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Research article

The effect of different media and temperature conditions for *Salmonella* bacteriophage preservation

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Abstract

This research aimed to determine the optimal media and temperature conditions for the long-term storage of bacteriophages. In this study, the viability of *Salmonella* phages in 50% glycerol, 10% sodium chloride-magnesium sulfate (SM) buffer, and 5% dimethyl sulfoxide (DMSO) media at room temperature, 4°C, -20°C, and -80°C for 12 months was determined. In 50% glycerol, at the end of the experiment, no significant difference was found between four temperature conditions on phage density, ranging from 6.20-6.23 log10 PFU/mL ($P>0.05$). Under 10% SM medium, phage preservation at room temperature provided the optimum density at 6.31 log10 PFU/mL. In addition, phages preserved in a 5% DMSO medium were of similar density values across all temperature treatments. Still, their availability after 12 month-storage (88.0-88.5%) was significantly lower ($P<0.05$) than that of 50% glycerol and 10% SM. Moreover, for phage lysis capacity, low temperatures (4°C, -20°C, and -80°C) were superior to room temperature used for preservation. Considering the density, lysis capacity, and practical convenience, storing phages at 4°C in a 50% Glycerol medium is recommended.

Keywords: Glycerol, Medium, Phage, Storage, Temperature

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INTRODUCTION

Nowadays, bacteriophages (or phages) are receiving more and more attention due to their great benefits. The use of phages as a biocide for plants and animals to replace antibiotics has been extensively studied (Doss et al., 2017; Tahir et al., 2017). However, to put phages into practice quickly and effectively, it is necessary to have a method of preservation that is both effective and economical for producers and customers. Phages are structural proteins, and therefore they are susceptible to protein denaturation factors. These include exposure to organic solvents, high temperature, pH, ionic strength, and interference effects (Malik et al., 2017). Preserving bacteriophages at low temperatures to maintain phages for a long time has been widely applied (Ackermann et al., 2004). Novik et al. (2009) have shown that refrigeration under 10% glycerol and 5% DMSO with a high freezing rate ensures high survivability and phage lysis capacity against Pseudomonas bacteria. In addition, Bonilla et al. (2016) also found SM buffer to be highly effective in preserving T4 bacteriophages, of which phages can be easily stored at 4°C in SM buffer and still maintain their activity for 96 days. Golec et al. (2011) showed that preserving the tail phage in DNA form in newly infected cells was a more reliable phage storage method than maintaining mature phages in lysate form, raw or pure. In addition, according to the study of Alvi et al. (2018), 4°C was the best temperature for phage preservation. The above results showed that each group of phages in each study has different storage conditions. Previously, Ackermann et al. (2004) stated that there is no fixed method for phage preservation due to differences in the sensitivity of individual phages to physical and environmental conditions of storage. Thus, a unique approach for each phage or the use of preservation methods is expected to ensure subsequent phage recovery. A combination of media and temperature for phage storage could be beneficial, particularly for *Salmonella* phages, which are more widely employed in the veterinary field as a therapy in intensively-reared animals. Therefore, the present study was carried out to determine appropriate preservation media and temperature conditions for the storage of *Salmonella* phages for up to 12 months.

MATERIALS AND METHODS

Media and growth conditions

Phages (*Myoviridae* family) were selected from those with a broad host spectrum that have been isolated in the laboratory at the Department of Veterinary Medicine, College of Agriculture, Can Tho University. Phages were stored in 3 mediums of 50% glycerol (Merck), 10% SM buffer (Merck) (5.8 g NaCl, 2 g $MgSO_4.7H_2O$, 50 mL 1M Tris-HCl, pH 7.4 in 1 liter of water) and 5% DMSO (Merck) at four temperature conditions: room temperature, 4°C, -20°C and -80°C.

