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## Research Note

# Comparison of the Single Agar and Double Agar layer methods for Enumeration of Bacteriophages

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Enumeration of bacteriophage is an important step for studying phage growth dynamics and efficacy of phage treatments. The 2-step double agar overlay method is commonly used for the enumeration of bacteriophages. The single agar layer method that involves a single step has been reported. Twenty-five enriched bacteriophage samples (24 coliphages and one vibriophage) were tested separately by both the single agar and double agar layer methods and the results indicated that there was no significant difference between the mean counts of phages obtained by single agar and double layer methods. Single agar layer method may be preferable for enumerating bacteriophages as it requires less media, shorter time, easier to perform and had better plaque visibility.

Bacteriophages have been increasingly explored as therapeutic agents for the control of bacterial infections in humans, terrestrial animals and aquatic animals (Rao & Lalitha, 2015; Culot et al., 2019; Mertz, 2019; Dec et al., 2020; Luong et al., 2020; Zbikowska et al., 2020). Use of bacteriophage in post-harvest applications for the control of bacteria on food and food contact surfaces is gaining importance. Estimating the number of phages is a requisite for understanding phage growth dynamics, quantify the number of phages in different samples, and know the phage numbers in phage formulations. Double agar layer method (Adams, 1959; Kropinski et al., 2009; Cormier & James, 2014) and Single agar method (Rao & Surendran, 2003) are used to enumerate the bacteriophages.

Double agar layer method method, as the name indicates, is a two-layer method. The basal layer was prepared by pouring 15-18 mL of sterile nutrient agar (peptone 5 $gL^{-1}$, sodium chloride 5 $gL^{-1}$, agar-agar 15 $gL^{-1}$) in a sterile petri plate and kept in a laminar flow chamber for 45 min to remove excess moisture. Afterwards, the liquid sample containing the bacteriophage (1 mL) and the specific host bacteria (1 mL) mixed with 8 mL of molten and cooled soft nutrient gar (peptone 5 $gL^{-1}$, sodium chloride 5 $gL^{-1}$, agar-agar 8 $gL^{-1}$) and carefully poured in sterile petri plates and incubated at 35±2° C for 6 h. Clear plaques were counted and reported as plaque forming units per millilitre (pfu $mL^{-1}$).

Single agar method is a single step method. Liquid sample containing the enriched coliphage (1 mL) and the specific host bacteria (1 mL) was mixed with 8 mL of molten and cooled soft nutrient agar (peptone 5g $L^{-1}$, sodium chloride 5g $L^{-1}$, agar-agar 8 g $L^{-1}$) and carefully poured into a sterile petri plate. The petri plates were incubated at 35±2p C for 6 h and the plaques (clear zone on bacterial lawn) were counted and reported as plaque forming units per millilitre (pfu $mL^{-1}$). Vibriophages were enumerated in the similar manner with minor changes such as using luminescent *Vibrio harveyi* as host bacterium, nutrient agar supplemented with 3% NaCl as the media and incubation conditions of 28 ±2°C temperature for a period of 8 h.

A total of 25 bacteriophage samples comprising of 24 enriched coliphage samples and one vibriophage

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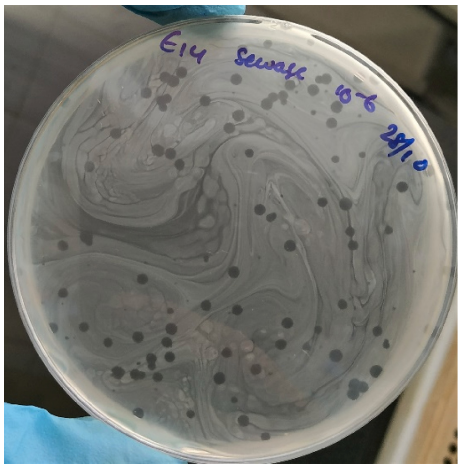
enriched sample were tested separately by both the single agar and double agar layer methods and the results are presented in Table 1. The coliphage counts ranged between 8.6 x $10^5$ and 7.2 x $10^8$ pfu $mL^{-1}$ in single agar whereas the coliphage counts ranged between 7.0 x $10^4$ and 4.8 x $10^8$ pfu $mL^{-1}$ in the double agar layer method. The mean coliphage count in the single agar method was 9.54 x $10^7$ ± 1.9 x $10^8$ pfu $mL^{-1}$ while the mean coliphage count in the double agar layer method was 4.97 x $10^7$ ± 1.1 x $10^8$ pfu $mL^{-1}$. There was no significant difference between the mean plaque counts of the 24 coliphage samples obtained by the single agar method and the double agar layer method ($p>0.05$ in Student's t-test, paired with 1-tailed distribution). The vibriophage counts obtained in the single agar method and double agar methods were 3.36 x $10^{11}$ pfu $mL^{-1}$ and 2.88 x $10^{11}$ pfu $mL^{-1}$, respectively. However, single agar layer method was found to be advantageous as it consumed lesser media, required lower time, easier to perform and the plaques were clear (Table 2). Hence the Single agar method may be preferred for enumerating phages in bacteriophage research and phage application studies.

