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Aqueous two-phase systems for the recovery and purification of phage therapy products: Recovery of salmonella bacteriophage ϕSan23 as a case study

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ABSTRACT

Contamination with *Salmonella* can cause significant economic and public health problems. Its prevention is accomplished by the use of antibiotics, but appearance of resistant strains has raised the need to search for alternatives. Recently, *Salmonella*-specific bacteriophages were identified and suggested as a therapy product in poultry. The large-scale production of this product requires the development of recovery/purification strategies to maximize product quality and minimize production costs. Aqueous two-phase systems (ATPS) have been used for different bioproducts, but few reports exist for the recovery of phages with this technique. This work analyzed the use of PEG-Salt and Ionic Liquid-Salt systems to characterize the partition of three phages samples: (1) Phage crude lysate containing phages, cell debris and bacterial cells (2) Centrifuged lysate containing phages, cell debris but reduced bacteria due to a centrifugation process (3) Filtered Lysate Phage containing phages and cell debris, without bacterial cells. Phage infectivity was measured in each of the ATPS components to determine the optimal system through recovery, purification fold and stability. Results indicate that phages partition to PEG 400 or ionic liquid phases, but in a PEG8000-Salt system, partition is favored to the salt-rich phase (55 and 3000-fold for phage crude lysate and filtered plate lysate, respectively) with a high recovery (60 and 90% for the same samples). Moreover, this salt phase provided the largest purification fold (up to 127-fold) and no significant reduction in infectivity. These findings serve as a platform for potential scale-up of bioprocesses for the recovery/purification of phage therapy products.

1. Introduction

Salmonella is a gram-negative bacterium responsible of foodborne gastroenteritis (salmonellosis) in humans [1]. This disease generates a significant economic burden to governments and general population for the expenses of medical care. It has been estimated that salmonellosis causes annually 1.3 billion cases and 3 million deaths [2]. Typically, the transmission of salmonellosis is associated with the consumption of contaminated poultry products [3,4]. Thus, there is a need to develop methods for the control of *Salmonella* in poultry, particularly in stages before packaging for human consumption.

The traditional approach for the control of *Salmonella* at pre-harvest level comprises the application of antimicrobials [5], but its constant use has caused the emergence of resistant bacteria strains [6,7]. This threat has created a niche for development of alternative treatments. Bacteriophages (or phages), which are specific viruses for a single bacteria species, or even specific strains, are a promising alternative to antimicrobials. Particularly, lytic viruses are promising as they kill their host when released from the cell [8]. Phages have a great potential in poultry products by acting as an alternative for the control of pathogenic bacteria [8]. As a potential therapy in poultry products, recently, a patent has been conceded for SalmoFree® [9] a mixture of six *Salmonella* lytic bacteriophages as a potential therapy in poultry products.

Concerning phage production, it is achieved by infection of their host bacteria and subsequent purification. This comprises the complete removal of the host bacteria, and their cell debris in case of lytic phages, and reduction of contaminants through several strategies. Different methods for recovery/purification of phages have been developed,

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including ion-exchange and affinity chromatography [10,11], filtration [12], isoelectric [13] and polyethylene glycol precipitations [14]. Depending on the applications and characteristics of the phages, a different recovery method is employed.

Downstream processing can account for a large part of productions costs [15]. Therefore, it is critical to develop alternatives for recovery/purification that are cost-effective. In this context, Aqueous Two-Phase Systems (ATPS) have demonstrated to be able to reduce costs when contrasted against conventional unit operations as chromatography [16]. ATPS are formed when hydrophilic compounds in aqueous solutions are mixed above a certain concentration or temperature [17]. This unit operation has been successful for recovery or purification of a wide array of bioproducts, such as antibiotics [18], antibodies [19], organic compounds [20], proteins [21] and stem cells [22]. Also, this technique has been used successfully to recover bacteriophage T4 [23] and M13 [24]. Previous works have employed polymer-salt systems to recover phages in one phase and selectively partition contaminants to the opposite phase with the objective of the construction of recombinant phage libraries.

The present work evaluates the potential application of ATPS for the recovery and purification of the *Salmonella*-specific bacteriophage ϕSan23, a component of the SalmoFree® cocktail. The strategy reported here, consisted in: First, to evaluate the stability of phages infectivity in each of the possible phases were the viruses could be recovered and second, to determine the partition behavior of phages and contaminants using phage samples with different degrees of purity. These samples comprises: (1) Phage crude lysate that contains phages, cell debris and bacterial cells (Broth Lysate); (2) Centrifuged lysate that contains phages, cell debris and less bacterial cells due to a centrifugation process (Broth Lysate + Centrifugation) (3) Filtered Lysate Phage that contains phages and cell debris, without any bacterial cells (Broth or Plate Lysate + Centrifugation + Filtration). This strategy was used in order to determine in which stage ATPS could be implemented in a future bioprocess. The findings obtained here serve as an initial step in the establishment of a bioprocess for the potential application of this recovery strategy for the generation of a phage therapy product.

