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Lysis activity of bacteriophages isolated from raw meat and their potential as biocontrol agents against *Escherichia coli*

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Abstract
The potential application of bacteriophages as biological agent in controlling pathogenic bacteria for improving food safety appears to gain interest and recognition quite recently. This study was to evaluate the lysis activity of bacteriophages isolated from raw meat. The lysis activity evaluated using disc diffusion method and *in vitro* challenge test in LB broth showed that the isolated bacteriophages have the ability to control the growth of *Escherichia coli* ATCC 25922, thus have potential as biological control agent for future use in food industry. The isolated bacteriophages have the ability to lyse *E. coli* ATCC 25922 cells culture in LB broth at 37 °C and 30 °C during 4 h of incubation. This finding is supported by the turbidity result which also indicates that the bacteriophages suppressed the growth of *E. coli* cells in LB broth. On the other hand, no lysis activity could be observed at 4 °C. However, more precise studies need to be done on all isolates to confirm on their potential as biological control of *E. coli* in food industry in the near future.

Keywords: bacteriophage, *Escherichia coli*, biological control, lysis activity

Introduction

Pathogenic *Escherichia coli* strains cause several types of human diarrhoea and their infection is a major cause of public health problems in developing countries (Ke Xin and Kwai Lin 2008). The ability of *E. coli* to survive well at a very long period of time outside their original place have make them a very useful indicator organism to evaluate the safety and quality in food industry (Samuel et al. 2011).

Bacteriophages or known as phages are bacterial viruses that can infect and propagate within bacteria and caused lysis to the bacterial cells. Their mechanisms are very specific towards target bacteria or referred to as host cell. Once the phage attacks the bacteria, its tail get attached to the host cells and serves as conduit for the phage genome insertion into the bacterial cells (Pieter and Rob 2010). The bacteriophage will inject its viral DNA into the host cell and kill it. Usually, phages are named in reference to their bacterial host.

The phage infection is studied using a layer of soft agar which allows the phage to diffuse rapidly. Upon bacterial cell lysis, the progeny phage particles are released and can diffuse and then infect neighbouring cells. This process will continue, and finally result in a development of circular area of cell lysis in a turbid lawn of cells, called a plaque (Nidham et al. 2007).

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Despite the long history of using bacteriophages in therapeutic and diagnostic applications for humans and animals, their potential applications in food production and processing is relatively a recent trend. One area that is gaining interest is their potential application as biological agent in controlling pathogenic bacteria for improving the food safety at various points in the food processing chain. Many researches have focused on using bacteriophages from various sources for food application including bacteriophages originated from sewage and faeces. Some studies have also been conducted to evaluate the prevalence of bacteriophages in foods and later to be applied in food industry. Even though no particular study and credential finding could show that bacteriophages isolated from other sources are not suitable for application in food industry but the public perception regarding their sources are of great concerns.

The US Food and Drug Administration (USFDA) has approved the phage preparation called LISTEX^{TM} P100 in 2007. The product is to be used against Listeria and can be applied in all food products. The USFDA has extended the use of LISTEX^{TM} to all products and has proclaimed GRAS (Generally Recognised as Safe) status for the use against Listeria in cheese. The same product had also been confirmed of its organic status under the European Union (EU) Law and can be used in the EU countries, in regular and organic products (EBI Food Safety 2007).

This study was conducted to evaluate the ability and potential of bacteriophages isolated from raw meat to kill or control the growth of *E. coli* by causing lysis to the cells.

Materials and methods

Escherichia coli strain from American Type Culture Collection (ATCC), the *E. coli* ATCC 25922 was used in this study as a representative of *E. coli* strain.

The bacteriophages were obtained from raw meat which are frequently found to be contaminated with *E. coli*. A 10 g of food sample was stomached with 90 ml peptone water in a sterile stomacher bag. A 50 ml sub-sample of the suspension was mixed with 2 ml of overnight *E. coli* ATCC 25922 culture as host culture. This mixture was incubated in an incubator shaker at 37 °C, 100 rpm for 4 h. Then, it was centrifuged at 10,000 rpm for 10 min. The supernatant was transferred to the sterile conical flask and filtered sterile to remove the unnecessary particles including bacterial cells.

Phage titration was done by combining a volume of 1 ml of filtered supernatant and 1 ml of *E. coli* host in 3 ml of soft Luria Bertani (LB) agar. This mixture was then poured onto hard LB agar plate. The plates were allowed to cool and incubated at 37 °C. The clear zone formation was recorded after 24 – 48 h. This clear zone area containing the phages is known as plaques.