Table 1. Enumeration of bacteriophages by Single agar and Double agar layer methods

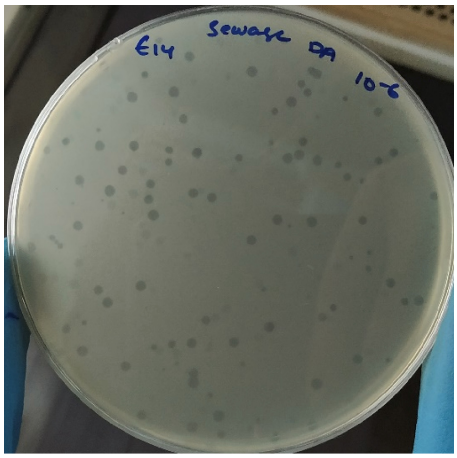
| Bacteriophage ID | Single agar Method (pfu $mL^{-1}$) | Double agar layer Method (pfu $mL^{-1}$) |
|---|---|---|
| Coliphage-E1-Sewϕ | 1.32 x $10^7$ | 1.56 x $10^7$ |
| Coliphage-E2-Sewϕ | 2.5 x $10^7$ | 2.2 x $10^6$ |
| Coliphage E3-Sewϕ | 1.2 x $10^7$ | 1.86 x $10^6$ |
| Coliphage E4-Sewϕ | 1.28 x $10^7$ | 2.14 x $10^7$ |
| Coliphage E5-Sewϕ | 1.55 x $10^6$ | 9.5 x $10^5$ |
| Coliphage E6-Sewϕ | 1.0 x $10^7$ | 8.0 x $10^6$ |
| Coliphage E7-Sewϕ | 3.4 x $10^7$ | 2.7 x $10^6$ |
| Coliphage E8-Sewϕ | 1.16 x $10^7$ | 1.36 x $10^7$ |
| Coliphage E9-Sewϕ | 1.24 x $10^7$ | 1.08 x $10^7$ |
| Coliphage E10-Sewϕ | 2.9 x $10^6$ | 1.08 x $10^7$ |
| Coliphage E11-Sewϕ | 2.88 x $10^7$ | 7.9 x $10^5$ |
| Coliphage E12-Sewϕ | 3.2 x $10^6$ | 1.04 x $10^7$ |
| Coliphage E13-Sewϕ | 1.26 x $10^8$ | 1.16 x $10^7$ |
| Coliphage E14-Sewϕ | 8.1 x $10^7$ | 7.2 x $10^7$ |
| Coliphage E15-Sewϕ | 2.26 x $10^8$ | 1.63 x $10^8$ |
| Coliphage E16-Sewϕ | 1.24 x $10^7$ | 1.44 x $10^7$ |
| Coliphage E17-Sewϕ | 1.28 x $10^8$ | 1.7 x $10^7$ |
| Coliphage E18-Sewϕ | 6.2 x $10^7$ | 2.93 x $10^8$ |
| Coliphage E19-Sewϕ | 1.12 x $10^6$ | 8.8 x $10^6$ |
| Coliphage E20-Sewϕ | 8.6 x $10^5$ | 5.2 x $10^5$ |
| Coliphage E21-Sewϕ | 9.2 x $10^7$ | 2.6 x $10^7$ |
| Coliphage E22-Sewϕ | 5.2 x $10^7$ | 7.0 x $10^4$ |
| Coliphage E23-Sewϕ | 7.2 x $10^8$ | 2.46 x $10^6$ |
| Coliphage E24-Sewϕ | 6.2 x $10^8$ | 4.8 x $10^8$ |
| Vibriophage-LV6ϕ | 3.36 x $10^{11}$ | 2.88 x $10^{11}$ |

Table 2. Comparison of the Single Agar and Double Agar layer methods

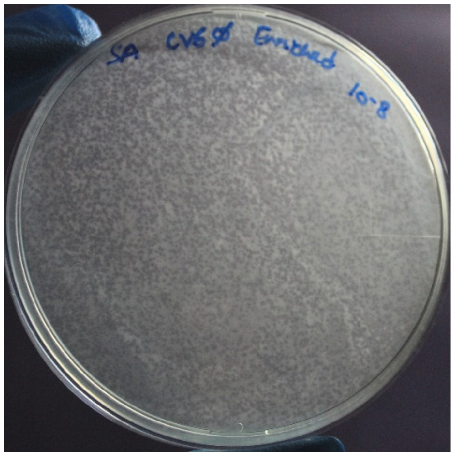
| | Single agar layer method | Double agar layer method |
|---|---|---|
| Media usage | Consumes only 8mL of nutrient media and is relatively economical | Consumes ~25 mL of nutrient media (15-18mL in basal layer and 8mL in top layer) |
| Time | Can be performed in lesser time (15 min) | Requires relatively longer time (60 min of which 45 min was for drying the bottom layer) |
| Labour | Less laborious as it involves a single-step process | More laborious as it involves a two-step process |
| Plaque visibility | Relatively better visual clarity in enumerating the bacteriophages (Fig. 1a), especially those that produce pin-point plaques (~1mm diameter) (Fig. 1c) | Relatively less clear plaques (Fig. 1b) and difficulty in visualizing pin-point sized plaques (Fig. 1d) |



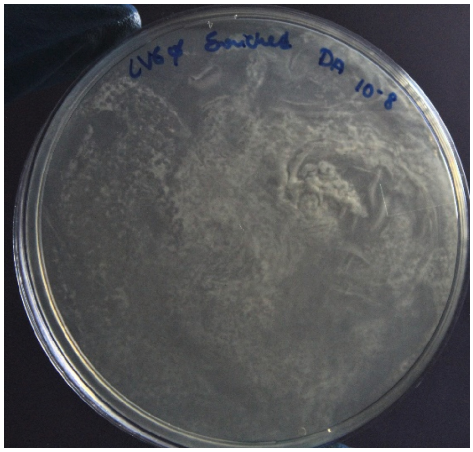

a) Plaques of coliphage on Single Agar




b) Plaques of coliphage on Double Agar




c) Plaques of vibriophage on Single Agar




d) Plaques of vibriophage on Double Agar

Fig. 1. Visualization of Plaque forming units of Coliphage E14-Sewϕand Vibriophage-LV6ϕ on Single Agar plate (a,c) and Double Agar plate (b,d)

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