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Development of a novel and highly efficient method of isolating bacteriophages from water

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ABSTRACT

Bacteriophages are widely used to the treatment of drug-resistant bacteria and the improvement of food safety through bacterial lysis. However, the limited investigations on bacteriophage restrict their further application. In this study, a novel and highly efficient method was developed for isolating bacteriophage from water based on the electropositive silica gel particles (ESPs) method. To optimize the ESPs method, we evaluated the eluent type, flow rate, pH, temperature, and inoculation concentration of bacteriophage using bacteriophage *f2*. The quantitative detection reported that the recovery of the ESPs method reached over 90%. The qualitative detection demonstrated that the ESPs method effectively isolated 70% of extremely low-concentration bacteriophage (10^0 PFU/100 L). Based on the host bacteria composed of 33 standard strains and 10 isolated strains, the bacteriophages in 18 water samples collected from the three sites in the Tianjin Haihe River Basin were isolated by the ESPs and traditional methods. Results showed that the ESPs method was significantly superior to the traditional method. The ESPs method isolated 32 strains of bacteriophage, whereas the traditional method isolated 15 strains. The sample isolation efficiency and bacteriophage isolation efficiency of the ESPs method were 3.28 and 2.13 times higher than those of the traditional method. The developed ESPs method was characterized by high isolation efficiency, efficient handling of large water sample size and low requirement on water quality.

1. Introduction

Bacteriophage is a generic term for a group of prokaryotic microbial virus that infects bacteria, actinomycetes, and mycoplasma, among other microorganisms. Bacteriophage was discovered > 100 years ago. Ernes Hankin firstly reported the presence of bacteriophage in the river water in India in 1896. Such substance can pass through porcelain filter, and possesses poor thermostability and strong antimicrobial activity. In 1915, Frederick Twort discovered fenestrae on the culture medium coated with *Staphylococcus aureus*. He described his observation as a virus infection. This discovery was officially named bacteriophage in 1917 by Félix d'Herelle (Golkar et al., 2014; Tiwari et al., 2014; Wittebole et al., 2014). Bacteriophage has a simple structure and only possesses external capsid protein and internal nucleic acids, including dsDNA, ssDNA, dsRNA, and ssRNA. The specificity of host bacterial lysis is the main characteristic of bacteriophage, which has attracted considerable attention and been widely applied in many areas, including bacteriophage treatment for multidrug-resistant bacterial infections (Fortuna et al., 2008; Henry et al., 2013; Klem et al., 2013; Saussereau et al., 2014; Viertel et al., 2014), inactivation of pathogenic bacteria in food (Atterbury, 2009; Hagens and Loessner, 2010), water (Pereira et al., 2011; Stenholm et al., 2008), and plants (Lim et al., 2013), and detection of bacteria (Derda et al., 2013).

Bacteriophages are widely distributed on the earth. They exist in almost every environment with bacteria, and their number reaches 10^{32}, about 10 times of bacteria (Labrie et al., 2010). The specificity of host bacterial lysis of bacteriophage restricts its application. Therefore, researchers invented the cocktail therapy (Cooper et al., 2014; Mendes et al., 2014; Sarker et al., 2012), which is a combination of different bacteriophages. At present, approximately 6000 strains of bacteriophage have been discovered and their morphology has also been well-described (Ackermann and Prangishvili, 2012). However, this number only accounted for a very small proportion of the total number of bacteriophage in nature. The more widely application of

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bacteriophages requires more bacteriophages. Most of studies introduce bacteriophage isolation from 5 g of soil or feces (Knezevic et al., 2009) and 100 mL to 1000 mL of water or sewage (Begum et al., 2010; Gomez-Donate et al., 2011). The low bacteriophage isolation efficiency in these studies may be attributed to the small sample size. The upgrade and optimization of particle and elution buffer used in the isolation continuously promoted the wider application of the isolation method, such as the effective recovery of adenovirus from water advanced by a novel celite method (McMinn et al., 2012). Similarly, developing a highly efficient bacteriophage isolation method from water is urgently needed to meet the high bacteriophage demand.

In this paper, a new and highly efficient isolation method for bacteriophage was developed based on electropositive silica gel particles (ESPs) under large water sample. Parameters in the ESPs method, such as eluent type, flow rate, pH, temperature, and inoculation concentration of bacteriophage, were discussed. Moreover, the developed ESPs method was compared with the traditional method.

2. Materials and methods

2.1. Materials

Silica gel (SG, 60–100 mesh) and brain heart infusion (BHI) were purchased from Qingdao Marine Co., Ltd. (Qingdao, China) and Becton Dickinson (USA), respectively. Tryptone, beef extract, glycine, aluminum chloride, sodium carbonate, sodium hydroxide, hydrochloric acid, and sodium thiosulfate were obtained from Sangon Biotech Co., Ltd. (China). The preparation of the five eluents was stated in the Supplementary information. PBS (0.01 mol/L, pH 7.4) was prepared in our laboratory.

Bacteriophage f_2 was purchased from American Type Culture Collection. A total of 42 strains of host bacteria and a yeast were used as test strains, which included 33 standard strains and 10 isolated strains. The sources of the strains were provided in the Supplementary information (Table S1).

2.2. Preparation of the host bacteria

43 host bacteria were respectively inoculated into the 43 test tubes (contained 10 mL BHI solution each), and incubated at 37 °C for 24 h. Then, 43 mL host bacteria culture (1 mL each) was aseptically removed to the sterile triangular flask, and thoroughly mixed. The solution was the host bacteria mixture solution which was prepared when needed.

