Plant Archives Volume 20 No. 1, 2020 pp. 359-362 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

BACTERIOPHAGE AS BIO-PRESERVING TO ENHANCE SHELF LIFE OF FRUIT AND VEGETABLES

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Abstract

Due to the growing need to keep crops fresh and undamaged, vendors need to find ways to fight corruption. One of the most important, least expensive and easiest to use methods is the phage, which eats bacteria that cause crop corruption. A group of fruits and vegetables were selected and the bacteria were isolated, sensitivity tests were carried out for most bacterial isolates, then five types of bacteria isolated from fruits and vegetables were identified for bacteriophages. Result show that the microbial load on the tomato has the highest reaching $11.4*10^5$ cfu/ml, followed by grape which was carrying 9.5*cfu/ml, then mushroom with 7.6*cfu/ml, both green pepper and lettuce were loads with 5.8*cfu/ml and 5.9*cfu/ml respectively. Finally spinach with 4.4*cfu/ml and apple with 3.5*cfu/ml. Revealing successful use of phage to prevent bacterial growth that causes spoilage of important crops may lead to higher economic income and control of the vegetable market.

Key words: Bacteriophage, Bio- preservation, Shelf life, Microbial resistance, Vegetables.

Introduction

The process of distasteful change of vegetables and fruits from its normal condition that make it unaccepcted by the consumer is known as vegetable spoilage; this economic issue was an obstacle in facing food insecure for many countries in the world, vegetable spoilage has negative effect for both. The farmers who cultivate crops besides paying high attention for them and for consumers of those crops who pay money for it. Vegetables and fruits loss reaches more than 40% globally. (Imedpub, 2018) After harvesting crops, oxygen boosts the environment of microbial growth especially aerobic bacteria which is the first responsible for the corruption of vegetables and fruits. (Imedpub, 2018) a bacteriophage is considered as bacterial eater for its efficiency to infect only bacteria; for very long time phage was used as therapy to treat bacterial infections as its specificity is higher than antibiotics with no side effects and no harm for human, animals and plant. These unique properties of bacteriophage attracted attention to use it in many fields to control microbial growth. Harper *et al*., (2014) phage just like other viruses require a host to express their genetic material and replication, for that phage will use one of its distinct life cycles to destroy bacteria either lytic or lysogenic cycle; lytic cycle includes expression of phage gene using the machinery of the bacterial cell to form protein coat around the multiplied viral genome forming a complete phage, lead bacterial cell to lyse (le.ac.uk, 2018) whereas in the lysogenic cycle the viral DNA will be attached to bacterial DNA, both DNA will be integrated this type of viral DNA is known as prophage and the bacteria will be called lysogenized, in prophage state, the viral DNA will be symbiotic with the bacterial DNA (Hamzah and Hasso, 2019). The phage will stay in a dormant state until the host bacteria situation deteriorates; at this point, prophage will be activated reproduction will take place and bacterial cell will be lysed (Courses, 2018) for their high effect on a bacterial cell in their both life cycle lytic and lysogenic; bacteriophage have been chosen to confront food borne pathogens that develop on vegetables crops and fruits to enhance shelf life to save agricultural resources. This technique becomes more popular rather than using materials like organic acids that change vegetables flavor and aroma, it is also an ecologically decent process, having the ability to face all microbial resistance challenge (Singh, 2018) this paper aims to find the best technique for vegetable conservation using bacteriophages as bio-preservative.

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Materials and Methods

Samples collection

A collection of fresh fruits and vegetables were collected from different grocery stores in Erbil, Iraq. The collection consisted of seven types of fruits and vegetables. All the collected crops were unpackaged and exposed to air; three copies of each type of collected crops were taken for laboratory test. The crops that collected for this investigation are from different sources, it may have been exposed to different conditions during transportation and storage; controlling these influences is however this study aim to find the best aim to find the best bio-effect on microbial growth on these crops.

