CHARACTERIZATION OF BACTERIOPHAGES SPECIFIC FOR Pseudomonas fluorescens (NCTC 10038): POTENTIAL BIOCONTROL TOOL

A.A. Machalela¹; L.S. Batalha²; M.T.P. Gontijo²; L.S. Ramos²; A.A.S.B. Freita²; M.E.S. Lopez²; R.C.S. Mendonça²

2-Departamento de Tecnologia de Alimentos – Universidade Federal de Viçosa – CEP: 36570-000 – Viçosa – MG – Brasil, Telephone: (+55-31) 3899-3801 – Fax: (+55-31) 3899-2227 – e-mail: (dramicrocta@hotmail.com).

ABSTRACT: Spoilage bacteria such as P. fluorescens are for the dairy industry a global problem and bacteriophages would be considered an alternative to control them. In this study 3 bacteriophages for P. fluorescens were isolated and they were tested against some conditions found in the food industry and antimicrobials products. Kinetic parameters growth for these bacteriophages was also determined. UFH-HD, UFV-HQ and UFV-SG bacteriophages can reach concentrations around 10⁹ PFU∙mL⁻¹ and each bacterial cell infected releases approximately 200 bacteriophage s per cycle. These bacteriophages were susceptible to UV light, LTLT pasteurization, alcohol 70%, alcohol gel, peracetic acid, acid descaling stainless and CIP alkaline detergent, but were resistant to several conditions such as sanitizers, HTST pasteurization, salts and acids, showing potential for use in the food industry.

KEYWORDS: bacteriophages, spoilage, P. fluorescens, milk, biocontrol.

1. INTRODUCTION

The milk and dairy products are one of the most produced and consumed foods around the globe. The estimate for world milk and milk products production in 2015 are 800689 thousand tonnes (FAO, 2015). Raw milk is rich in water and, in smaller quantities, carbohydrates, proteins, lipids and some vitamins and minerals. Thus, the nutritional quality, its high content of moisture and nearly neutral pH makes this food susceptible to physicochemical alterations and spoilage due to microorganism growth (Corrêa et al. 2011).

The cold storage of milk is a regular practice in the food industry. Although this practice is very useful to prevent the proliferation of most microbes, psychrotrophic bacteria can multiply under refrigeration temperature. The main genus present is Pseudomonas, being P. fluorescens the most important strain that causes deterioration in milk. Despite the use of pasteurization, which is efficient to prevent pathogens, Pseudomonas bacteria remain viable after the most common time/temperature binomial pasteurization (Jay, 2005).

An alternative for raw milk conservation is the use of specific bacteriophages combined with low or high temperature to prevent or minimize these contamination problems. Lytic bacteriophages are ubiquitous entities with a limited lytic spectrum able to reduce bacteria in several environments (Hungaro et al, 2014). Therefore, the phages are used as a tool to prevent the proliferation of bacteria and to insure longer shelf life of foods. This work aimed to isolate and characterize bacteriophages specific for P. fluorescens with potential to use in the dairy industry.
2. METHODOLOGY

2.1. Isolation, Propagation and Titration

Lytic bacteriophages were isolated from residual water of a small dairy plant in the Universidade Federal de Viçosa – Brazil. The sample was centrifuged at $13000 \times g$ at 4 ºC for 10 min and the supernatant was collected and added in Tryptic Soy Broth (TSB) inoculated with *P. fluorescens* (Wall et al., 2010). The suspension was incubated in a shaker at 130 rpm at 30 ºC for 24 hours. The suspension was purified and the presence of the bacteriophage was confirmed using the micro-drops technique according to Hungaro et al., 2013. Propagation and titration process was performed according to Sambrook and Russel (2001).

2.2. Lytic Spectrum

The host range of bacteriophages was determined against *P. aeruginosa* (ATCC 25619), *E. coli* O157:H7 (CDC EDL – 933), *Salmonella* Enteritidis (ATCC 13076), S. Tiph (ATCC 14028), *Listeria monocytogenes* (ATCC 15313), *Staphylococcus aureus* (ATCC 25904), *Acetobacter acetii* (ATCC 15973), *Leuconostoc mesenteroides* (ATCC 10830), *Lactobacillus sp.*, *Pediococcus acidilactic* (CRG – IZ 009), *Gluconobacter oxydans* (ATCC 23774) and *P. fluorescens* (NCTC 10038) using the micro-drops technique as described above.

