 loads per sample may be required
- Add 300 µL of Solution S4 and centrifuge for 30 s at 10000 ×g
- Discard the flow through. Cnetrifuge again for 1 min at 10000 ×g
- Carefully place the spin filter in a new clean tube (provided). Avoid splashing any Solution S4 onto the spin filter
- Add 50 µL of Solution S5 to the centre of the white filter membrane. Centrifuge for 30 s.
- Discard the spin filter. DNA in the tube is now application ready. No further steps are required

Part Two - Requirements

This section describes concisely and in a step by step manner exactly what requirements, equipment or materials, including personal protection are required for this apparatus or process.

Specific

- Mo Bio UltraClean Soil DNA Isolation Kit

General

- Vortex
- Vortex adapter or flat-bed vortex pad

- Ice
- Microcentrifuge
- (heating block)

Part Three – Safety and points to note

This section describes concisely and exactly what hazards are present during any phase of operation of this apparatus or process and any personal protection that may be required.

- Do not exceed 10000 ×g or tubes may break
- Solution S5 contains no EDTA
- .If Solution S1 has precipitated, heat solution to 60 °C until dissolved before using

REFERENCES

Mo Bio Laboratories, Inc UltraClean Soil DNA Isolation Kit Instruction Manual – Alternative Protocol, for maximum yields

A.XII DNA Extraction Techniques

QIAGEN QIAamp® DNA Stool Mini Kit: For DNA purification from stool samples

SCOPE; This standard operating procedure (S.O.P.) describes the procedure for efficient and safe operation DNA extraction from cyanobacteria using the QIAGEN QIAamp® DNA Stool Mini Kit

PURPOSE; The purpose of this SOP is to document a procedure for safe and efficient set-up and operation of the QIAGEN QIAamp® DNA Stool Mini Kit as applied to microalgae and cyanobacteria and also provides safety information considered important to the methods.

RESPONSIBILITIES; Kyleigh Victory is responsible for the efficient and safe operation of the QIAGEN QIAamp® DNA Stool Mini Kit

CONTENT/DESCRIPTION

Part One – Extraction of DNA from freeze-dried cyanobacterial cells

This section describes concisely and in a step by step manner exactly how to extract DNA from microalgae and cyanobacteria using the QIAGEN QIAamp® DNA Stool Mini Kit, Protocol for Isolation of DNA from Stool for Pathogen Detection

- Weigh 120 mg of sample into a 2 mL microcentrifuge tube (not provided) and place tube on ice
- Add 1.4 mL Buffer ASL to each sample. Vortex continuously for 1 min or until sample is thoroughly homogenised
- Heat the suspension for 5 min at 70 °C
- Vortex for 15s and centrifuge sample at full speed for 1 min to pellet particles
- Pipet 1.2 mL of the supernatant to a new 2 mL microcentrifuge tube (not provided) and discard the pellet
- Add 2 InhibitEX tablet to each sample and vortex immediately and continuously for 1 min or until the tablet is completely suspended. Incubate

the suspension for 1 min at room temperature to allow inhibitors to adsorb to the InhibitEX matrix

- Centrifuge sample at full speed for 3 min to pellet inhibitors bound to InhibitEX
- Pip*et al*l of supernatant into a new 1.5 mL microcentrifuge tube (not provided) and discard the pellet. Centrifuge the sample at full speed for 3min. Transfer of small quantities of pellet material will not affect the procedure
- Pipet 15 µL Proteinase K into a new 1.5 mL microcentrifuge tube (not provided)
- Pipet 200 µL supernatant from step 8 into the 1.5 mL microcentrifuge tube containing Proteinase K
- Add 200 µL Buffer AL and vortex 15 s
- Incubate 70 °C for 10 min. Optional: centrifuge briefly to remove drops from inside of the tube lid
- Add 200 µL of ethanol (96-100%) to the lysate, and mix by vortexing. Optional: centrifuge briefly to remove drops from inside of the tube lid
- Label the lid of a new QIAamp spin column placed in a 2 mL collection tube. Carefully apply the complete lysate to the QIAamp spin column without moistening the rim. Close the cap and centrifuge at full speed for 1 min. Place the QIAamp spin column in a new 2 mL collection tube, and discard the tube containing the filtrate
- Carefully open the QIAamp spin column and add 500 µL Buffer AW1. Centrifuge at full speed for 1 min. Place the QIAamp spin column in a new 2 mL collection tube, and discard the collection containing the filtrate
- Carefully open the QIAamp spin column and add 500 µL Buffer AW2. Centrifuge at full speed for 3 min. Discard the collection tube containing the filtrate
- Transfer the QIAamp spin column into a new, labelled 1.5 mL microcentrifuge tube (not provided) and pipet 200 µL Buffer AE directly onto the QIAamp membrane. Incubate 1 min at room temperature, then centrifuge at full speed for 1 min to elute the DNA

Part Two - Requirements

This section describes concisely and in a step by step manner exactly what requirements, equipment or materials, including personal protection are required for this apparatus or process.

Specific

- QIAGEN QIAamp® DNA Stool Mini Kit

General

- Vortex
- Microcentrifuge
- heating block

Reagents

- 96-100 % v/v Ethanol

Part Three – Safety and points to note

This section describes concisely and exactly what hazards are present during any phase of operation of this apparatus or process and any personal protection that may be required.

- Ensure Buffers AW1 and AW2 have been prepared according to the instructions on the labels
- Mix all buffers before use
- If a precipitate has formed in Buffer ASL or AL, dissolve by incubating at 70 °C
- Prepare a 70 °C water bath or heating block before commencing extraction
- For detection of cells difficult to lyse, such as some bacteria and parasites, the lysis temperature in step 3 can be increased to 95 °C
- All centrifugation steps should be carried out at room temperature (15-20 °C) at 20,000 ×g (~14,000 rpm).

- To optimise DNA yield, repeat the elution step using a further 200 µL of Buffer AE
- Thorough vortexing of the samples ensure maximum DNA concentration in the final eluate
- Do not add Proteinase K directly to Buffer AL
- Close each spin column to avoid aerosol formation during centrifugation. If the lysate has not completely passed through the column after centrifugation, centrifuge again until the spin column is empty
- Residual Buffer AW2 in the eluate may cause problems in downstream applications. An optional step of placing the spin column in a fresh collection tube and centrifuging for 1 min at full speed is recommended.

REFERENCES

QIAGEN QIAamp® DNA Stool Mini Kit Handbook – Protocol for Isolation of DNA from Stool for Pathogen Detection

A.XIII Preparation of ready-to-use GeneRuler™ 100bp DNA Ladder

SCOPE: This standard operating procedure (S.O.P.) describes the procedure for efficient and safe application of the ready to use GeneRuler™ 100 bp DNA ladder.