Fruits and vegetables were collected from stores in sterilized plastic sack, then crops have been transferred to lab same day to take bacterial samples from them for culturing using a sterilized cotton swab; swabs were inoculated on numerous differential media which included: MacConkey agar, Blood agar, Manitol salt agar & EMB agar. After incubation isolates were taken from cultural media for morphological and biochemical analysis to identify types of microbial growth on selected fruits and vegetables.

Sensitivity test

Most bacterial isolates were subjected to susceptibility test, the disk-diffusion method of Kirby-Bauer on Mueller-Hinton agar was used to determine the susceptibility of bacteria to groups of antibiotics encompass: penicillin, ampicillin, ciprofloxacin, gentamycin, Cephaloridine, erythromycin; inhibition zone measured according to CLSI standard table (Hsueh *et al*., 2010).

Bacteriophage isolation & Purification

Five types of bacteria isolated from fruits and vegetables were identified for bacteriophages. These bacterial isolates were incubated separately in nutrient broth for 24h. at 37°C; 5ml were taken from these pre-incubated bacteria then mixed with another 5 ml of deca-strength phage broth and 40ml of water sample that must contain the bacteriophage; these tubes incubated in 37°C for 48h. This step is known as enrichment method used to amplify the number of bacteriophage that present in sewage sample. After incubation tubes were centrifuged at 2500rpm. For 10 min. to separate host cell from viruses in order to get a pure solution of bacteriophage, plaque assay was done to determine bacterial lyse that shown as plaques on the plate surface.

Result

Microbial loads that found on fruit and vegetable which were selected for this research have been listed in table 1.

It is observed that the microbial load on the tomato has the highest among the rest of the crops, reaching $11.4*10^5$ cfu/ml, followed by grape which was carrying 9.5*cfu/ml, then mushroom which was carrying with 7.6*cfu/ml, both green pepper and lettuce were approximately equals in their microbial loads with 5.8*cfu/ml and 5.9*cfu/ml respectively. The lowest microbial load were observed on spinach with 4.4 *cfu/ml and apple with 3.5*cfu/ml. The classical method of bacterial identification showed the presence of the following gram-positive bacteria: *Staphylococcus aureus*, *Bacillus* sp., *Lactobacillus* sp., Streptococcus sp. While the Gram-negative bacteria were included: *E.coli*, *Klebsiella* sp., *Enterococcus faecalis*. The results of the sensitivity test for isolated bacteria were listed in table 2.

All the bacterial isolates that exposed to susceptibility were sensitive to penicillin except *Staphylococcus aureus* and *Klebsiella* sp. which were resistance in addition to *Enterococcus faecalis* that showed medium resistance to penicillin. Four bacterial isolates showed a sensitive reaction to ampicillin those isolates include: *Bacillus* sp., *Lactobacillus* sp., *Streptococcus* sp. and *Enterococcus faecalis* while *Staphylococcus aureus* was resistant to ampicillin and both *E. coli* and *Klebsiella* sp. were Medium sensitive to ampicillin. *Bacillus* sp. *Lactobacillus* sp. *Streptococcus* sp. and *Enterococcus faecalis* were resistance to ciprofloxacin while *E.coli*, *Klebsiella* sp. and *S. aureus* bacteria were sensitive to ciprofloxacin. *S. aureus*, *Bacillus* sp., *E.coli* and *Enterococcus faecalis* were sensitive to gentamicin while

Table 1: List of selected crops for study with their scientific names.

Collected crops	Scientific names
1. Tomato	*Solanum lycopersicum*
2. Green pepper	*Capsicum annuum*
3. Spinach	*Spinacia oleracea*
4. Grapes	*Vitis vinifera*
5. Apple	*Malus domestica*
6. Lettuce	*Lactuca sativa*
7. Mushrooms	*Agaricus bisporus*

Table 2: Approximate range of macabre load on selected fruits and vegetable.

Selected crops	Bacterial load (10^5 cfu/ml)
1. Tomato	*Solanum lycopersicum*
2. Green pepper	*Capsicum annuum*
3. Spinach	*Spinacia oleracea*
4. Grapes	*Vitis vinifera*
5. Apple	*Malus domestica*
6. Lettuce	*Lactuca sativa*
7. Mushrooms	*Agaricus bisporus*

Table 3: Antimicrobial susceptibility test for bacterial isolates.