Later, the above LB plates with confluent lysis (agar plates where the borders of the plaques touching each other) were flooded with 4.0 ml of sterile PBS and left at 4 °C overnight, to allow the bacteriophages to diffuse into buffer. Phages were harvested by pipetting the buffer off the plates. The buffer was centrifuged at 12,000 rpm, 4 °C for 10 min using centrifuge (Beckman Coulter) to precipitate the bacteria. The supernatant was added with 1.8 ml of polyethylene glycol and sodium chloride. The mixture was kept on ice for 30 min.

Second centrifugation was carried out at 12,000 rpm and the supernatant was discarded and the phages which were found as a glassy pellet were re-suspended in 0.5 ml buffer. The phages will later be used during evaluation of lysis activity.

The overnight culture of *E. coli* ATCC 25922 was mixed with soft LB agar and quickly poured onto LB agar in plates and allowed to solidify. The sterile disc of approximately 5 mm in diameter was then gently placed onto the surface of solidified LB agar. The isolated phages was applied onto the disc and incubated at 37 °C. The

clear zone formation was recorded (in mm) after 24 – 48 h of incubation. Later, the bacteriophages that created the highest inhibition zone were selected for evaluation of the lysis activity against *E. coli* ATCC 25922 in LB broth (*in vitro* challenge test).

The medium (LB broth) in four different flasks was inoculated with an overnight culture of *E. coli* ATCC 25922 respectively and incubated in incubator shaker at 100 rpm, 37 °C for 1 h. Then, bacteriophages were added to the culture as follows: (a) Two flasks were added with two different single phages, respectively (b) One flask was added with combination of two bacteriophages, and (c) One flask was added with bacteriophages cocktail (combination of five different phages). Another 2 flasks were reserved as positive control (*E. coli* culture without addition of phages) and negative control (only phage was added).

This challenge test was performed at three different temperatures: 37, 30 and 4 °C. The bacterial count was taken every hour for 4 h and plated in triplicate to get its viable cell count number. The cell count was recorded as cfu/ml. Approximately 3 ml of the culture was also taken every hour for 4 h for turbidity observation using spectrophotometer and the result was recorded as OD value.

Results and discussion

Ability of the bacteriophages to lyse E. coli ATCC 25922 cells and create inhibition zone on LB agar

This study can be considered as an initial stage to evaluate the ability of the isolated bacteriophages as a potential agent to fight and to lyse the control host, *E. coli* ATCC 25922. The presence of bacteriophages was indicated by a clear plaque formation on the LB agar. Once a bacterial host cell (*E. coli*) is infected with phage, the cell will be lysed, releasing the phage progeny which can diffuse to neighbouring cells and infect them as well, lysing them, and the process will continue. Ultimately, this process will result in a circular area of cell lysis called plaque.

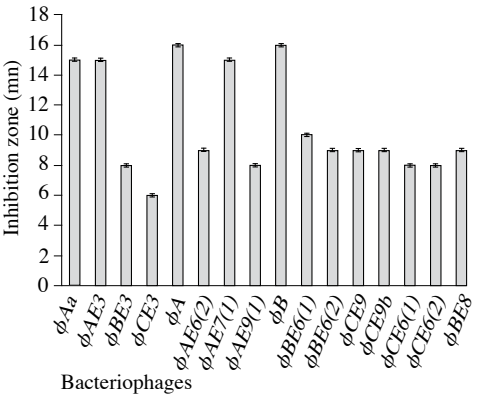

Figure 1. Inhibition zone (mm) formed by lysis activity of the isolated bacteriophages against control host E. coli ATCC 25922 on LB agar

Figure 1 shows that all 16 isolated bacteriophages are able to lyse the host bacteria and inhibited the cells by creating clear zone ranging from 6 mm to 16 mm. Five isolates [ΦA, ΦB, ΦAa, ΦAE7(1) and ΦAE3] that created the highest inhibition zone were selected for evaluation of the lysis activity against *E. coli* ATCC 25922 in LB broth (*in vitro* challenge test).

In vitro challenge test and turbidity evaluation of the E. coli ATCC 25922

This study was conducted using five isolates [ΦA, ΦB, ΦAa, ΦAE7(1) and ΦAE3] that have created the highest inhibition zone using disc diffusion method. This study investigated the ability of bacteriophages (either alone or in combination) to lyse the bacterial cells *in vitro* using LB broth at three different temperatures which were at 4, 30 and 37 °C. The change in turbidity of the broth also evaluated and measured as optical densities reading (OD).