PURPOSE: The purpose of this SOP is to document a procedure for safe and efficient application of the ready to use GeneRuler™ 100 bp DNA ladder and also provides safety information considered important to the methods.

RESPONSIBILITIES: Kyleigh Victory is responsible for the efficient and safe application of the ready to use GeneRuler™ 100 bp DNA ladder.

CONTENT/DESCRIPTION

Part One – Preparation of the ready to use GeneRuler™ 100 bp DNA ladder

This section describes concisely and in a step by step manner exactly how to prepare the ready to use GeneRuler™ 100 bp DNA ladder for application during gel electrophoresis of DNA.

GeneRuler™ DNA ladders are designed and recommended for sizing and approximate quantification of PCR products or other wide range double-stranded DNA fragments in agarose or polyacrylamide gels. The ladders are composed of chromatography-purified individual DNA fragments. GeneRuler™ DNA ladders are available in two formats: conventional (in a storage (TE) buffer), and a convenient ready-to-use format: premixed with 6X Loading Dye Solution for direct loading onto agarose or polyacrylamide gels.

Prepare the following mixture for loading on agarose gel:

- Combine 1 µl of GeneRuler™ DNA ladder (0.5 µg), 1 µl of 6X Loading Dye Solution and deionised water up to 6 µl
- Vortex gently before use
- Load 6 µl of the prepared DNA ladder on a 5 mm lane of agarose gel.
- Use 0.2 µg (0.4 µl) of the GeneRuler™ DNA ladder per 1 mm polyacrylamide gel lane width:

Prepare the following mixture for loading on polyacrylamide gel:

- Combine 2 µl of GeneRuler™ DNA ladder (1 µg), 0.5 µl of 6X Loading Dye Solution and deionised water up to 3 µl.
- Vortex gently before use;
- Load 3 µl of the prepared DNA ladder on a 5 mm lane of polyacrylamide gel.
- Do not heat before loading.

Recommendations for Loading

For accurate sizing, use the same loading dye solution for sample and ladder DNA.

- Apply the ready-to-use GeneRuler™ or O'GeneRuler™ DNA ladder:
- 1 µl (0.1 µg) per 1 mm of an agarose gel lane width;
- 1-2 µl (0.1-0.2 µg) per 1 mm of a polyacrylamide gel lane width.
- Use 0.1 µg (0.2 µl) of the GeneRuler™ DNA ladder per 1 mm agarose gel lane width:

Part Two - Requirements

This section describes concisely and in a step by step manner exactly what requirements, equipment or materials, including personal protection are required for this apparatus or process.

Reagents

- GeneRuler™ Storage and Loading Buffer (for ready-to-use ladders):
- Ready to use GeneRuler™ 100 bp DNA ladder
- deionised water

General

- 20 µL Micropipette
- Vortex

Part Three – Safety and points to note

This section describes concisely and exactly what hazards are present during any phase of operation of this apparatus or process and any personal protection that may be required.

- GeneRuler™ 100bp DNA Ladders are supplied with 6X Loading Dye Solution.
- Bands composed of equalized amounts of DNA for easier quantification.
- Range: 10 fragments (in bp): 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100.
- Storage Buffer (TE buffer): 10 mM Tris-HCl (pH 7.6) and 1 mM EDTA.
- GeneRuler™ Storage and Loading Buffer (for ready-to-use ladders): 10 mM Tris-HCl (pH 7.6), 10 mM EDTA, 0.005% bromophenol blue, 0.005% xylene cyanol FF and 10% glycerol.

APPENDIX B GENERAL METHODOLGY

B.I Microalgal Cultivation Techniques

B.I.1 Isolation Techniques

A comprehensive review of microalgal cultivation techniques, including factors that affect successful isolation from field samples is presented below. Sample collection methods, isolation equipment and techniques were considered prior to selection, to optimise the likelihood of successful isolation of the cyanobacterial strain of interest.

B.I.1.1 Factors affecting Isolation

The primary objective of isolation and purification techniques is to ". . . create a viable culture of a single species of phytoplankton, free of other organisms . . ." (Guillard 2005). Rippka (1988) draws a distinction between isolation and purification, defining isolation as ". . . the separation of individual clones of cyanobacterial species from other photosynthetic organisms . . ." (i.e. a unialgal culture); purification removes all other detectable contaminants (axenic culture). Purification to axenic conditions s far less complex when commenced from a unialgal culture (Guillard 2005), and is therefore rarely attempted prior to isolation of a species from a field sample (Rippka 1988). Consequently, understanding the natural environment in which the species exists, and the influence of seasonal variables such as temperature fluctuation is essential for a successful isolation (Castenholz 1988; Andersen and Kawachi 2005). Early methods of isolation depended largely on chance, as field samples often didn't contain species of interest (Acreman 1994). Ferris and Hirsch (1991) identifies one of the most frustrating aspects of dealing with cyanobacteria as being the repeatedly arduous and time-consuming labour involved in producing axenic cultures contrasting with the low success rate.

Selection of an appropriate culture medium is critical, as a single cell will not survive if isolated into full strength medium, and many species will often survive the initial isolation but lose viability after successive transfers to fresh media (Andersen and Kawachi 2005). It is imperative to subculture regularly into fresh media, designed to promote growth of the species of interest (Castenholz 1988; Andersen and Kawachi

2005), to remove the toxic build up of algal waste products and prevent conditions becoming anoxic due to bacterial contamination (Andersen and Kawachi 2005). Long-term culture using the initial isolate should be avoided, due to the high risk of bacterial and fungal contamination (Shirai 1989).

The requirement for obtaining an axenic culture over a unialgal culture is determined by the type of investigations to be undertaken. Ohtake *et al* (1989) notes that the establishment of axenic cultures is necessary for physiological, biochemical and genetic studies (Ohtake *et al*. 1989; Shirai *et al*. 1991). There are two key pathways to purification of a culture. Physical separation of the target cells from unwanted organisms occurs in one or a series of steps. Fungal contaminants and zooplankton may be removed through differential filtration (Andersen and Kawachi 2005; Guillard 2005). Organisms that attach to the surface of microalgae can be dislodged by ultrasound followed by centrifugation. Sonication and vortexing apply shear force to the surface of the cells, detaching contaminating organisms (Guillard 2005); however, sonication at high frequency produces damaging intracellular forces which may destroy target cells. Alternatively, the contaminating organisms are killed using directed chemical agents (e.g. antibiotics) or UV radiation (Gerloff *et al*. 1950; Castenholz 1988; Rippka 1988), which leave the target cells intact. If several types of contaminant are present, it may be necessary to proceed in stages, using a variety of methods to eliminate the individual contaminants successively (Guillard 2005). Use of detergents to aid in separating cells and particles, sonication, vortex mixing or fragmentation apply to special circumstances of purification. These techniques usually have a more direct application and are often essential to success of the purification attempt.

