A Laboratory Practicum on Screening for Lytic Bacteriophages from Soil Samples

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ABSTRACT Bacteriophages are viruses that explicitly infect and lyse bacteria. They are ubiquitous and play vital roles in various biotechnological applications such as phage-based bacterial detection and bacteriophage therapy. This paper describes a simple bacteriophage screening protocol for teaching in secondary and tertiary institutions. This laboratory practicum was designed to enable students explore the presence of bacteriophages from soil samples. The tendency of many bacteriophages to produce plaques that are small or excessively turbid on agar plates limits their proper visualisation and enumeration. One percent of TTC (2,3,5-Triphenyltetrazolium chloride) was incorporated during the pour plate technique to enhance visibility of bacteriophage plaques on Petri dishes. The isolated bacteriophages were then amplified in growth medium containing targeted bacterial hosts. The results showed presence of six bacteriophages with distinctive plaque morphologies. Students were then taken through bacteriophage amplification and storage procedures. A survey was conducted to evaluate student's learning experience both prior and after laboratory classes. Analysis from the survey showed that only 26% of students attempted all questions from the pre-laboratory bacteriophage practicum questionnaire whereas 74% failed the survey. On the other hand, 53% of the students attempted all questions for the post-laboratory phage practicum questionnaire and 16% failed the survey.

KEYWORDS: Bacteriophages; biocontrol; enrichment culture; phage isolation; plaque assay

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INTRODUCTION

Bacteriophages (phages) are viruses which specifically infect and lyse bacteria (Matsuzaki, *et al.*, 2005). Phages are widely known for their abundance in the biosphere and the crucial role they play in the evolution of their bacterial hosts and the emergence of new pathogens (Chai, *et al.*, 2010). Since their discovery over a century ago, phages have primarily been exploited in many areas such as in phage therapy, biocontrol, bacterial detection, drug delivery, drug discovery and nanotechnology (O'Sullivan, *et al.*, 2016). In recent years, the interest of therapeutic application of phages has rekindled mainly due to the emergence and rapid spread of antibiotic-resistant pathogens and the so-called "superbugs" such as methicillin-resistant *Staphylococcus aureus* (MRSA), carbapenem-resistant *Acinetobacter baumannii* and *Enterobacteriaceae* and *Clostridium difficile* among many others (Casey, *et al.*, 2018).

The widespread availability of phages in the environment makes their isolation possible from any material that supports bacterial growth. The study of viruses is an important topic in any microbiology course. However, it is unfortunate that many science laboratory practicums do not involve students in exploring the availability and diversity of viruses such as phages in the environment (Allen and Gyure, 2013). It is often difficult for lecturers and students to visualize viruses without the use sophisticated equipments, which are not always available in educational institutions (Allen and Gyure, 2013). In most cases, expensive equipment such as transmission electron microscopy (TEM) and scanning electron microscope (SEM) are often used as an indirect

method of visualizing and studying the morphology of viruses, as well as characterizing their diversity.

This study was designed for students who were taking an industrial microbiology module of an undergraduate biotechnology degree in Swinburne University of Technology Sarawak Campus, Kuching, Malaysia. The aim of the practicum was to introduce bioprospecting to students and demonstrate to them, the abundance of phages in the environment. A simple and inexpensive protocol was designed to isolate phages from environmental soils using enrichment culture technique. Students' learning experience was determined through anonymous survey questions which were answered by the students before and after the laboratory class.

MATERIALS AND METHODS

Soil Sample Collection

Soil samples were collected from Fairy Cave Nature Reserve situated in the township of Bau (N 01°22'53.39" E 110°07'02.70") and also a palm oil plantation located in Lundu (N 01°31'51.4" E 109°56'04.9") Sarawak, Malaysia. The students were grouped and then assigned soil samples (one sample per group) prior to the experiments. Each group collected soil samples at a depth of 5-25 cm using sterile tools. Soil samples were then placed inside sterile polystyrene containers and stored in an ice box (at the sampling site) before being transported to the laboratory for further analysis.

