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Isolation of Bacteria from *Psophocarpus Tetragonolobus* and Biofilm Formation

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Abstract: Vegetables are easily spoiled by different microorganisms with bacterial and fungal species during processing. Usually, vegetables are serving as carrier for pathogenic bacteria, parasites and viruses and lead to food borne illness outbreaks. The winged bean (*Psophocarpus tetragonolobus*) species is goes to the genus *Psophocarpus*, a tropical legume plant commonly known as the Goa bean, Manila bean, four-angled bean or four-cornered bean and Mauritius bean. The ability of bacteria to attached on surfaces and form a distinct biofilm can lead to food spoilage. Biofilm formation relies on three main parameters including the bacterial cells, the surface attached, and the surrounded medium but the attachment of bacteria to the surface is the initial process of biofilm. This research conducted to isolate bacteria from the winged bean and to determine biofilm formation. Throughout the study, four different types of bacteria were isolated from winged bean such *E. coli, Listeria, Salmonella,* and *Shigella*. Then, all the isolated bacteria were further analyzed for biofilm formation. As the results, only *E. coli* bacteria show the ability to form a biofilm on winged bean at room temperature. The other bacteria such as *Listeria, Salmonella, and Shigella* have not shown the biofilm formation. This could be due to the environment, temperature or the sample size which not being favourable factors for the formation. However, there is an urgent need to ensure the microbiological safety in food industries, especially in processed foods, like fresh-cut vegetables, in such a way that the lack of heat treatment does not promote the growth of potentially pathogenic microorganisms

Keyword: *Pathogenic bacteria, Psophocarpus tetragonolobus, biofilm, temperature, food industry*

1. Introduction

Vegetables are easily spoiled by different microorganisms after harvest. Usually, vegetables are serving as carrier for pathogenic bacteria, viruses, and parasites and lead to food borne illness outbreaks. The fresh vegetable has latent food-borne pathogens including *Salmonella*. Green leafy vegetables including spinach, lettuce and cabbage were found to dock *Salmonella* [1].

An appropriate handling during harvesting, packaging, transportation and marketing can highly reduce vegetable spoilage [2]. Spoilage bacteria can be defined as microorganisms that are able to cause the worsening of food and affect the quality [3]. Different bacterial species might source spoilage in food products depends on the processing and preservation method. For example, light preservation and atmosphere change may promote the growth of other species as lactic acid bacteria (LAB), Enterobacteriaceae, *Clostridium* spp and *Bacillus* spp. [4].

Winged bean, *P. tetragonolobus* which knows as legume was grown exclusively in Southeast Asia such as Malaysia and Thailand. This vegetable grows as a plant by climbing the stems and leaves, before the seeds are ironic with protein compared to soybean in nutritional value and composition. Winged bean usually consumed raw as salad (locally known as *ulam*).

Attachment of pathogens and other bacteria to food can lead to spoilages. Bacteria depend on its ability to attach to host cells. The adhesion capacity of vegetables and water-

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borne pathogens that could develop biofilms such as *Salmonella* spp., *Pseudomonas fluorescens, Bacillus cereus* and *Aeromonas hydrophila* can decreased product shelf-life and lead to the transmission of diseases [5].

Biofilms can be defined as a different kind of bacterial communities that attached to a surface and surrounded itself, produced extracellular polymeric substances (EPS) matrix. Bacteria can form biofilms on surfaces of food processing that possibly leading to contamination of food product. Biofilms formed by the carbohydrate-protein interaction between surface adhesions of microorganisms and the surface of food [6].

This study designed to isolate bacteria from winged bean and determine the biofilm formation of bacterial diversity in this plant.

2. Materials and Method

2.1 Sample collection

In this study, winged beans were collected and analyzed. The microbiological parameters examined are Total Plate Count (TPC), *Salmonella* spp., *Shigella* spp., *E. coli*, and *Listeria* spp.Winged beans were placed in sterile plastic bags and stored at room temperature for five days. Then it was cut into small pieces and weighed accordingly.

2.2 Bacteria isolation from the winged beans

Total plate count method was applied in isolation of bacteria from the winged beans. The standard technique with the specific dilution was transferred and spread aseptically onto the nutrient agar surface. The plates were incubated at 35°C for 24-48 h and colonies counting before calculated the total aerobic microorganism per gram.