B.II Sample collection

Sample collection plays a significant role in the success of establishing in vitro cultures of cyanobacteria for future research. Samples collected from diverse habitats will likely present different algal isolates (Lee and Shen 2004). Generally samples should be stored in darkness or low light conditions, as light can cause severe damage to samples outside their normal nutrient environment (Castenholz 1988). The growth stage of the cells within the sample will ultimately determine the success of species isolation and cultivation in the laboratory; cells in the early exponential phase are

more likely to survive as a viable laboratory culture than those in the stationary phase or that are highly susceptible to environmental changes (Andersen and Kawachi 2005). Some species will not survive more than a few hours following sample collection, requiring immediate isolation into a suitable culture environment (Andersen and Kawachi 2005). The presence of zooplankton and other organisms will influence the success of the isolation, as zooplankton will rapidly consume microalgal cells. Gerloff *et al* (1950) noted green algae and diatoms will rapidly outgrow cyanobacteria in some circumstances. Multiple isolation methods can be employed if sampling from an unknown environment, or if the target organism is unknown (Andersen and Kawachi 2005). Application of a sound sterile technique is vital to avoid contamination of the culture, particularly if collecting an unfamiliar species.

B.II.1 Isolation Equipment

Selection of isolation equipment will be determined by accessibility to resources, financial limitations, and application of the species involved. For sample observation, cell enumeration and taxonomic identification, dissecting or inverted microscopes are the most widely used (Andersen and Kawachi 2005). Dissecting microscopes should have good optical lenses with magnification up to 80x and a clear glass stage. Dark field illumination is superior to transmitted light, but is significantly more expensive; reflected light is not recommended for any but the largest organisms (Andersen and Kawachi 2005). Inverted microscopes should have a long working-distance condenser for expediency when using a micropipette. Multiwell plates are the preferred sample dish, though microscope slides can also be used. Compound microscopes are not recommended for isolation of algal cells as the small working distance between the objective lens and sample dish prohibits the use of micropipettes (Andersen and Kawachi 2005).

Membrane filters, used to separate algae from other organisms and organic matter, are of 2 basic types. The first has intertwined organic fibres, leaving passages of different effective diameter (Andersen and Kawachi 2005). The second type is composed of thin, flat sheets of polycarbonate that have been perforated by a random distribution of circular holes with uniform diameter. Membrane filters are designed to separate two size fractions at a designated porosity. Woven screens of stainless steel or nylon are also available with square openings as small as 1 µm (Andersen and Kawachi

2005). Further isolation techniques are frequently required following filtration as this technique does not isolate single cells.

A variety of materials are used for isolation and culture vessels to cultivate microalgae in a laboratory. Borosilicate glassware was almost exclusively used, with Erlenmeyer flasks being the most popular choice (Castenholz 1988); however plastics with modifiable properties are rapidly replacing glass containers (Andersen and Kawachi 2005). Plastics may be pre-treated prior to use with chemical disinfecting agents or antibiotics, and tissue-culture grade plastic can be coated with substances that will enhance growth of algae. Regardless of material, all equipment should be sterilised before use.

B.II.2 Single Cell Isolation Techniques

Single cell isolation of cyanobacteria may be achieved through a variety of methods. Acknowledged techniques include single cell isolation by micropipette, agar streak plates and serial dilution (Andersen and Kawachi 2005); these methods are described in detail in this section. Liquid enrichment cultures are extensively used as a preliminary isolation step (Andersen and Kawachi 2005), and are established, as described by Rippka (1988), by ". . . imposing a positive selection criterion on members of a population most apt to proliferate in the environment provided . . ." (Rippka 1988). Lee and Shen (2004) concur, though add that the environment ". . . be inhibitory or lethal for non-target organisms . . .". Gerloff *et al* (1950) successfully isolated a unialgal culture of *M. aeruginosa* through repeated subculturing in enriched media. Rao *et al* (1994) also obtained unialgal cultures of cyanobacteria through repeated subculturing. However, the intrinsic disadvantage with this method is the lack of adequate representation of species variation.

B.II.3 Single Cell Isolation by Micropipette

The objective of single cell isolation by micropipette is to extract a cell from a sample while observing under a microscope, deposit the cell without damage into a sterile drop of water or media, retrieve the cell and transfer it to a second sterile drop (Lee and Shen 2004; Andersen and Kawachi 2005). This process is repeated until a single cell has been isolated, free of all contaminating organisms and other algal cells, and is

placed into a sterile container of fresh culture medium (Andersen and Kawachi 2005). Micropipette isolation balances 2 essential but opposing factors: cell damage by excessive handling (negative) and clean isolation of a single cell (positive). Repeated handling of robust organisms is not of particular concern, but for delicate organisms, the micropipette technique will alleviate handling distress (Andersen and Kawachi 2005).

Isolation by micropipette is performed with a Pasteur pipette or glass capillary tube, (Andersen and Kawachi 2005). The micropipette is formed by heating the glass pipette in a flame, rotating the tube to ensure even softening. At melting point, the pipette is removed from the flame and the ends pulled gently in opposite directions to produce a thin tube. A straight tip is preferable to a curved tip, as the straight tip is easier to use when discharging a captured cell into a sterile "rinsing" drop. Forceps are used to sever the tip cleanly; tips with jagged edges will not draw up cells efficiently (Andersen and Kawachi 2005). The diameter of the final micropipette should be at least twice that of the cells; if the opening is too small, the cell may be damaged by fluid shearing forces as it is drawn into the tube. If the diameter is too large, picking up a single cell becomes difficult and unwanted material is captured along with the target cell. Sterile drops, either distilled water or fresh culture media, are used for rinsing the cells and should be prepared on a microscope slide before beginning the isolation (Andersen and Kawachi 2005).

A flexible latex tube is attached to a mouthpiece on one end of the micropipette. A small amount of water is drawn into the micropipette to act as a cushion – if a "dry" pipette is immersed in the sample, violent capillary action results, potentially damaging the cells and drawing unwanted material into the capillary tube. The operator places their thumb over the end of the "mouthpiece". The micropipette tip is placed near the target cell and the operator removes their thumb to allow the capillary action to draw up the cell. The tip is removed from the sample and immersed in the first droplet and the cell discharged by gentle blowing into the mouthpiece. This process is repeated for each droplet to "rinse" the cell before final discharge into a sterile culture vessel containing a small amount (e.g. 10 mL) of media. Excessive isolation should be avoided as it often results in cell damage (Andersen and Kawachi 2005).