The bacteriophages are able to lyse and control the growth of *E. coli* cells (*Figure 2*). The *in vitro* challenge test results indicated that the culture added with bacteriophages cocktail showed better result in controlling the growth of *E. coli* cells compared to the single or combination of two bacteriophages. At 37 °C, the reduction of cell growth was up to 3 log phase for

the addition of phage cocktail while only 2 log reduction (single phage ΦA) and 1 log reduction for single phage ΦB and combination of phage ΦA and ΦB. This observation was witnessed after 4 h of incubation. As for 30 °C, approximately the same pattern was observed after 4 h of incubation and the usage of bacteriophages cocktail showed better result in eliminating the *E. coli* cells if compared to the usage of single or combination of two phages. This result is in line with a few studies including McLaughlin (2007). They revealed that in most situations, using a single species of bacteriophages is insufficient to eliminate the growth of entire bacterial population in broth due to the development of bacteriophage-insensitive mutants (BIMs).

However, at 4 °C, no reduction in cell number of the *E. coli* culture can be observed after 4 h of incubation (*Figure 2*). The same pattern was also observed by O'Flynn et al. (2004) as he conducted the similar challenge test using bacteriophages. They reported the reduction in bacterial cell number at 37 °C and 30 °C, but the bacterial cells were not reduced by the addition of isolated bacteriophages either using alone or in combination at chilled temperature. O'Flynn et al. (2004) concluded that it is

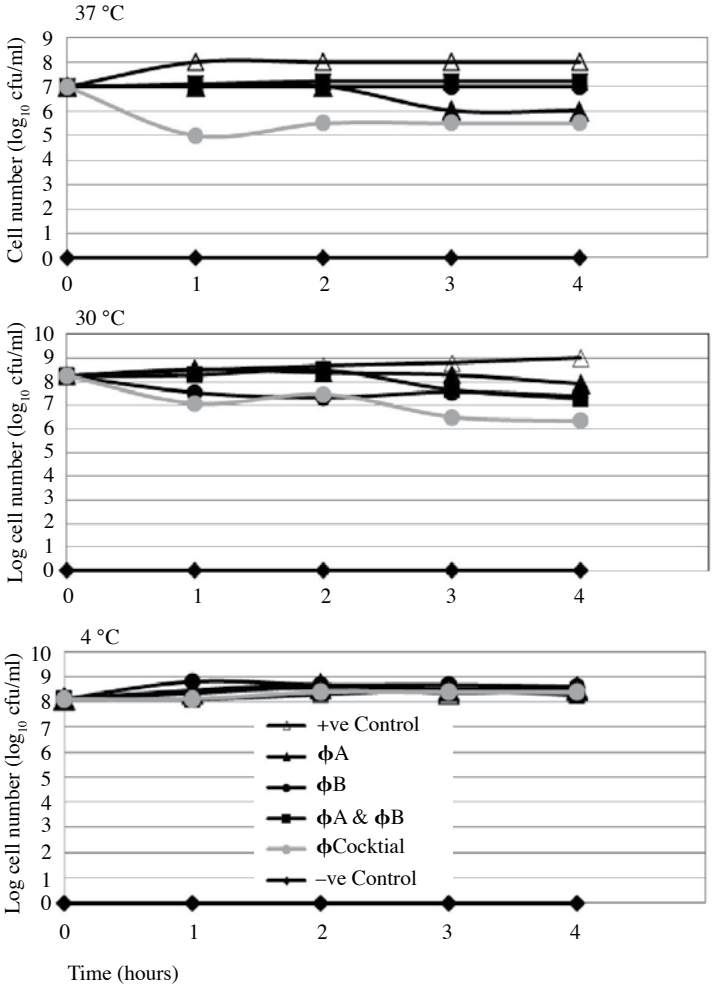

Figure 2. Lysis activity of bacteriophage against control host E. coli ATCC 25922 at 37, 30 and 4 °C in vitro