2.2.1 *E. coli*

10 g of sample was homogenized with 90 ml of dilution water using stomacher. The 1.0 ml portion was transferred into 3 x 10 ml double strength LST tubes. Then, the tube was incubated at 35±1ºC for 48 hrs. Confirmation test were carried out for all presumptive positive gassing tubes. The LST tubes were gently shake and transferred a loop for each suspension to EC broth tubes. The EC tube was incubated at 45.5±0.2ºC for 48 hrs. A loop of suspension from all positive tube were streaked on L-EMB agar and incubated for 18-24 hours at 35±1ºC. The plates were examined for *E. coli* colonies.

2.2.2 *Salmonella* spp.

25 g of sample was suspended in 225 ml sterile peptone water (SPW) and blended for 2 min using stomacher. The sample mixture was incubated for 24 h at 35 ºC for pre-enrichment. Then, for the enrichment of *Salmonella* spp., 0.1 ml of sample mixture was moved to 10 ml Rappaport-Vassiliadis (RV) medium and incubated for 24 h at 35 ºC.

Enrichment broths were used to streak on Hektoen Enteric (HE) agar and were incubated at 35 ºC for 24 h then observed for presence typical *Salmonella* colonies.

2.2.3 *Shigella* spp.

25 g of sample was suspended with 225 ml of *Shigella* broth medium + Novobiocin (0.5 μg ml^{-1}) in stomacher bag. The sample was homogenized for 1 min and kept at 41.5°C for 24 h in an anaerobic condition. Different selective plating media were used for *Shigella* spp. isolation including XLD agar as intermediate selectivity medium and a *Salmonella-Shigella* agar (SSA) as a highly selective media. Then, *Shigella* broth was sub-cultured on XLD agar and SS agar before incubated at 37°C for 18–24 h [7].

2.2.4 *Listeria* spp.

25 g of the vegetable sample was inoculated into 225 ml of Buffered *Listeria* Enrichment Broth (BLEB), with supplement (Acriflavin, Nalidixic acid, and Cycloheximide) and blended for 1 minute in a stomacher at high speed then incubated at 37°C for 48 h. After incubation the mixture was streaked on PALCAM agar and further incubated for 24-48 h at 35°C.

2.3 Biofilm formation

Bacteria that isolated from Winged Bean were grown in TSB medium for 24 h at 37 °C. For the screening of surface-associated biofilm, 10 μL of bacterial suspension were diluted with 90 μL TSB and added to sterile 96-well polystyrene plates wells. TSB wells were used as a negative control for each screening. The plates then incubated at 37 °C for 24 h. Flowing the incubation time, the

wells in the microtiter plate were inverted upside down to discard the medium and were washed with distilled water for three times (200 μL/well). Wells were allowed for air drying, and were stained with 50 μL of 0.5% crystal violet (solution in water) for 10 min. 200 μL of distilled water was used in removing the excess stain by rinsing each well. The crystal violet and biofilm cells was decolorized with 50 μL of 99% ethanol, the absorbance of all plates were obtained using microplate reader with wavelength at 595 nm. Result for average optical density (OD) was calculated for triplicate including the negative controls [8].

2.3.1 Biofilm calculation

It is recommended that biofilm formation results are stated as averaged numbers as the OD values were calculated for all tested strains including negative controls in triplicate for three times [9]. The results were presented as shown in Table 1.

Table 1 Classification of biofilm formation

Biofilm formation			
Strong	Moderate	Weak	No
>4 ODc	<4 ODc	<2 ODc	OD≤ODc

3. Result and Discussion

3.1 Interpretation of the results

All the results were tabulated in table 1 and 2. Different types of bacteria were isolated from winged beans such as *Listeria* spp., *E. coli*, *Salmonella* spp. and *Shigella* spp. and Table 1 shows the OD readings of the bacteria after isolation using microtiter reader while Table 2 shows the biofilm formation of different types of bacteria at 595 wavelengths. From Table 2, it was determined that only *E. coli* was able to form biofilm at room temperature. The rest of the bacteria do not show the possibility of forming a biofilm. The results were calculated based on the OD readings that obtained through using microtiter reader. The blanks readings known as ODc where ODc = average OD of the negative control + (3x standard deviation of negative control). The final OD value of the tested bacteria was expressed as OD=average OD of the bacteria – ODc. An only *E.coli* bacterium forms the biofilm where the OD reading was 0.112. TPC readings also fall under category weak biofilm producer where the OD reading was 0.199.