B.II.4 Agar Streak Plate

Agar isolation is the preferred method microalgae because axenic cultures can be directly established without further treatment (Andersen and Kawachi 2005). Purification with agar is only effective for microalgae capable of growth on the surface of, or embedded within, the agar (Guillard 2005). While it was long believed agar was unsuitable for growing *Microcystis* species (Rippka 1988), Shirai *et al* (1991) successfully developed a solid medium for this purpose, though they note that the medium is not efficient at isolating axenic cultures from some strains. Ordinary agars often contain agarose and agaropectin that may be contaminated with various impurities (Guillard 2005), and many contain water-soluble lytic agents that inhibit algal growth (Allen and Gorham 1981; Shirai 1989; Watanabe 2005). Agar concentration and composition are thus vital for successful isolation of single cells (Eloff 1981). However, Eloff (1981) observed that small inocula of *Microcystis* cells rarely survived on agar. Culture medium solidified with agar may be used for algal purification, but should be sterilised prior to mixing with agar to prevent formation of growth inhibiting agents (Court *et al*. 1981; Shirai 1989).

A field sample is streaked across the surface of the agar plate and incubated until colonies of cells appear. Incubation times vary greatly between species, from a few days for soil and freshwater algae, to many months for marine species. Cells can be retrieved from the original plate using an inoculation loop, and either streaked onto a new plate, or dislodged from the loop in liquid culture media. Some species prefer to be embedded within the agar. As the agar reaches gelling temperature, a liquid suspension of microalgal cells is mixed with the agar (Watanabe 2005). A micropipette tip can be driven into the agar to retrieve the single cells (Andersen and Kawachi 2005). Bacterial and fungal contamination often occurs on agar streak plates; however, provided plates are correctly streaked, unialgal cultures can still be obtained. Antibiotics or other selective agents are commonly included to eliminate high bacterial cell numbers (Guillard 2005). Parker (1982) reported the selective growth of *Microcystis* sp. on agar containing Na_2S. Lysozyme, an enzyme that hydrolyses linkages in the peptidoglycan layers of bacterial cell walls may be included when isolating some species of microalgae (Guillard 2005), but is not suitable for isolation of cyanobacteria due to the inherent similarities between the cell walls (Guillard 2005).

B.II.5 Serial Dilution

Serial Dilution is most often used when attempting to isolate random microalgal species from field samples (Andersen and Kawachi 2005). The aim of this technique is to deposit a single cell into a suitable vessel (e.g. test tube, flask or well) through repeated dilutions of the sample, thereby establishing a single cell isolate (Andersen and Kawachi 2005). The number of dilutions required will be determined by the cell concentration (if known), or alternatively by performing a sequence of dilutions until a single cell is isolated. Dilutions may be made with distilled water, culture medium, seawater or a combination, depending on the origin of the sample. Particular compounds may be added to the isolation tubes at higher concentrations to select specifically for a species that requires these nutrients (Andersen and Kawachi 2005). Serial dilution is most effective for isolation of organisms abundant in field samples, but is not appropriate for purification as bacteria outnumber other organisms in natural samples (Guillard 2005). The serial dilution technique can be employed for purification from unialgal cultures, and is largely successful when employed in conjunction with washing by repeated aseptic filtration or centrifugation (Guillard 2005).

B.III Chemical Structure Analysis Techniques

B.III.1 High Pressure Liquid Chromatography

High Pressure (Performance) Liquid Chromatography is one of the most powerful analytical tools available for separation, identification and quantification of components within an aqueous sample (Waters 2007). HPLC can be applied to diverse industries, including pharmaceuticals, food, forensic science and environmental assessment. Reverse phase HPLC is applied to separate compounds based on their hydrophobic character, making it an ideal instrument for isolation of peptides (Guzzetta 2001). RP-HPLC is often performed in isocratic mode, holding solvent conditions constant, and is applied for characterisation of a compound and to identify closely related degradation products. Columns are commonly packed with silica particles, and may be of varying lengths and diameters (Guzzetta 2001); column selection will depend on the analysis to be conducted and sample type involved.

Physical separation between proteins, small peptides and free amino acids is specifically crucial as analytical methods are often sensitive to the latter substances (Barbarino and Lourenco 2005). To overcome these impediments, a number of provisions are available. For example, when applying HPLC to elute samples, Trichlorofluoroacetic acid (TCA) is often included in the mobile phase to precipitate the protein and avoid any adverse influence of substances that may interfere with protein quantification and purification methods (Barbarino and Lourenco 2005).

B.III.2 Mass Spectrometry

Mass spectrometry (MS) is an analytical tool used to measure the molecular mass or molecular formula of a sample (Bailey and Bailey 1995; Ashcroft 2004), or more specifically, the mass-to-charge ratio of ions in a sample. Mass spectrometry is generally utilized to resolve the composition of a physical sample, by generating a mass spectrum representing the masses of sample components. The technique encompasses diverse applications, including: identification of unknown compound molecules or their fragments; structure elucidation of a compound by observing the fragmentation pattern; quantifying the amount of a compound in a sample using a mass spectrometer with multiple analysers (mass spectrometry is not intrinsically quantitative); and determination of other physical, chemical and biological properties of compounds when combined with other techniques (Ashcroft 2004).

A typical mass spectrometer comprises three parts: an ion source, a mass analyzer and a detector (Ashcroft 2004). Samples are analyzed by bombarding a sample with an electron beam, causing the compound to rupture into a variety of positive fragments (Bailey and Bailey 1995). Each ion is observed as a peak on the mass spectrum, the intensity of the peak describes the relative abundance of the ion (Bailey and Bailey 1995). Structural information can be determined by fragmenting specific sample ions inside the mass spectrometer, and analyzing the fragment ions; this function is normally undertaken by Tandem (MS/MS) Mass Spectrometers, instruments that comprise more than one analyzer, and thus can be used for structural and sequencing investigations (Ashcroft 2004). Fragmented ions are pieced back together to generate a structural motif of the intact compound. Intrinsic and characteristic fragmentation patterns resulting from MS/MS allow specific compounds to be identified within complex mixtures (Ashcroft 2004).