2. Materials and methods

2.1. Microorganism

Salmonella enteritidis s25pp was donated by Dr. Pilar Donado from the Colombian Integrated Program for the Antimicrobial Resistance Surveillance (COIPARS – CORPOICA). This strain was isolated from the broiler carcass in a Colombian poultry farm [25]. *Salmonella* bacteriophage ϕSan23 is a *Myoviridae* phage that belongs to the phage collection of the Microbiological Research Center at Universidad de los Andes, Bogota, Colombia [9]. *Salmonella* strain was stored with Glycerol 10% (v/v) at −80 °C and phages suspensions at 4 °C.

2.2. Reagents

Polyethylene glycols with average molecular weights of 400 and 8000 (PEG400 and PEG8000, respectively), 1-ethyl-3-methylimidazolium acetate (EMIM Ac), Lysogeny Broth, Dibasic Sodium Phosphate (Na_2HPO_4), monobasic sodium phosphate (NaH_2PO_4), dibasic potassium phosphate (K_2HPO_4) and Monobasic Potassium Phosphate (KH_2PO4) were purchased from Sigma Aldrich (St. Louis, MO, USA). Bacteriological agar was purchased from BD (Sparks, MD, USA). All chemicals were of analytical grade and all solutions were prepared using Milli Q water. PEG 8000 50% (w/w) stock solution was prepared for ATPS formation. PEG 400 and EMIM Ac are liquids at room temperature and were used at 100% (w/w). Potassium Phosphate solution (40%, w/w, pH 7.0) was prepared in a ratio 18:7 K_2HPO_4/KH_2PO_4. Sodium Phosphate solution (40% w/w, pH 7) was prepared in a ratio 8:7 Na_2HPO_4/NaH_2PO_4. Adjustment of pH was made by addition of orthophosphoric acid or sodium hydroxide as needed.

SM buffer without gelatin (10 mM Tris Base pH 7.4, 10 mM $MgSO_4$, 100 mM NaCl) was used to store phages. Soft Lysogeny Broth agar was used (4 g/L bacteriological agar, 10 g/L tryptone, 5 g/L yeast extract and 5 g/L NaCl) to perform overlay assays. Culture media and components were obtained from Sigma Aldrich (St. Louis, MO, USA).

2.3. Propagation and quantification of bacteriophages

Phage samples were prepared from broth (BL) and plate lysates (PL). For the plate lysate method, serial dilutions of a stock of purified phage suspension were prepared and plated by the method of double layer agar described by Sulakvelidze and Kutter [26]. After incubation and lysis plaques formed (preferably confluent or total lysis in the culture plates), 2 mL of SM buffer were poured to the soft agar surface and aseptically transferred into a 15 mL centrifuge tube (this will help to collect phage produced by infection of *Salmonella* in the plates). Subsequently, 300 µL of chloroform were added and mixed in a vortex for 1 min. Then, centrifugation for 20 min, 4500 rpm, 4 °C was performed, the supernatant was filtered through a 0.22 µm filter and transferred into a new sterile 15 mL tube. This suspension in the experimental design corresponds to the Plate Lysate + Centrifugation + Filtration (PL + C + F).

Broth lysates (BL) were made by growing *S. enteritidis* s25pp in 50 mL of Lysogeny Broth until an $OD_{600\ nm}$ of 0.1, where 50 µL of a 1×10^9 UFP/mL phage suspension was added and incubated for 17 h at 37 °C and 50 rpm. Then, a 20 min centrifugation at 8500 rpm was performed to reduce the remaining bacteria cells. The supernatant was filtered using a 0.22 µm membrane filter to ensure complete removal of *Salmonella* cells. This lysate corresponds to Broth Lysate + Centrifugation + Filtration (BL + C + F). The same process was done for Broth lysate + Centrifugation (BL + C) without the filtration step, it is important to note that centrifugation do not remove completely Salmonella cells, this is the reason why for ATPS analysis this is an additional matrix studied for biomass removal and phage recovery. Finally, Broth lysate (BL) corresponds to a lysate without any process after the incubation (raw extract containing phages, bulk biomass after phage lysis and cell debris) (Fig. 1).

Phage quantification for ATPS phases analysis and also for stability analysis were performed using spot tests with the *Salmonella enteritidis* strain s25pp [26]. For this, 100 µL of bacteria in the exponential growth phase was added to 3 mL Lysogeny Broth soft agar and then poured onto Lysogeny Broth plates for a homogenous Salmonella growth. Plates were dried for 30 min at room temperature to form the overlay. Serial dilutions of the phage suspension in SM buffer were prepared and 5 µL of each dilution were placed as a drop in a single spot onto the bacteria overlay afterwards. Subsequently they were allowed to dry for 15–20 min and incubated for 24 h at 37 °C. All quantifications were performed in duplicate. The PFU/mL was calculated by considering the dilution factor and the sample volume of the drop/spot with visible separated lysis plaques. Phases from systems loaded with phages and biomass were centrifuged at 1500 rpm for 10 min at 25 °C and the supernatants were used for phage quantification, this was performed in order to remove cells that could interfere with the measurements.

2.4. Quantification and bacterial culture conditions

For the experiments, a weekly subculture of *S. enteritidis* s25pp, from a vial stored at −80 °C, was grown for 24 h, 37 °C in plates with Lysogeny Broth agar and stored at 4 °C. This subculture was used for the production of the lysates as explained before.

The concentration of *S. enteritidis*, for samples that were analyzed with bacterial cells present, was determined by inoculating Lysogeny Broth agar plates with 10 µL of 10-fold serial dilutions of the sample in