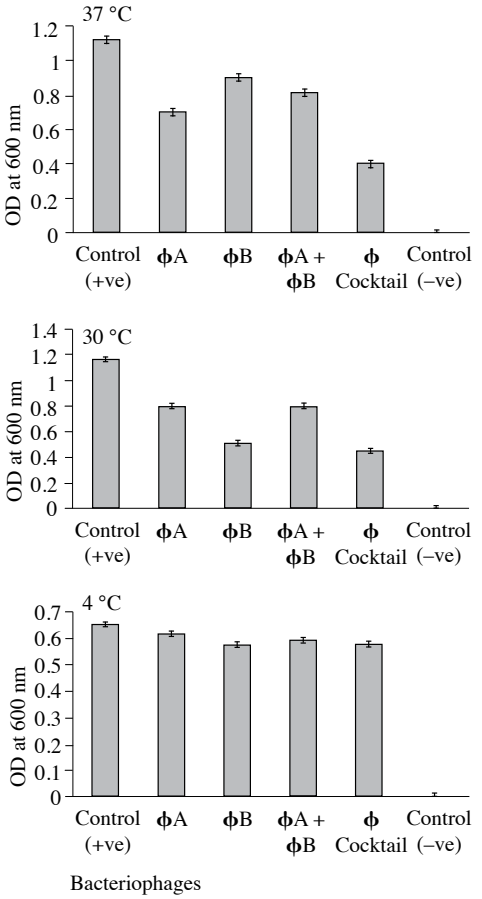

Figure 3. Turbidity of control host E. coli ATCC 25922 following the infection by various bacteriophages in Luria Bertani broth at 37, 30 and 4 °C

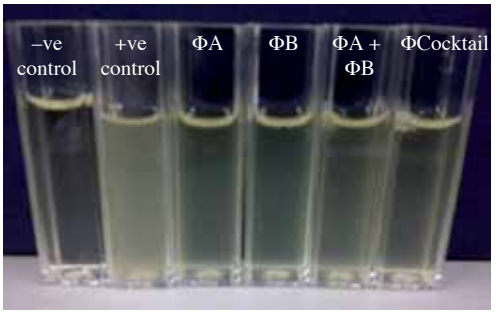

Plate 1. Visual observation of the turbidity of the broth with different treatments

due to the inability of bacterial cells to grow at low temperature, thus no lysis activity by the bacteriophages.

The turbidity of the broth was also evaluated and the results (*Figure 3*) showed that the OD results were lower for all bacteriophages added culture than the positive control (*E. coli* culture in LB broth not added with any bacteriophages). The negative control (bacteriophages in broth without *E. coli* culture) remained clear as the blank. As the bacterial cell grew in the broth, the turbidity will increase and make the broth cloudy, thus giving a very high OD result, approximately 1.11 and 1.16 for *E. coli* (positive control) that grew at 37 °C and 30 °C respectively. However, for culture treated by bacteriophages, the turbidity of the broth would remain clear or lower as some of the bacterial cells have been lysed by the bacteriophages. McLaughlin and Brooks (2008) discovered that the OD of the bacteriophage treated culture was greater than the control and concluded that this was due to the assimilation of nutrients from killed bacterial cells. *Plate 1* shows the visual observation of the turbidity of the broth with different treatments.

Conclusion

Bacteriophages isolated from raw meat are able to lyse *E. coli* ATCC 25922 cells culture in LB broth at 37 °C and 30 °C during the *in vitro* challenge test. However, no lysis activity can be observed at 4 °C. The turbidity results were also in line with the *in vitro* finding, indicating that the bacteriophages suppressed the growth of *E. coli* cells in broth. These results have shown promising potential of the bacteriophage isolates as biological control against *E. coli*.

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Abstrak

Potensi dan kegunaan bakteriofaj dalam mengawal pertumbuhan bakteria patogen untuk keselamatan makanan agak baru disedari dan semakin mendapat perhatian. Kajian ini dijalankan untuk memencilkan bakteriofaj daripada daging mentah. Kebolehan bakteriofaj untuk memecahkan (lisis) sel bakteria dilaksanakan dengan menggunakan kaedah sebaran cakera dan juga kajian *in vitro* menggunakan kaldu LB. Kajian menunjukkan bakteriofaj mempunyai kebolehan mengawal pertumbuhan *E. coli* ATCC 25922, seterusnya berpotensi sebagai agen pengawal biologi untuk kegunaan masa hadapan dalam industri makanan. Bakteriofaj berkenaan mempunyai kebolehan untuk memecahkan sel bakteria *E. coli* ATCC 25922 di dalam kaldu LB pada suhu 37 °C dan 30 °C pada 4 jam inkubasi. Keputusan ujian kekeruhan juga menunjukkan bakteriofaj berkenaan dapat menekan pertumbuhan *E. coli* dalam kaldu LB. Namun begitu, aktiviti pemecahan sel ini tidak dapat dikesan pada kajian yang dijalankan pada suhu 4 °C. Kajian terperinci masih perlu dijalankan untuk memastikan potensi bakteriofaj ini untuk kegunaan industri makanan pada masa depan.