B.III.3 LC/MS

Secondary metabolites are often components of complex mixtures and hence require sensitive, refined techniques to provide structural information on constituents (Hostettmann and Wolfender 2001). Liquid Chromatography Mass Spectrometry (LC/MS) combines the separation efficiency of HPLC with the detection properties of MS. The interface between the two instruments eliminates the solvent to generate gas phase ions, which are transferred to the optics of MS detector (Hostettmann and Wolfender 2001; Waters 2007). The mass spectrometer then measures the exact mass of the components present, as described above. LC/MS is a highly valuable technique for obtaining molecular weight and structural information of a compound, particularly where quantitative information is of little importance (Waters 2007). Analysis of small non-polar metabolites is achievable with suitable configuration of LC/MS (Hostettmann and Wolfender 2001).

B.III.4 Nuclear Magnetic Resonance

Nuclear Magnetic Resonance is defined as "spectroscopy in which a compound is placed in a magnetic field and exposed to radio frequency radiation, providing information regarding the carbon and hydrogen structure of the (organic) compound" (Bailey and Bailey 1995). NMR enables elucidation of compound structure with respect to the position and number of Hydrogen atoms within a molecule (Bailey and Bailey 1995), and is a highly powerful tool in the discipline of natural products chemistry, where often a completely novel compound possessing unexpected structural features requires clarification (Neri and Tringali 2001). NMR is essentially able to distinguish between H atoms in different chemical environments within a molecule, by absorbing radio frequency energies at different applied magnetic fields (Bailey and Bailey 1995; Neri and Tringali 2001). To quantify small amounts of compound with precision, 1H-NMR offers several significant advantages (Dagnino and Schripsema 2005). The position of adsorption is relatively constant for Hydrogen in particular chemical structures; consequently, the number of signals recorded correspond to the number of different "types" of hydrogen in a molecule (Bailey and Bailey 1995). Similarly, the integral of a 1H NMR signal peaks in the spectrum are in proportion to the number of H+ contributing to the signal; accordingly the signals of different compounds can be directly compared (Dagnino and Schripsema 2005).

Modern NMR may provide significant assistance in isolation and purification of natural compounds in combination with the "hyphenated techniques", LC/MS, LC/UV and LC/NMR, in which the crude extract is separated by liquid chromatography then analysed on-line by MS, UV or NMR spectral methods (Neri and Tringali 2001)

APPENDIX C MICROALGAL CULTIVATION MEDIA

C.I Preparation of JDM BG_{11} Media

SCOPE: This standard operating procedure (S.O.P.) describes the procedure for efficient and safe preparation of BG_{11} culture media.

PURPOSE: The purpose of this SOP is to document a procedure for safe and efficient set-up and preparation of BG_{11} culture media, and also provides safety information considered important to the methods.

RESPONSIBILITIES; Kyleigh Victory is responsible for the efficient and safe preparation of BG_{11} media.

CONTENT/DESCRIPTION

Part One - Preparation of Media

This section describes concisely and in a step by step manner exactly how to prepare BG_{11} media prior to culturing. BG_{11} media composition as follows:

- Prepare the metals mix in 2 components.
- In a 500 mL volumetric flask, combine 6.7 g Ferric citrate with 14.9 g Na_2EDTA in 500 mL deionised water. Adjust pH to 7.0 and boil mixture to dissolve.
- Note: requires a large quantity of 5N NaOH and colour darkens at around pH 7.0. Check pH after boiling and readjust to pH 7.0 if necessary.
- Prepare the trace metals mix as follows:
- Combine 2.86 g H_3BO_3, 1.81 g $MnCl_2.4H_2O$, 0.222g $ZnSO_4.7H_2O$, 0.39 g $Nz_2MoO_4.2H_2O$, 0.079g $CuSO_4.5H_2O$ and 0.05 g $Co(NO_3)_4.6H_2O$ in a 500 mL volumetric flask.
- Mix in 500 mL deionised water, then combine with Fe-EDTA mix to make 1 L metals stock solution. Store in plastic bottles at 4°C. Use 1 mL per 1 L of 1X JDM media.

- Prepare stock solutions of K_2HPO_4 (3.05g), $MgSO_4.7H_2O$ (7.5 g), and $CaCl_2.2H_2O$ (3.6 g) in 100 mL each of deionised water. Store at 4 °C.
- Add 1 mL each of above stock solutions to 1 L deionised water, 1.5 g of $NaNO_3$ and 1 mL metals stock solution, cap volumetric flask and invert to mix. Ensure $NaNO_3$ has dissolved.

Part Two - Requirements

This section describes concisely and in a step by step manner exactly what requirements, equipment or materials, including personal protection are required for this apparatus or process.

General

- 500 mL volumetric flasks
- Laboratory scales
- Measuring cylinders

Reagents

- $NaNO_3$ (sodium nitrate)
- K_2HPO_4 (potassium phosphate)
- $MgSO_4.\ 7H_2O$ (magnesium sulphate heptahydrate)
- $CaCl_2\ 2\ H_2O$ (calcium chloride dihydrate)
- ferric citrate
- Disodium EDTA. $2H_2O$
- H_3BO_3 (boric acid)
- $Na_2MoO_4.\ 2H_2O$ (sodium molybdate)
- $CuSO_4.\ 5H_2O$ (copper sulphate)
- $ZnSO_4.\ 7H_2O$ (zinc sulphate heptahydrate)
- $Co(NO_3)_2.6H_2O$ (cobalt nitrate)
- $MnCl_2.4H_2O$ (manganese chloride)
- 5N NaOH
- Distilled, deionised water

Part Three – Safety and points to note

This section describes concisely and exactly what hazards are present during any phase of operation of this apparatus or process and any personal protection that may be required.

- Refer to the relevant MSDS document for more information.
- Personal protective equipment, most importantly goggles, must be worn at all times when handling chemicals.
- Autoclave immediately to stop fine precipitate forming.
- Do not leave old JDM media in large 8L mixing bottles. 2 x JDM without buffer is not stable after autoclave; 1 x JDM is stable
- Sodium nitrate is an irritant. Wear rubber gloves when handling.
- Calcium chloride dihydrate may cause irritation. Use in an area with good ventilation.
- Disodium EDTA dihydrate is irritating to the eyes, skin and respiratory system. Wear rubber gloves when handling. Use in a mechanically ventilated area.
- Manganese chloride is an irritant. Use in a well-ventilated area. Wear protective gloves when handling.
- Zinc sulphate heptahydrate is an irritant. Use in a well-ventilated area. Wear protective gloves when handling.
- Cobalt nitrate is an irritant. Use in a well-ventilated area. Wear protective gloves when handling.
- Sodium molybdate is an irritant. Use in a well-ventilated area. Wear impervious protective clothing including boots, gloves and lab coat.