Phage quantifications

Phage proliferation: From the isolates available in the laboratory, the phages were proliferated in the ratio of phage: *Salmonella*: TSB (Tryptic Soy

Broth including casein peptone, dipotassium hydrogen phosphate, glucose, sodium chloride, and soya peptone) being 1: 2: 30 with a *Salmonella* (*Salmonella* Typhimurium - ATCC®14028™) population of 10^8 CFU/mL (OD_{600} from 0.3 to 0.5). The phages were incubated at 37°C for 24 hours. After that, chloroform solution was added to the proliferated phage biomass at the rate of 1 chloroform: 10 phages, vortex, incubated for 2 hours, then centrifuged at 6,000 rpm at 4°C for 15 minutes and collected phages. Media for storing, namely 50% glycerol, 10% SM buffer, and 5% DMSO, were sterilized before adding the phage solution. Each sample (1 mL) was stored in 2 ml Eppendorf ampoules with five replicates. The bacteriophage density before and after storage was determined based on a plaques count method. After one month, two months, three months, six months, and 12 months, phages in different storage conditions were detected for phage density determination.

Phage density: The phage concentration was diluted by introducing 0.1 mL of each phage solution to a sterile Eppendorf tube containing 0.9 mL of clean distilled water and shaking to homogenize the mixture. This step was repeated until the phage concentration reached 10^{-5}. Then, 0.1 mL of each diluted phage was spread on each sterile Petri dish and 0.1 ml of *Salmonella* host bacteria suspension (10^8 CFU/mL). In the next step, about 10-12 mL TSB medium with 0.6% agar was added, melted, and let cool in a water bath at 50°C for at least 30 minutes. The next step was to shake the dish to homogenize the mixture, then let it stand until the agar cooled down at 37°C for 24 hours. After 24 hours, plaques were observed and counted (Poxleitner et al., 2017).

The phage density was calculated using the following formula (González-Menéndez et al., 2018). PFU / mL = N × 1 / DF × 1 / V, in which: N is the number of countable plaques; DF is the dilution factor; V is the phage volume (mL).

Statistical analysis

All data were statistically analyzed using the Minitab 16.2 software (Minitab, 2010). The General Linear Model $Y_{ij}=\mu+F_i+e_{ij}$ was used to evaluate the effect of experimental factors, where Y_{ij} is the value phage density, μ is the overall mean, F_i is the effect of different media or temperature conditions, and e_{ij} is the random error. The differences between treatments were determined using Tukey's pairwise comparisons ($P<0.05$).

RESULTS

Phage density at different preservation temperature conditions

Table 1 shows the phage density investigated at room temperature, 4°C, -20°C, and -80°C in the 50% glycerol, 10% SM, and 5% DMSO. Under 50% glycerol medium, phages were not affected by any temperature conditions after one month, but their density decreased after two and three months. However, at the end of the experiment, no significant difference was found between four temperature conditions on phage density ($P>0.05$). For 10% SM medium, phage preservation at room temperature was slightly inferior in terms of density compared to those of other media. Finally, phages stored in 5% DMSO media maintained comparable densities across all temperature treatments.

Table 1 Phage density (log_{10} PFU/mL) in 3 media at different storage temperature conditions

Medium	Time	Room temperature	4°C	-20°C	-80°C	SE means	P
50% glycerol	1 month	6.92	6.92	6.90	6.91	0.010	0.459
	2 months	6.91[a]	6.91[a]	6.54[b]	6.53b	0.009	0.001
	3 months	6.53[a]	6.54[a]	6.39[b]	6.36b	0.012	0.001
	6 months	6.37	6.38	6.37	6.32	0.022	0.186
	12 months	6.20	6.20	6.21	6.23	0.008	0.058
10% SM	1 month	6.94c	7.01[ab]	6.97[bc]	7.01[a]	0.010	0.001
	2 months	6.99	6.95	6.97	6.99	0.015	0.078
	3 months	6.60[a]	6.56[b]	6.62[a]	6.60[a]	0.010	0.001
	6 months	6.44a[b]	6.42[b]	6.45[ab]	6.46[a]	0.008	0.010
	12 months	6.31[a]	6.22[b]	6.29[a]	6.31[a]	0.006	0.001
5% DMSO	1 month	6.48	6.49	6.51	6.50	0.008	0.055
	2 months	6.47	6.50	6.51	6.50	0.022	0.622
	3 months	6.11	6.15	6.12	6.10	0.020	0.244
	6 months	5.95	6.03	5.95	5.94	0.030	0.160
	12 months	5.79	5.78	5.80	5.81	0.014	0.455

[a,b,c] Values within a row with different superscripts are significantly different (P≤0.05)

When analyzing the lysis capacity of phages after 12 months of preservation, it was discovered that phages preserved at low temperatures (4°C, -20°C, and -80°C) performed slightly better (Figure 1), of which plaques formed in 10% SM medium at room temperature were fainter than those created from cold-stored phages.