2.3. Molecular and Morphological Characterization

A suspension of bacteriophage (750 μL) was treated with 3.75 μL of RNAse for 10 min. After that, 37.5 μL of Proteinase K and 1.35 μL of SDS was added to the suspension. DNA extraction was made according to Sambrook and Russell (2001) with phenol-chloroform (1:1) and chloroform (1:1).

The restriction assay was performed using the endonucleases *Hind III*, *Ecor I*, *Xba*I, *Alu I*, *Hae III*, *Hinf I* and *Xho I*. The digestion was performed at 37 ºC for 4 hours and the reaction was eluted in agarose gel at 90V for 1 hour. The fragments of DNA were visualized under UV light transiluminator and scanned using Quantum ST4-1000/26mX software.

The bacteriophages were stained with uranyl acetate 5% and observed in Microscopy Transmission Electron (MET) in Núcleo de Microscopia e Microanálise – Universidade Federal de Viçosa.

2.4. Growth Kinetic Parameters

The efficiency of phage adsorption in the host was determined using *P. fluorescens* cell culture in exponential growth phase (OD: 0.22). The cell culture and each bacteriophage was standardized to MOI = 0.01. 100 μL of the phage suspension and 100 μL of the bacteria were added in 800 μL of TSB at 30 ºC. The number of free bacteriophages was determined by titration as above described. The aliquot was collected at an interval of 1 minute and centrifuged at 10000 × g for 5 min and posterior filtration through membrane (0.22 μm). The data were plotted in a graph of natural logarithm of the number of bacteriophages versus time. Growth parameters were determined by the change in the total number of free phages in different growth time. The study was performed based on the adapted methodology of Pajunen et al. (2000) with agitation to 100 rpm at 25 ºC and MOI = 0.01. Free bacteriophages were determined at 0, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23 and 25 min. Each time, aliquots of 1 mL were filtrated in a membrane (0.22 μm), plated and incubated at 30 ºC for 18 hours and titrated.
2.5. Stability of Phages to Light, Sanitizers, Acids, Salts and Thermal Process

For testing the bacteriophages stability to light (15 and 30 days) the methodology adapted of Iriarte et al (2011) was used. The phages (10⁹ PFU/mL) were exposed to UV light (KII – 108 SCT T8.30 W.GER) at a 64 cm distance and separately to fluorescent light (AH: TUB – T10-40 w) at 1.37 m of distance. The stability of phages against acetic acid 1% and the lactic acid 0.3% during 24, 48 and 72 hours of exposure in several concentrations are shown in Table 1. The viability in alcohol 70%, alcohol gel, per acetic acid, acid descaling stainless and CIP alkaline detergent was tested as well. Resistance of the bacteriophages under salt was evaluated using adapted methodology of Mylon et al (2011). They were tested against sodium citrate (500 mg L⁻¹), sodium chloride (500 mg L⁻¹) and calcium chloride (450 mg L⁻¹) after 24, 48 and 72 hours of exposure. The stability of the bacteriophages was tested also under different temperatures according to Tey et al (2009) adapted methodology. Phages suspension was incubated at -20, 10 and 24 ºC for 30 days. The resistance during LTLT and HTST pasteurization process was also tested.

3. RESULTS AND DISCUSSION

Bacteriophages with specificity to P. fluorescens could be found in different places in the dairy industry. In fact, some bacteriophages are a big problem in this industry, especially those that affect the bacterial fermentative processes (Marcó et al., 2012). The bacteriophages were isolated after cleaning and sanitization processes of places and equipment of dairy industry. Thus, these bacteriophages showed strength to environmental stresses and also suggest that phages can be isolated of nutrient-poor environments (Nilsson, 2014). The bacteriophages were called UFV-HD, UFV-HQ and UFV-SG. After purification and propagation the bacteriophages reached concentrations approximately of 10¹⁰ PFU·mL⁻¹.

A total of 12 bacterial strains were tested to determine the host range of these bacteriophages, showing specificity only for P. fluorescens. Lytic activity over other bacterial strains tested including lactic bacteria (LAB) was not observed. In this way the use of these bacteriophages represents an advantage in the dairy industry if P. fluorescens control is necessary. Since, the great concern by the dairy industry is the possibility of bacteriophages infecting LAB, representing a yield and quality loss of ferment products (Marcó et al., 2012).

Some typical conditions evaluated in this research and that are usual in the food industry did not affect the bacteriophages survival as shown in Table 1 and 2. The bacteriophages in the current study were sensitive to UV light. UV light can affect genetic material of virus and generate damages such as pyrimidine dimers (Ikehata and Ono, 2011) avoiding their replication.