Bacterial Host Strains

The bacteria used in this practicum served as bacterial hosts for phage isolation and propagation. The bacterial hosts were *Escherichia coli* (ATCC® 29425™), *Staphylococcus aureus subsp.aureus* (ATCC® 25923™), *Streptococcus pneumoniae* (ATCC® 55143™), *Pseudomonas aeruginosa* (ATCC® 15442™), *Klebsiella pneumoniae* (ATCC® BAA-24735™) and *Vibrio parahaemolyticus*. These bacterial strains were purchased from American Type Culture Collection (ATCC) Manassas, Virginia, the United States of America, except for *Vibrio parahaemolyticus* which was acquired from Swinburne University of Technology Sarawak Campus microbiology strain collections. The bacteria were aseptically grown under batch cultivation conditions according to the ATCC instructions and stored on agar plates at 4°C prior to the commencement of the experiments. The bacterial hosts used in the practical classes are categorised as biosafety level 1 (*Escherichia coli, Streptococcus pneumoniae*) which are non-pathogenic with none or minimal potential hazard to laboratory personnel and the environment, while the biosafety level 2 (*Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Vibrio parahaemolyticus*) are known pathogenic agents of moderate potential hazard to personnel and the environment. Hence, the instructors and students were mandated to handle all bacterial cultures with appropriate biosafety procedures and strictly conduct proper laboratory safety practices.

Enrichment Culture and Phage Isolation

A gramme (1 g) of each soil sample was inoculated into separate sterile conical flasks (250 mL capacity) containing 100 mL of sterile Brain-heart infusion (BHI) media (37.0 $g.L^{-1}$ Oxoid, Basingstoke, UK). 4 mL of sterile 10 mM $CaCl_2$ (Sigma-Aldrich (M) Sdn Bhd) was added to each conical flask and the contents were aerobically incubated for 1 hr at 37°C. 5 mL of each bacteria host used in this study were cultivated for their respective mid-exponential phases and then inoculated into each conical flask. The samples were incubated overnight in an incubation shaker (CERTOMAT® CT plus – Sartorius) under aerobic batch conditions at 37°C and 150 rpm. 1 mL of 1% (v/v) TTC (2,3,5-triphenyl tetrazolium chloride) (HiMedia, Laboratories Pvt. Ltd) and 1.2 mL of

sterile 10 mM $CaCl_2$ solutions were added into 100 mL liquefied nutrient agar (28.0 $g.L^{-1}$, 45°C, Oxoid, HiMedia, Laboratories Pvt. Ltd) prepared in a Schott bottle (250 mL). 5 mL from previously cultured broth was inoculated separately into each Schott bottle and the contents were mixed gently and poured out into Petri dishes avoiding the formation of any bubbles. The plates were allowed to air dry in a biological safety cabinet (Class II, type A2, Thermo Scientific™) for 15 min and then placed in an incubator (Incucell, MMM Medcenter Einrichtungen GmnH) without inversion under aerobic conditions for 24 hr at 37°C. The plates were incubated without inversion as suggested by Sambrook and Russell (2001) to encourage sweating of fluid onto the surface of the dish, allowing the phages to spread easily.

Phage Amplification and Storage

A sterile straw was used to excavate the agar part containing the plaque from each Petri plate and then dropped into a universal bottle containing 4 mL BHI broth supplemented with 100 µL of 10 mM $CaCl_2$ solution. Immediately, 1 mL of the respective bacteria host was inoculated and the bottles were incubated for 24 hr under aerobic batch conditions at 37°C and 150 rpm. After bacteria lysis was observed, each solution was centrifuged using a centrifuge machine (Eppendorf, 5424R) at 8000 g for 5 min and the supernatant containing phage particles was filtered through a 0.22 µm syringe filter. Glycerol stock of the phage isolates was performed following a modified procedure of Fortier and Moineau (2009) for phage preservation. Each group of students transferred 100 µL of phage lysate into two sterile cryogenic vials. A drop of chloroform was added to the first tube and then stored in the refrigerator at 4°C. In the second tube, 200 µL of 75% (v/v) glycerol in phage buffer (PB) was added and the cryogenic vial was gently mixed by inverting the tube a few times and then frozen at -80°C.

Survey Questionnaires

Survey questions on laboratory phage practical were administered to students before the start of the first laboratory class and at the end of the last laboratory class. The pre-survey questionnaire was prepared to test students general knowledge on bacteriophages such as the history, their structure, their mode of action and applications. On the other hand, the post-survey questionnaires focused on the technical knowledge resulting from the laboratory experience. Both the pre and post-survey questionnaires were provided to 19 undergraduate Biotechnology students enrolled for HES2210-Industrial Microbiology course. The data obtained from the survey questions were used to analyse the student's learning experience and the grading rubric was developed to measure their performance, which was presented as N to HD (0-100%). Supplementary materials used for the preparation of this laboratory practicum which includes student laboratory protocol, provisional guides for laboratory technicians and instructions for preparation of materials, grading rubric, survey questions and timeline guides of the practice can be obtained from the authors.