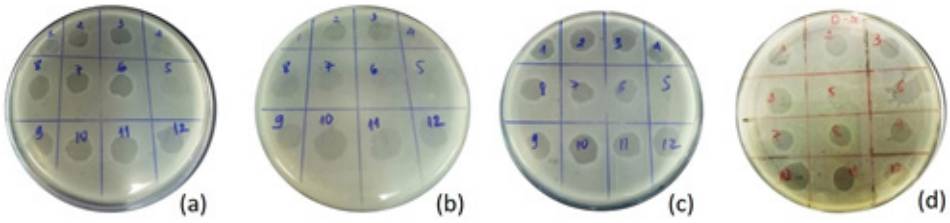

Figure 1 Plages of phages in 10% SM medium before preservation (a) and 12 months of storage under room temperature (b), 4°C (c) and -20°C (d).

Evaluation of phage density on the various preservation media

Most of the time, at room temperature and 4°C of preservation, phage density in 10% SM was lower than in 50% glycerol and 5% DMSO (Figure 2). At -20°C and -80°C, sudden drops in phage number ($P<0.05$) were found in 50% glycerol and 5% DMSO medium during the second and third month of storage, respectively. At the end of the preservation period, a lower density value in 5% DMSO was also recorded (88.3-88.5%). Phage density was generally reduced by 10-12% at 12 months of storage for all tested media and temperature levels.

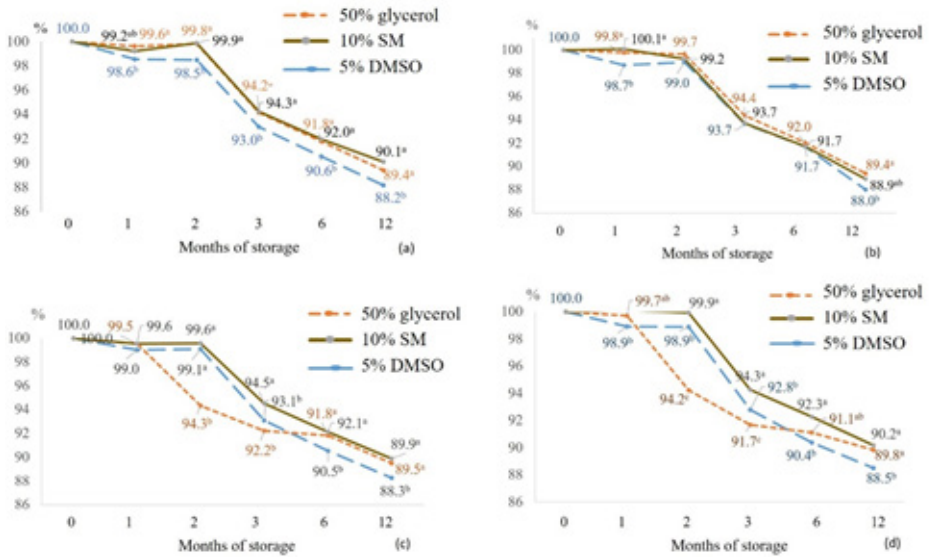

Figure 2 Percentage of phage density preserved at room temperature (a), 4°C (b), (-20°C (c) and -80°C (d).

DISCUSSION

Previous studies have shown that phages are sensitive to organic solvents, pH, temperature, and salinity (Moghimian et al., 2016; Litt and Jaroni, 2017). Bacteriophages are susceptible to denaturation leading to loss of function when exposed to adverse conditions (Vagenende et al., 2009); therefore, finding the appropriate environment and temperature to preserve them is also an essential factor in determining their effectiveness when put into practice. According to Tovkach et al. (2012), gelatine, magnesium ion, and glycerol are additives that, when added, can help increase the stability of bacteriophages during storage. The present findings show that the phage densities when stored in 50% glycerol and 10% SM media at four temperature levels were both higher than in the 5% DMSO medium. DMSO is commonly used as a stabilizer to freeze viral particles (Wallis and Melnick, 1968). DMSO and glycerol are known as organic solvents to stabilize proteins in their native form (Nagao et al., 2000). Glycerol acts by retaining water in the cells, avoiding excessive dehydration caused by exposure to concentrated solutions; the higher the concentration of glycerol means, the higher its level of protection (Mazur and Kleinhan, 2008). Besides, SM buffer in the presence of gelatin strongly