Bacterial species	penicillin	ampicillin	ciprofloxacin	gentamycin	Cephaloridine	erythromycin	Methicillin
S. aureus	R	R	S	S	R	S	S. for 43% R. for 57%
Bacillus sp.	S	S	R	S	R	S	
Lactobacillus sp.	S	S	R	M	R	S	
Streptococcus sp.	S	S	R	M	R	S	
E.coli	S	M	S	S	R	S	
Klebsiella sp.	R	M	S	M	R	M	
Enterococcus faecalis	M	S	R	S	R	S	

Klebsiella sp., *Lactobacillus* sp. and *Streptococcus* sp. showed medium sensitivity to gentamicin. All bacterial isolates were exposed to this test showed sensitive reaction to Cephaloridine. Finally the entire bacterial isolates were sensitive to erythromycin except *Klebsiella* sp. was medium sensitive to erythromycin. Methicillin was used with *Staphylococcus aureus* only to diagnose the presence of MRSA which is known to be pathogenic and resistance to some common antibiotics.

After inoculation between bacteria extracted from crops and phages, the lysis zone took three to five days to form. The zone formed by phage was approximately (1.5-2) mm in diameter, the double agar layer method that detect PFU were used for counting, table 4. The count was found after phage enrichment using double agar layer method.

Discussion

Microbial presence on fruits and vegetables is obvious, despite the little knowledge about the ecosystem of microbes that inhabit raw and fresh fruits and vegetables before and after harvesting. Many pathogenic bacteria prove themselves on the surface of the growing crop, these pathogenic are from various sources such as soil or irrigation water or animals that dwelled fields or from composted or sewage even insects have been recorded as a source of crop contamination in the field (Berger *et al*., 2010). In addition to agricultural practices, the presence of a fungus, which usually begins to exist after harvest, can increase the chance of growth of pathogenic bacteria due to the change in pH caused by the fungus (Beuchat, 2002; Hamzah *et al*., 2019). It is a proven fact that paper crops are more likely to be infected with pathogenic bacteria than other crops harvested and packaged, Leafy vegetables are representing a health hazard and have been linked to many epidemics around the world. This explains the relatively high microbial load found on lettuce and spinach in this study (Hooshang, 2012). Many of the techniques used to inhibit bacterial growth on fruit and vegetable crops are not perfect and this is what drives the search for an alternative bio preservation like phage Because of its long history of safe and successful use as well as its strong effect on bacteria (García *et al*., 2008). Due to its increased marketing as an appropriate solution to the bacterial resistance to antibiotics, farmers and fruit traders can use it as a routine measure to reduce the losses incurred by farmers and consumers due to damage caused by bacteria that endemic to fruit and vegetable surfaces (González-Menéndez *et al*., 2018). The large biological diversity in the high density and stable bacterial communities leads to the support of genetic exchange between them leading to the emergence of resistant strains of pathogenic bacteria (Schwartz *et al*., 2003; Dirwal *et al*., 2019). Because of the limited therapeutic potential of methicillin-resistant *Staphylococcus aureus*, the focus has occurred for this bacteria, where because of beta-lactam ring there are no antibiotics that can overcome these bacteria except vancomycin. The presence of this resistant type of bacteria on fruits and vegetables explains the necessity to use a useful bio-preserving technique to overcome hazardous food-borne pathogens (Hryniewicz, 1999).

Table 4: The count was found after phage enrichment.

Bacteria	Best titer
1. *Staphylococcus aureus*	$2.5 * 10^4$ (pfu/ml)
2. *Bacillus sp.*	$3.2 * 10^4$ (pfu/ml)
3. *Lactobacillus sp.*	$4.4 * 10^3$ (pfu/ml)
4. *Streptococcus sp.*	$2.7 * 10^4$ (pfu/ml)
5. *E.coli*	$3.5 * 10^3$ (pfu/ml)

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