- To pour agar plates, 2 x JDM is buffered with 100 mM TES-KOH (for final concentration of 50 mM). 1M TES-KOH pH 8 stock solution must be autoclaved to stop fungal growth, then stored at 4°C

REFERENCES

(Andersen and Kawachi 2005)

C.II Preparation of B-12

SCOPE: This standard operating procedure (S.O.P.) describes the procedure for efficient and safe preparation of B-12 culture media.

PURPOSE: The purpose of this SOP is to document a procedure for safe and efficient set-up and preparation of B-12 culture media, and also provides safety information considered important to the methods.

RESPONSIBILITIES; Kyleigh Victory is responsible for the efficient and safe preparation of B-12 media.

CONTENT/DESCRIPTION

Part One - Preparation of Media

This section describes concisely and in a step by step manner exactly how to prepare B-12 media prior to culturing. B-12 media composition as follows:

Compound	mg/L deionized distilled water
$NaNO_3$	100
K_2HPO_4	10
$MgSO_4 . 7H_2O$	75
$CaCl_2. 2 H_2O$	40
Na_2CO_3	20
ferric citrate**	6
Disodium EDTA. $2H_2O$	1
vitamin B12	0.1

** Autoclave separately

CB media composition as follows:

Compound	mg/L deionized distilled water
$Ca(NO_3)_2. 4H_2O$	150

KNO_3	100
$MgSO_4$. $7H_2O$	40
β-disodium glycerophosphate	50
bicine	500
biotin	0.0001
vitamin B12	0.0001
thiamine hydrochloride	0.01
PIV metals	3.0

PIV metals:

Compound	mg/L deionized distilled water
$FeCL_3$. $6H_2O$	19.6
$MnCL_2$. $4H_2O$	3.6
$ZnSO_4$. $7H_2O$	2.2
$CoCl_2$. $6H_2O$	0.4
Na_2MoO_4 . $2H_2O$	0.25
Disodium EDTA. $2H_2O$	100

Notes:

- pH of B-12 and CB media adjusted to 9.0
- B-12 and CB are used both as a liquid media, and as the base for solid media which is sterilized at 121°C for 15 min.

Part Two - Requirements

This section describes concisely and in a step by step manner exactly what requirements, equipment or materials, including personal protection are required for this apparatus or process.

Reagents

- $NaNO_3$ (sodium nitrate)
- K_2HPO_4 (potassium phosphate)
- $MgSO_4.7H_2O$ (magnesium sulphate heptahydrate)
- $CaCl_2.2\ H_2O$ (calcium chloride dihydrate)

- Na_2CO_3 (sodium carbonate)
- ferric citrate
- Disodium EDTA.$2H_2O$
- vitamin B12
- $Ca(NO_3)_2.4H_2O$ (calcium nitrate)
- KNO_3 (potassium nitrate)
- β-disodium glycerophosphate
- bicine
- biotin
- thiamine hydrochloride
- $Na_2MoO_4.2H_2O$ (sodium molybdate)
- $FeCl_3.6H_2O$ (iron (III) chloride hexahydrate)
- $MnCl_2.4H_2O$ (manganese (II) chloride tetrahydrate)
- $ZnSO_4.7H_2O$ (zinc sulphate heptahydrate)
- $CoCl_2.6H_2O$ (cobalt (II) chloride hexahydrate)
- thioglycolate (TGC) medium
- NaCl (pH 7.0)
- Distilled, deionised water

Part Three – Safety and points to note

This section describes concisely and exactly what hazards are present during any phase of operation of this apparatus or process and any personal protection that may be required.

- Refer to the relevant MSDS document for more information.
- Personal protective equipment, most importantly goggles, must be worn at all times when handling chemicals.
- Sodium nitrate is an irritant. Wear rubber gloves when handling.
- Sodium carbonate is an irritant to skin and mucous membranes. Use in an area with good ventilation.

- Calcium chloride dihydrate may cause irritation. Use in an area with good ventilation.

- Disodium EDTA dihydrate is irritating to the eyes, skin and respiratory system. Wear rubber gloves when handling. Use in a mechanically ventilated area.

- Calcium nitrate is an irritant. Use in a well ventilated area. Wear protective gloves when handling.

- Bicine is an irritant. Use in a mechanically ventilated. Wear chemical-resistant gloves when handling.

- Iron (III) chloride hexahydrate is a corrosive. Wear appropriate gloves when handling.

- Manganese chloride is an irritant. Use in a well-ventilated area. Wear protective gloves when handling.

- Zinc sulphate heptahydrate is an irritant. Use in a well-ventilated area. Wear protective gloves when handling.

- Cobalt (II) chloride hexahydrate is an irritant. Use in a well-ventilated area. Wear protective gloves when handling.

- Sodium molybdate is an irritant. Use in a well-ventilated area. Wear impervious protective clothing including boots, gloves and lab coat.

APPENDIX D DNA SEQUENCE ALIGNMENT ANALYSIS OF *MCY* GENES

D.I *mcy*A

1	5’	TTTTAGGGGC	TTTTTATATT	TACGCATCAG	AGGGACTTCG	TTACGGGTTT	3’
51	5’	AGTCTCTAAT	GGACGGTTAG	AAGCTGCCGA	TGGTGAAAAG	ATATTAGGTT	3’
101	5’	TATTTATGAA	TACTTTGCCC	CTACTTTTAT	ATTTATCTGG	AGGCCCTTGG	3’
151	5’	AGCGATTTAG	TTAAGCAGGC	TTTTGATGTT	GAAAGAAAAT	GCTTATCTTG	3’
201	5’	GCGAAGATAT	CCCCTCGCTG	AGTTACAAAA	ATCAGGTCAA	CCTTTGTTTG	3’
251	5’	ATACGGCTTT	TTTTTATAAA	ACACTAAACG	CTATTTCGGG	GATTATGTCT	3’
301	5’	CCCCCTACGA	CTTCCAGAGA	GCGGTCGAGG	ATAAAGCCAC	CGATCCCCTC	3’
351	5’	TACTATGATG	CAAGGGGTGA	TAAACTCTCG	ATCGCCGTCA	ACGACCTACA	3’
401	5’	CGAACACATC	ACCGCCAAAA	TCGAAGAGTT	ACAGATCGAA	AATGTCGAGA	3’
451	5’	TAGAAGACCG	TTTACAAAGG	GAATTAAGAA	GGGATTACTA	CATCCTGACG	3’
501	5’	GCCCCGCAGC	GGTTAGAGGC	GCTGGCCCGT	GATTTCGTTG	AACATTACAG	3’
551	5’	CACCGCTTGG	GAAACAGGCA	AAGCCATGGT	GATTTCTATC	TACAGAGCCA	3’
601	5’	CCTAAGTAAG	ACATCATAGT	TATTCGGATA	TTATGGATTA	ATTCCGA	3’