influences the lyophilization of phage (Puapermpoonsiri et al., 2010). Manohar and Ramesh (2019) believed that gelatin could form supporting polymers to maintain the morphology of the phage during lyophilization. Research by Puapermpoonsiri et al. (2010) also showed that gelatin had better stabilizing properties against Staphylococcus phage (Siphoviridae) and Pseudomonas phage (Myoviridae). Furthermore, in all experimental conditions, phage density remained more than 88% compared with before storage; however, the lysing ability of phages in cold conditions was better than at room temperature. Practically, phage density can be affected by temperature, time, and preserved medium composition. The viability and storage can be inactivated by the destruction of its structural elements such as head, tail, and shell or by altering the structure of DNA (Ackermann et al., 2004).

With temperature, it was previously stated that the stability of the phage increases as the storage temperature decreases (González-Menéndez et al., 2018). At low temperatures (-80°C and -196°C), all phages showed good viability after 24 months regardless of the stabilizer, and in the remaining temperatures (20°C/25°C, 4°C, and -20°C), the phages were less stable. Golec et al. (2011) also concluded that tail phages could be stored inside infected cells at -80°C without phage and host viability loss. In the present study, after 12 months of storage at -80°C, the phage densities in all three 50% glycerol, 10% SM, and 5% DMSO media were relatively high (88.3-89.9%). However, the evidence of temperature influence on phage density was weak and unclear over different preservation periods.

The viability of phages can be maintained for up to 20 months when lyophilized and stored at 4°C; however, the phage's survival ability decreases to 10 months at 37°C (Manohar et al., 2019). In the current study, when stored at 4°C, the phage density in all three media decreased after 12 months of storage, but the phage density in all three media was relatively high, fluctuating from 88.0% to 89.4%. In accordance with the current study, Leung et al. (2018) demonstrated that two types of bacteriophages, PEV2 (Podovirus) and PEV40 (Myovirus), along with varying amounts of trehalose (70% and 60%) and leucine (30% and 40%), could be successfully preserved for one year at 4°C and 20°C using vacuum packaging. This is supported by Alvi et al. (2018), who concluded that the optimal storage of bacteriophages was at 4°C compared with those stored at -20°C and -80°C, and there was no reduction in the density of samples held at 4°C for one year.

In the present work, in a 50% glycerol medium, phages were more stable when stored at room temperature and 4°C. According to Clark's study (1962), when the phage was preserved for 2 years at room temperature (24-28°C) and 4°C in a 50% glycerol preservative solution, dried, and in lyophilization, the phage densities of all stored samples decreased over time regardless of storage conditions. In addition, Ackermann et al. (2004) concluded that various phages lyophilized with the addition of 50% glycerol could persist for many years in vacuum tubes. However, in the report of Olson et al. (2004), the addition of 5-10% glycerol to bacteriophage suspensions could ensure viability for 30 days at -20°C or -70°C. Also mentioning the effect of storage medium, Jepson and March (2004) observed no phages in the SM buffer at 42°C after 84 days. In contrast, at 37°C, no phages were detected after 120 days. Recently, Bonilla et al. (2016) stated that many phages could be easily

stored at 4°C in an SM buffer while maintaining viability during repeated use. If long-term storage of bacteriophage is required, it should be kept under liquid nitrogen conditions with DMSO (5%).

CONCLUSION

Phage preservation in 50% glycerol and 10% SM media ensured better density than 5% DMSO at all temperatures tested. At room temperature and 4°C, phage density was more stable than at -20°C and -80°C when stored in a 50% glycerol medium. For phage lysis capacity, low temperatures (4°C, -20°C, and -80°C) were superior to room temperature used for preservation. In terms of economic efficiency and convenience of storage, phage preservation in the 50% glycerol medium at 4°C is recommended for practical application.

AUTHOR CONTRIBUTIONS

This work was conducted with the contribution of all authors. LNNP, LHA and NTN designed the experimental procedures. LNNP, HTL, PKNH and HTL performed the experiments. LNNP, CTHT, TTHM, NHX and NTN interpreted the data and prepared the manuscript. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

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