RESULT AND DISCUSSION

Phage Isolation

Six phages were isolated by the students from soil samples based on formation of clear plaques (Figure 1) on their respective bacterial lawns. The plaques were either round or irregular in shape, with sizes ranging from 0.4-0.7 cm. 1% TTC solution was incorporated into the liquefied nutrient agar during phage screening process using the pour plate method to serve as a rapid method to detect the antibacterial activity of phages by improving plaque resolution. The metabolic activity of viable active cells can break down TTC to form (TPF) a red coloured compound (Kumar, *et al.*, 2011). Formation of clear plaques on the agar plates as seen in Figure 1A and 1B suggested that the

bacterial host was completely susceptible to the phage present in the soil sample. Most reported studies on phage isolation have utilised sewage samples as an optimal screening resource (Lobocka, *et al.*, 2014). The study of phage isolation from limestone caves and palm oil plantation regions are very limited. A major technical challenge faced during the phage isolation process was that some of the students did not allow the plates to air dry completely in the biological safety cabinet (BSC) prior to an overnight incubation at 37°C (Figure 1C). This made it difficult to distinguish a clear plaque formation on the bacterial lawn, which normally appears round in shape.

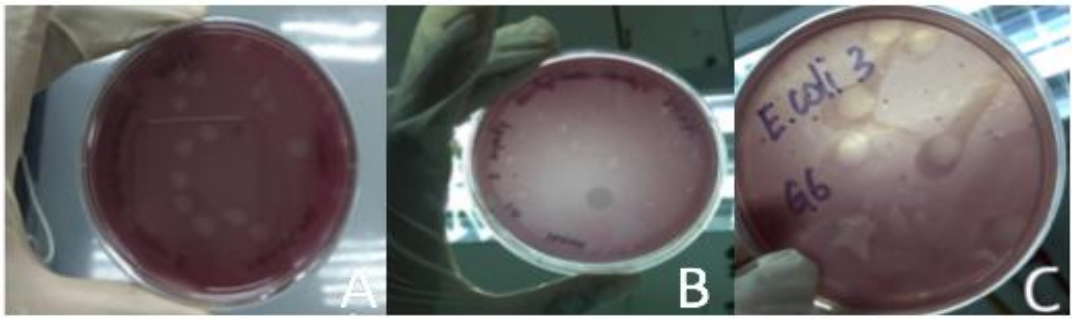

Figure 1. Plaque appearance of bacteriophages infecting *Vibrio parahaemolyticus* (A), *Staphylococcus aureus* (B) and *Escherichia coli* (C) following a 24-hr incubation at 37°C.

Students' Learning Experience

The survey results showed that students scored better grades in post-laboratory survey questions when compared to pre-laboratory survey questions (Figure 2). The majority (74%) of the students who attempted the pre-laboratory survey questions failed, whereas only 5% of the students passed with distinction (D). This implies that most of the students went to the laboratory unprepared, having little or no vehement knowledge regarding bacteriophages. Analysis of the student's learning outcome was performed and the data was presented as a percentage of students who attempted the pre and post-laboratory phage practicum survey questions. The results indicated that 26% of the students attempted all the questions in the pre-laboratory phage practicum, whereas 53% of the students attempted all the questions in the post-laboratory phage practicum (Figure 2A and 2B). This suggested that the students were more inclined to attempt the survey questionnaires after their practical activity. The results obtained in this survey demonstrated that majority of the students did not do proper reading, including supporting materials which were provided in the lab manual prior to the commencement of the experiment as instructed by the lab demonstrator. However, when the responses for post-laboratory phage survey questions were analysed, a remarkable increase in the grades was observed. The percentage of students who previously failed the survey dropped to 16%, while most (58%) of the students who took this survey scored a pass (P). The highest score obtained by the students in both the pre and post-survey questions were 78% and 86%, whereas the lowest scores were 4% and 20% (Figure 2C and 2D). This analysis showed that students learning interest had significantly improved as a result of the laboratory practicum. This practicum enabled students to learn a quick, inexpensive and effective method of isolating diverse bacteriophages from the environment. It was a way for students to gain realistic hands-on experience on how bacteriophages can be sourced from our surroundings. Students reported to have find the experience enjoyable and at the same time accelerating a greater retention of the course material. Nonetheless, students reported working as a team boosted their confidence and motivation to tackle scientific problems.