(a)

Sequences producing significant alignments		**High Score**	**E value**
emb|CAO90227.1|	*mcy*A [*Microcystis* aeruginosa PCC 7806]	155	2e-36
gb|AAL82382.1|	*Mcy*A [*Microcystis* aeruginosa	155	2e-36
dbj|BAA83992.1|	*mcy*A [*Microcystis* aeruginosa]	155	2e-36
gb|AAF00960.1|AF183408_8	*Mcy*A [*Microcystis* aeruginosa PCC 7806]	155	2e-36
gb|ABS83327.1|	*Mcy*A [uncultured cyanobacterium]	152	1e-35
emb|CAD56441.1|	*Mcy*A protein [*Microcystis* sp. 199]	152	1e-35
emb|CAD56445.1|	*Mcy*A protein [*Microcystis* sp. IZANCYA5]	152	1e-35

(b)

AAL82382	1545	LSLLNNQRDIVTGLVSNGRLEAADGEKILGLFLNTLPLRLELSGGPWSDLVKQAFDVERE	1604
*mcy*A	10	LFIFTHQRDFVTGLVSNGRLEAADGEKILGLFMNTLPLLLYLSGGPWSDLVKQAFDVERK	189
AAL82382	1605	CLSWRRYPLAELQKSGQPLFDTAFNFIHFHVYQGII	1640
*mcy*A	190	CLSWRRYPLAELQKSGQPLFDTAFFYKTLNAISGIM	297

(c)

Figure D-1: DNA sequence analysis of the PCR product *mcy*A from *Microcystis flos-aquae*.

(a) DNA sequence of *mcy*A from obtained from *M. flos-aquae*. (b) Results of a BLASTX DNA sequence analysis of the PCR product *mcy*A amplified from MIC FEB05. The *mcy*A gene of *M. flos-aquae* was most similar to the *mcy*A gene of *M. aeruginosa*. (c) DNA sequence alignment of *mcy*A gene from *M. flos-aquae* and AAL82382. The sequences are 86% similar

D.II *mcy*B

1	5’	CCGGTAGAGC	TGCCACGCTT	CCAGTAACGA	ACACTTACAG	AGTCTTCTGG	3’
51	5’	GAGACCATAA	AGCCTATATT	TTCACCCTCA	TCCAGAAATT	CAACCAAACC	3’
101	5’	GTCAATCCCG	ACCAACCCTA	CAACTCCCGA	AACGATATCA	TCATTATCAG	3’
151	5’	CGACGAAGCC	CACCGAACCC	AGTACGGAAC	CCTAGCCAGC	AACCTCCGGG	3’
201	5’	CTGCACTTCC	AGGCGCTGGT	TTTATCGGTT	TTACCGGCAC	CCCTCTAGTC	3’
251	5’	AGCAACGACG	AGATCACTAA	ACGCTATTTC	GGGGATTATG	TCTCCACCTA	3’
301	5’	CGACTTCCAG	AGAGCGGTCG	AGGATAAAGC	CACCGTTCCC	CTCTACTATG	3’
351	5’	ATGCAAGGGG	TGATAAACTC	TCGATCGCCG	TCAACGACCT	CAACGAACAG	3’
401	5’	ATCGCCGCCA	AAATCGAAGA	GTTAGAGATC	GAAAATGTCG	AGATAGAAGA	3’
451	5’	CCGTTTAGAA	AGGGAATTAA	GAAGGGATTA	CTACATCCTG	ACGGCCCCGC	3’
501	5’	AGCGGTTAGA	GGCGCTGGCC	CGTGATTTCG	TTGAACATTA	CAGCACCGCT	3’
551	5’	TGGGAAACAG	GCAAAGCCAT	GGTGATTTGT	ATCTACAGAG	CCCGTACGCG	3’
601	5’	CAAATCAA				3’	

(a)

Sequences producing significant alignments		**High Score**	**E value**
ref|YP_001052675.1|	type I site-specific deoxyribonuclease, H	217	4e-55
ref|YP_390604.1|	type I site-specific deoxyribonuclease, HsdR	214	2e-54
ref|YP_878479.1|	HsdR [Clostridium novyi NT] >gb|ABK61228.1| ...	192	1e-47
ref|ZP_01877891.1|	HsdR [Roseovarius sp. TM1035] >gb|EDM33275	190	6e-47
ref|NP_869939.1|	HsdR [Rhodopirellula baltica SH 1] >emb|CAD7	181	3e-44
ref|ZP_00970077.1|	COG0610: Type I site-specific restriction-	161	2e-38
ref|ZP_02038347.1|	hypothetical protein BACCAP_03976 [Bactero...	161	3e-38
ref|ZP_01042537.1|	Type I site-specific deoxyribonuclease Hsd...	159	1e-37
ref|YP_001530120.1|	type I site-specific deoxyribonuclease, H	158	2e-37
ref|YP_912147.1|	protein of unknown function DUF559 [Chlorobi.	156	7e-37

(b)

ABN63806	353	CRADSGKTLKELIGQQKAFVFTLIQKFNEKVDPLKPYSERDDIIVITDEAHRTQYGTLSL	412
*mcy*B	11	CHASSNEHLQSLLGDHKAYIFTLIQKFNQTVNPDQPYNSRNDIIIISDEAHRTQYGTLAS	190
ABN63806	413	NMRNALPNASFIGFTGTPLFKDDEITKKVFGDYVSTYDFQRAVEDKATVPLYYDARGEEL	472
*mcy*B	191	NLRAALPGAGFIGFTGTPLVSNDEITKRYFGDYVSTYDFQRAVEDKATVPLYYDARGDKL	370
ABN63806	473	IFTDEDGNEHTVADPKGINERIAEKLEELEIDDVDVQQRLERELKRDYHIITSTSRLDQI	532
*mcy*B	371	SIAVND----------LNEQIAAKIEELEIENVEIEDRLERELRRDYYILTAPQRLEAL	517
ABN63806	533	AQDFVAHYANGWETGKAMFVCL	554
*mcy*B	518	ARDFVEHYSTAWETGKAMVICI	583

(c)

Figure D-2: DNA sequence analysis of the PCR product *mcy*B from *Microcystis flos-aquae*.