### Table 1 – Viability loss rate of phages by exposure to typical conditions observed in the food industry.

<table>
<thead>
<tr>
<th>Factors</th>
<th>HD</th>
<th>HQ</th>
<th>SG</th>
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<tbody>
<tr>
<td>UV light (Log PFU·min⁻¹)</td>
<td>0.36</td>
<td>0.37</td>
<td>0.33</td>
</tr>
<tr>
<td>Fluorescent light (Log PFU·day⁻¹)</td>
<td>0.02</td>
<td>0.006</td>
<td>0.013</td>
</tr>
<tr>
<td>Lactic Acid 0.3% (Log PFU·h⁻¹)</td>
<td>0.00</td>
<td>0.002</td>
<td>0.002</td>
</tr>
<tr>
<td>Acetic Acid 1.0% (Log PFU·h⁻¹)</td>
<td>0.021</td>
<td>0.00</td>
<td>0.021</td>
</tr>
<tr>
<td>Sodium dichloroisocyanurate (200 mg · L⁻¹) (Log PFU·min⁻¹)</td>
<td>0.02</td>
<td>0.00</td>
<td>0.006</td>
</tr>
<tr>
<td>Hydrogen peroxide (60 mg · L⁻¹) (Log PFU·min⁻¹)</td>
<td>0.00</td>
<td>0.00</td>
<td>0.006</td>
</tr>
<tr>
<td>Sodium hypochlorite (0.25%) (Log PFU·min⁻¹)</td>
<td>0.006</td>
<td>0.00</td>
<td>0.006</td>
</tr>
<tr>
<td>Sodium citrate (500 mg · L⁻¹) (Log PFU·h⁻¹)</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
</tr>
<tr>
<td>Sodium chloride (500 mg · L⁻¹) (Log PFU·h⁻¹)</td>
<td>0.002</td>
<td>0.002</td>
<td>0.004</td>
</tr>
<tr>
<td>Calcium chloride (450 mg · L⁻¹) (Log PFU·h⁻¹)</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
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</table>

These bacteriophages have not shown significantly sensitive to fluorescent light, which is good for usage in the food industry, which is widely illuminated and excessive light does not affect the phage viability. In the other hand, these viruses were resistant to lactic and acetic acid, sanitizers and salts (Table 1) suggesting that viral proteins are stable in these conditions and their viability is not influenced for these factors. Bacteriophages lost viability in presence of alcohol 70%, alcohol gel, peracetic acid, acid descaling stainless and CIP alkaline detergent.

In relation to temperature, bacteriophages for *P. fluorescens* showed sensibility (Table 2). Bacteriophages were susceptible to (-20, 10 and 24 °C) when stored for 30 days. Considering the pasteurization process, bacteriophages lost the viability in LTLT process and reduced around to 2.6 Log PFU·mL⁻¹ when the process was HTST.

The viral genome usually is packaged into a protein capsid or glycoproteins (Gelderblom, 1996) and proteins can be affected by temperature, acids and other parameters. Denaturation of capsid proteins or glycoproteins can be related and would explain the loss of viability of bacteriophages after exposure to stress conditions as described above.

<table>
<thead>
<tr>
<th>Table 2 – Bacteriophage viability after exposure to several Temperature/Time</th>
<th>HD</th>
<th>HQ</th>
<th>SG</th>
</tr>
</thead>
<tbody>
<tr>
<td>-20 °C storage for 30 days (Log PFU·mL⁻¹)</td>
<td>4.32 ± 0.2</td>
<td>4.04 ± 0.3</td>
<td>4.38 ± 0.2</td>
</tr>
<tr>
<td>10 °C storage for 30 days (Log PFU·mL⁻¹)</td>
<td>1.02 ± 0.1</td>
<td>0.04 ± 0.1</td>
<td>0.28 ± 0.3</td>
</tr>
<tr>
<td>24°C storage for 30 days (Log PFU·mL⁻¹)</td>
<td>1.02 ± 0.3</td>
<td>0.04 ± 0.3</td>
<td>0.25 ± 0.1</td>
</tr>
<tr>
<td>Pasteurization (62°C/ 30 min)</td>
<td>Non-viable</td>
<td>Non-viable</td>
<td>Non-viable</td>
</tr>
<tr>
<td>Pasteurization (72°C/ 15 s)</td>
<td>3.12 ± 0.2</td>
<td>2.24 ± 0.3</td>
<td>2.48 ± 0.3</td>
</tr>
</tbody>
</table>

Food industry should be a place with high quantity of factors that can affect the viability of bacteriophages, resulting in decreasing of their activity over *P. fluorescens*. However, these bacteriophages can be considered highly stable and can be used to the biocontrol of *P. fluorescens* under the various conditions used in food processing. The similar results in bacteriophages characterization, lytic spectrum and the fact that these bacteriophages were isolated in the industry (different equipment and places) suggest that UFV-HD, UFV-HQ and UFV-SG can be the same species of bacteriophage.