2.3. Preparation of ESPs

ESPs were prepared according to the previously reported method with some modifications (APAH, 1998; Li et al., 1998). In brief, 6.675 g of $AlCl_3$ was completely dissolved by slowly adding 950 mL of distilled water. Then, 45 mL of 2 mol/L Na_2CO_3 was slowly added to the $AlCl_3$ solution, producing a milky $Al(OH)_3$ gel. The pH of the gel solution was adjusted to 7.2 with 0.1 mol/L NaOH or HCl solution. The final volume of the $Al(OH)_3$ gel solution was 1000 mL. The solution was centrifuged at 1100*g* for 15 min after 24 h at room temperature. The supernatant was discarded, and the sediment was resuspended in 1 L of 0.14 mol/L NaCl solution. After 24 h, the $Al(OH)_3$ gel solution was washed three times by applying the aforementioned method and autoclaved at 121 °C for 15 min. SG (1375 g) was slowly added into the 1000 mL of $Al(OH)_3$ gel solution and stirred to mix the solution completely. The gelatinous substance was then placed in an oven at 60 °C for 36 h. The dried substances were the ESPs, which were sealed and stored at room temperature. The difference between SG and ESPs was respectively tested using a scanning electron microscope (SEM, Hitachi S-4800, Japan), a laser scattering particle size distribution analyzer (Horiba LA-960, Japan) and a ZETA potentiometer (Anton Paar SurPASS, Austria).

2.4. Preparation of eluents

Five eluents were prepared according to the previously reported method with some modifications (APAH, 1998; Li et al., 1998; Mendez et al., 2004; Watt et al., 2002). (1) Three-time nutrient broth (3 × broth) - Add 30 g of tryptone, 15 g of beef extract and 15 g of sodium chloride to 1 L of reagent water and heat to dissolve. (2) Three percent (w/v) of beef extract (3% beef extract) - Add 30 g of beef extract to 1 L of reagent water and heat to dissolve. (3) 0.5 mol/L glycine (Gly) – Add 37.5 g of Gly to 1 L of reagent water and mix to dissolve. (4) Glycine and three-time nutrient broth (Gly + 3 × broth) - Add 30 g of tryptone, 15 g of beef extract, 15 g of sodium chloride, 37.5 g of Gly and 20 sodium hydroxide to 1 L of reagent water and heat to dissolve. (5) Glycine and 3% (w/v) of beef extract (Gly + 3% beef extract) - Add 30 g of beef extract and 37.5 g of Gly to 1 L of reagent water and heat to dissolve. Five eluents are all adjusted pH to 10.2–10.5 with 1.0 N hydrochloric acid or 1.0 N sodium hydroxide, if necessary. Autoclave at 121 °C and 15 psi for 20 min.

2.5. Development of the ESPs method

ESPs (800 g) were added slowly into 83.5 mm (inner diameter) × 400 mm (height) filter cylinder containing 1.5 L of sterile purified water. The ESPs were stirred with a glass rod to eliminate bubbles. The upper covers of the filter cylinder were screwed and connected to a vessel. Sodium thiosulfate (0.5 mL of 10% sodium thiosulfate per 1 L of tap water) was added into the sterile containers containing 100 L of tap water to neutralize residual chlorine in the water. The water sample was completely mixed by spiking with a certain amount of bacteriophage f_2. The prepared water sample was pumped into the filter cylinder through a peristaltic pump.

Filtered water was collected into a wastewater tank. During this period, the flow rate of the water sample was observed. After the water sample was completely filtered, a certain amount of eluent was added to elute the ESPs through the peristaltic pump. The eluate was collected in a sterile triangular flask and its pH was adjusted to 7.0. Some PEG 6000 was added into the eluate, while ensuring the final concentration reached 13%. PEG 6000 was then dissolved completely using a magnetic stirrer. The solution was stored at room temperature for 30 min and centrifuged at 15,000*g* for 30 min at 4 °C. The supernatant was discarded, and the sediment was resuspended with 100 mL of PBS. The bacteriophage f_2 in the prepared 100 mL of eluate was tested quantitatively or qualitatively according to the different amounts of bacteriophage f_2 added into the water sample.

The quantitative detection of bacteriophage f_2 in the eluate was conducted according to Reference (Fisher et al., 2011). The quantitative result was used to calculate the bacteriophage f_2 recovery (R: $R = (A/B) \times 100\%$, where B and A are the amounts of bacteriophage f_2 before and after the concentration, respectively).

The qualitative detection included the following steps. (1) BHI solution (10 ×, 10 mL) was added into 100 mL of eluate, and then 1 mL of *E. coli* 285 solution was added. The resulting solution was evenly mixed and incubated for 24 h at 37 °C. (2) The culture was centrifuged at 4000*g* for 10 min. (3) The method was modified (Salter et al., 2010). The supernatant (1 mL) was evenly mixed with 100 μL of *E. coli* 285 solution. The solution remained static for 5 min and was detected for the presence of bacteriophage plaques by the double agar layer method (Mooijman et al., 2001). Tap water (100 L) without bacteriophage f_2 served as the negative control, which was tested by qualitative method. Furthermore, the parameters in the ESPs method, such as flow rate, temperature, pH, eluent type, and volume, were optimized.

2.6. Isolation of bacteriophage from surface water

Water sampling and detection: Three sampling sites were set in the upstream (N 39° 13′ 50.43″, E 117° 06′ 13.59″), midstream (N 39° 08′