The use of plaque assay served as a simple and effective technique that allowed students to have a better understanding of how bacteriophages infect and lyse their bacterial hosts. Students noted that the addition of 1% TCC solution enhanced visualisation of the plaques formed on the lawns of their respective bacterial hosts. In some plates, bacterial colonies were seen as red dots,

indicating that the bacteria were able to reduce TCC from white to red colouration. Formation of plaques (clear zones) on the lawns of their respective bacterial hosts signified that the bacteria had undergone necrosis (death due to infection by bacteriophages). Many of the students expected all the plaques to have a common circular morphology; they were surprised to obtain plaques formations having various sizes and shapes. Additionally, students were able to apply the knowledge gained during plaque assay and its essence as a preliminary method of characterising phages based on size and shape.

Another notable experience shared by the students while conducting the experiments, was an improvement in their aseptic techniques. Students emphasised that, to enforce sterility and minimise contamination, they frequently sprayed ethanol (75%) on their respective working benches. A few other students accentuated, to avoid cross-contamination, new sterile pipette tips were used each time pipetting of the sample solutions were carried out. Furthermore, students stressed that their technical and communication skills improved admirably as they were able to present their data in a more scientific manner following the laboratory practicum. Technical skills acquired during practical classes are the basis for many types of research carried out in microbiology and biotechnology. Thus, it is imperative to emphasize the importance of students to record their observations carefully in laboratory notebook because small details may be of significant value during report writing and presentations. It is also recommended that students are taught the usage of plaque assay as a method of determining plaque forming units (pfu) which measures the number of infectious virus particles. More laboratory practicums that discusses students' understanding or learning outcome should be reported such as that performed by Masnoddin, *et al.*, (2018) and Awang-Kanak, *et al.*, (2016) which assessed students' understanding on basic plant tissue culture and basic Mendelian Genetics problems at Foundation Science, University Malaysia Sabah. These studies are essential to instructors as they provide further information on students learning behaviors, challenges they encounter during laboratory activities, as well as ways of improving student's learning experiences.

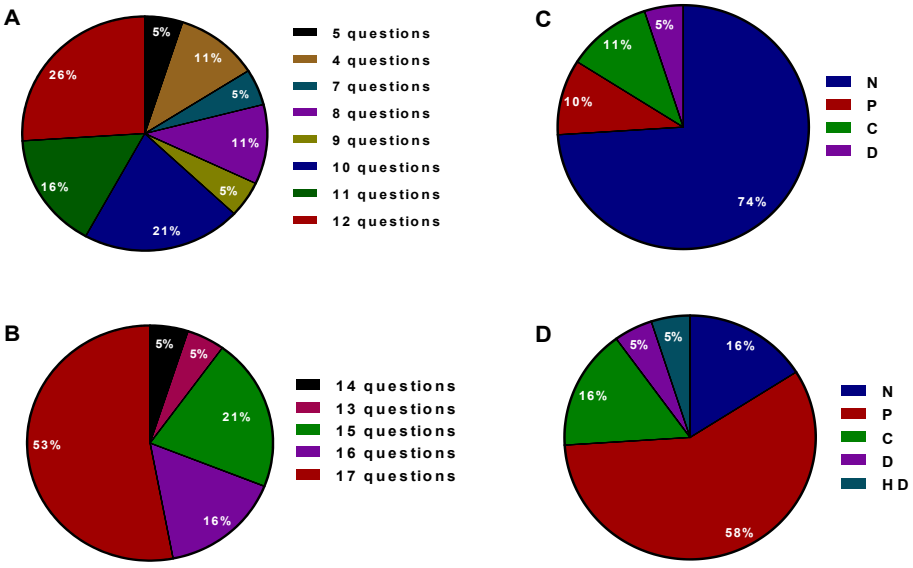

Figure 2. A group of charts showing the percentage of students who attempted the questions before (A) and after (B) the phage practicum, as well as grade performance in the survey questions before (C) and after (D) the practical activities. The score description used for the grades were HD (80-100%); D (70-79%); C (60-69%); P (50-59%); N (0-49%), following Swinburne University of Technology 's grading rubric.

CONCLUSION

Six phages were isolated from cave and palm oil plantation soil samples with the aid of 1% (v/v) TTC solution. Each isolated phages presented a distinctive plaque morphology. In the absence of sophisticated equipment such as TEM and SEM for visual analysis of viruses, a simple and inexpensive method (plaque assay) can be utilised. Although the exercise demonstrated in this study focused on some bacterial pathogens that are food poisoning causative agents. However, the protocol can also be adapted to non-pathogens and plant pathogens. The use of survey questions in this current study, during pre- and post-phage laboratory classes vividly highlighted important learning gap. Students had an improved understanding of bacteriophage concept and laboratory knowledge on isolation process in post-phage laboratory class survey questions.

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