### 3.2. Bacteriophages belong to Caudovirales Orden

The DNA of bacteriophages was sensitive to *Alu I* and *Hae III* as shown in Figure 1. The restriction profile does not show differences among bacteriophages for *P. fluorescens*, suggesting that the enzymes used recognized similar sites in the genome of UFV-HD, UFV-HQ and UFV-SG bacteriophages.

*Figure 1 – Morphological and Molecular Characterization of bacteriophages (a) UFV-SG (b) UFV-HD (c) UFV-HQ (d) Restriction fragment length polymorphism (RFLP).*
The Table 3 shows size characteristics of bacteriophages aim of this study. In general, bacteriophages in this study presented icosahedral heads and contractile tails in accordance to microscopy electron transmission. These parameters suggest that all bacteriophages belong to Caudovirales order and that exist differences among them. UFV-SG and UFV-HD bacteriophages can be classified into Podoviridae family and UFV-HQ into Myoviridae family. Despite the morphological differences, the similarity in the profile restriction and the host range, sequencing is necessary to final conclusions about taxonomy classification.

Table 3 – Length of bacteriophages structures UFV-SG, UFV-HD and UFV-HQ

<table>
<thead>
<tr>
<th>Structures</th>
<th>HD</th>
<th>HQ</th>
<th>SG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Head (nm)</td>
<td>68.2 by 54.5</td>
<td>66.7 by 51.8</td>
<td>81.8 by 72.7</td>
</tr>
<tr>
<td>Tail (nm)</td>
<td>59 by 18.1</td>
<td>88.8 by 7.4</td>
<td>54.5 by 18.1</td>
</tr>
</tbody>
</table>

3.3. *P. fluorescens* Phages: Adsorption and Growth Characteristics

Figure 2A shows the adsorption dynamic of bacteriophages UFV-HD, UFV-HQ and UFV-SG every minute in contact with *P. fluorescens*. These bacteriophages present a rapid adsorption during the first 2 minutes. After this time more than 90% of the bacteriophages were adsorbed and the adsorption time decreased and the final number of free bacteriophages was less than 10%

Bacteriophages follow an exponential growth. After 20 minutes each infected bacteria can release about 212 for UFV-HD; 245 for UFV-HQ and 240 for UFV-SG viral particles. The fast action of these phages can be an advantage for usage against *P. fluorescens* as biocontrol in the food industry.

Figure 2 – Adsorption and kinetic parameters of bacteriophages. A) Adsorption rate: 90% of free bacteriophages are adsorbed in the first 2 minutes; B) Latency of these bacteriophages was around to 7 minutes. The lytic cycle of phages was about 20 minutes.

Latent period is a time that regulates phage generation time and the burst size (Abedon et al., 2001). The latent period (Figure 2B) was faster in relation to other bacteriophages that have typical latent periods around 21-120 minutes (Lu and Breidt, 2015). Abedon et al, (2001) related that the bigger burst sizes are associated with higher latencies and vice versa.

The generation time for *P. fluorescens* strains is 52 min at 30°C (Ludewig et al., 2009) and bacteriophages specific for these bacterial cells must have a lower replication time to avoid the division cell before the end of the lytic cycle. Kinetics parameters, burst size and adsorption rate are influenced by the host physiology (Abedon et al., 2001). For this reason, viral cycles must happen in
short time when compared with bacterial generation time, in order to guarantee the efficiency in the reduction of the microbial population. The efficiency in the biocontrol is the most important reason to know growth parameters of bacteriophages with potential for usage in the food industry.

4. CONCLUSION

In this study it was possible to isolate phages with potential for biocontrol of P. fluorescens from the dairy industry. UFV-HD, UFV-HQ and UFV-SG belong to Caudovirales order and reached about $10^{10}$ PFU·mL$^{-1}$. These bacteriophages showed a short time of adsorption and considerable burst size indicating high replication rate. Lactic bacteria were not infected by these bacteriophages, and their use may avoid losses in relation to fermented dairy products if applied in biocontrol to spoilage Pseudomonas. The viability to HTST pasteurization and resistance to various factors present in the dairy industry showed that these bacteriophages can be used as a potential tool to increase shelf life to pasteurized milk and other products in dairy industry.

5. REFERENCES