(a) DNA sequence of *mcy*B from obtained from *M. flos-aquae*. (b) Results of a BLASTX DNA sequence analysis of the PCR product *mcy*B amplified from MIC FEB05. The *mcy*B gene sequence of *M. flos-aquae* did not resemble any known sequences of *mcyB* genes of other *Microcystis* sp. but demonstrated 61% homology to a type 1 site specific deoxyribonuclease from *Shewanella baltica* OS155. (c) DNA sequence alignment of *mcy*B gene from *M. flos-aquae* and ABN63806. The sequences are 61% similar

D.III *mcy*C

1	5' AAGGCGATTC	TAAGGGGGCA	AGAAAGGCTT	TGGATTTTGC	AGCATATCAG	3'
51	5' CGAATCTTCT	AGTGCTTATA	ATATTAGGCT	TGCCATGAGA	ATTAATGGCA	3'
101	5' AGTTAGATGT	TTCTATATTA	ACAGCAGCCT	TTCAAGAACT	GGAAGTCATTA	3'
151	5' TCAGCGACAA	ATCCCACCGA	ACCCAGTAC	GGAACCCTAG	CCAGCAACCT	3'
201	5' CCGGGCTGCA	CTTCCAGGCG	CTGGTTTTAT	CGGTTTTACC	GGCACCCCTC	3'
251	5' TAGTCAGCAA	CGACGAGATC	ACTAAACGCT	ATTTCGGGGA	TTATGTCTCC	3'
301	5' ACCTACGACT	TCCAGAGAGC	GGTCGAGGAT	AAAGCCACCG	TTCCCCTCTA	3'
351	5' CTATGATGCA	AGGGGTGATA	AACTCTCGAT	CGCCGTCAAC	GACCTCAACG	3'
401	5' AACAGATCGC	CGCCAAAATC	GAAGAGTTAG	AGATCGAAAA	TGTCGAGATA	3'
451	5' TAAGACCGTT	TAGAAAGGGA	ATTAAGAAGG	GATTACTACA	TCCTGACGGC	3'
501	5' CCCGCGGCGG	TTAAAGACGC	TGGCCCGAGA	TTTCATTGAA	CATTACAGCA	3'
551	5' CCGCTTGAGA	AAAAGGCAAA	GCCATGGTGA	TTTGTATCTA	CGAGCATAAA	3'
601	5' TC					3'

(a)

Sequences producing significant alignments		**High Score**	**E value**
gb|AAL82384.1|	*Mcy*C [*Microcystis* aeruginosa]	84.0	5e-15
dbj|BAA83994.1|	*mcy*C [*Microcystis* aeruginosa]	84.0	5e-15
gb|AAF00962.1|AF183408_10	*Mcy*C [*Microcystis* aeruginosa PCC 78	84.0	5e-15

(b)

AAL82384	21	VSHGQRRLWILQHISESSSAYNIRLAMRINGKLDVSILTAAFQEL--VNRHEILRTTFTS	78
*mcy*C	7	ILRGQERLWILQHISESSSAYNIRLAMRINGKLDVSILTAAFQELEVIISDKSHRTQYGT	186
AAL82384	79	VGGNIKQVI	87
*mcy*C	187	LASNLRAAL	213

(c)

Figure D-3: DNA sequence analysis of the PCR product *mcy*C from *Microcystis flos-aquae*.

(a) DNA sequence of *mcy*C from obtained from *M. flos-aquae*. (b) Results of a BLASTX DNA sequence analysis of the PCR product *mcy*C amplified from MIC FEB05. The *mcy*C gene sequence of *M. flos-aquae* was similar to the *mcy*C genes of *Microcystis aeruginosa*. (Genbank accession number AAL82384). (c) DNA sequence alignment of *mcy*C gene from *M. flos-aquae* and AAL82384. The sequences are 63% similar

D.IV *mcy*D

1	5’	CCGGGGGGTG	CGACGTTGAC	GGGGATTAGG	ATTTTTAAAG	TGAAGATGAG	3’
51	5’	GAGGTATTTT	ATGGTGTTGT	AACGCTAGAA	CGGTTTTAAT	TAATCCGGCA	3’
101	5’	ATTCCGGCAG	CACCTTCTAA	ATGGCCTAAA	TTCGTTTTTA	CTGAACCAAT	3’
151	5’	AATTAAGGGC	CGATTAGGGG	AGCGTTGAAA	CAACGCCGTC	CGATTATGA	3’
201	5’	GGTTTGACGG	CTGATACCAT	GGAGCGTTCG	ACGGCCTCTT	AGGGCGAGGA	3’
251	5’	GGAGAAGATC	AGACCACGAC	TCTTCCCTTA	ATATTACGCC	CTCCTTTACG	3’
301	5’	AATGGTGGAA	GATACAATAG	TCTTGAGCCG	AGGACCTGAA	TTATGCCTCA	3’
351	5’	TTTTTTA					3’

(a)

Sequences producing significant alignments		**High Score**	**E value**
emb|CAO90228.1|	*mcy*D [*Microcystis* aeruginosa PCC 7806]	113	3e-24
gb|AAY17517.1|	polyketide synthetase [*Microcystis* aeruginosa PCC	113	3e-24
gb|AAY17518.1|	polyketide synthetase [*Microcystis* sp. FACHB-569]	113	3e-24
gb|AAF00959.1|AF183408_7	*Mcy*D [*Microcystis* aeruginosa PCC 7806]	113	3e-24
dbj|BAB12210.1|	polyketide synthase [*Microcystis* aeruginosa]	113	3e-24
gb|AAR92050.1|	microcystin synthetase [*Microcystis* viridis NIES	109	6e-23
gb|AAR92048.1|	microcystin synthetase [*Microcystis* aeruginosa PC	109	6e-23
gb|AAR92049.1|	microcystin synthetase [*Microcystis* sp. 205]	109	6e-23
gb|AAR92047.1|	microcystin synthetase [*Microcystis* aeruginosa NI	109	6e-23

(b)

CAO90228	2439	IELESMSAVFGQRSPNRPLIIGSVKTNLGHLEGAAGIAGLIKTVLALQHHKIPPHLHFKN	2498
*mcy*D	207	VKPHNRTALF-QRSPNRPLIIGSVKTNLGHLEGAAGIAGLIKTVLALQHHKIPPHLHFKN	31
CAO90228	2499	PNPR	2502
*mcy*D	30	PNPR	19

(c)

Figure D-4: DNA sequence analysis of the PCR product *mcy*D from *Microcystis flos-aquae*.

(a) DNA sequence of *mcy*D from obtained from *M. flos-aquae*. (b) Results of a BLASTX DNA sequence analysis of the PCR product *mcy*D amplified from MIC FEB05. The *mcy*D gene sequence of *M. flos-aquae* demonstrated homology to the *mcy*D gene of *Microcystis aeruginosa*. PCC7806 (Genbank accession number CAO90228) (c) DNA sequence alignment of *mcy*D gene from *M. flos-aquae* and CAO90228. The sequences are 85% similar