Table 2 Optical density by microtitter reading

Sample	**1st**	**2nd**	**3rd**	**mean**
Blank (control)	0.053	0.059	0.062	0.058
***Salmonella* spp.**	0.103	0.102	0.102	0.103
E. coli	0.134	0.234	0.156	0.135
TPC	0.299	0.248	0.240	0.274
***Listeria* spp.**	0.092	0.078	0.089	0.086
***Shigella* spp., (XLD agar)**	0.083	0.131	0.147	0.120
***Shigella* spp., (SS agar)**	0.107	0.078	0.131	0.105

Table 3 Biofilm formation by bacteria

Sample	**Mean**	**SD**	**ODc**	**Biofilm formation**
Blank (control)	0.058	0.005	0.063	-
***Salmonella* spp.**	0.102	-	-	0.039
E.Coli	0.175	-	-	0.112
TPC	0.262	-	-	0.199
***Listeria* spp.**	0.086	-	-	0.023
***Shigella* spp. (XLD agar)**	0.120	-	-	0.057
***Shigella* spp. (SS agar)**	0.105	-	-	0.042

3.2 Microbiological quality results

3.2.1 Total Plate Count

TPC in food is one of the microbiological sign in food quality. These reveal the sample exposed to any contamination and in overall is the presence of good conditions for the growth of microorganisms. This parameter was used to indicate if the industrial process of cleaning, disinfection, temperature control, transportation and storage have been performed sufficiently. From the observation, total plate count that obtained was 2.3×10^{-8} cfu/g. The mixed vegetable salad showed the highest percentage; this may be attributed to the processing, handling, cutting, and cross contamination.

3.2.2 *E. coli*

Escherichia coli, a gram-negative bacteria showing good growth with dark blue-black colonies as the acid acts upon the dyes with metallic green sheen in EMB agar which indicate vital fermentation of lactose and acid

production showed precipitation of green metallic pigment [10]. There was definite growth of *E. coli* observed in the EMB agar. The total fecal coliform that observed was more than 1.6 $10^{-}4$. This means the winged beans were contaminated with *E. coli*.

E. coli able to grow well at 37ºC and this lead they found in the guts of mammalians. It is also more tolerant of low pH than of high pH which is approximately pH 7.0 and can grow in any low to medium concentration of salt. The operations as cutting, shredding, dicing or peeling create surfaces upon which enteric pathogens can more easily attach.

3.2.3 *Salmonella* spp.

Positive growth was observed in *salmonella* where the blue-green to blue colonies appear on HE agar and pink colonies on XLD agar. *Salmonella* can form a biofilm on fresh produce and long storage time (one week at 4°C and 25°C). In addition, the same journal stated that the capability of *Salmonella* to produce a biofilm on plastic cutting board also and this eventually will transfer the bacteria to the vegetables also.

WHO (2002) recorded that the effect of microbiological threats on food safety for *Salmonella* is a worldwide major public health concern. This is due to the contamination of this bacterium that rise from contaminated water during washing vegetables or the vegetables were handled by the carrier or infected workers, contaminated irrigated water with sewage and organic manures widely used by farmers in fertilization vegetables. *Salmonella* can invade plant tissues through the cut surface of leaves [11].

3.2.4 *Shigella* spp.

Shigella positively growth observed on both XLD and *Salmonella-shigella* agar, *Shigella* on SS agar produced colonies with black centers and a clear halo while *Shigella* on XLD agar will be in red colonies, black centers. The possible factors of the contamination can be due to the pathogen range that might occur *via* fingers, flies or the utensils used such as cutting surfaces. In addition, the bacterial contamination of vegetables occurs due to consumed vegetables without any treatment. Contamination of vegetables with different pathogens can occur directly or indirectly *via* processing equipment, transportation, and human handling. The usage of good food hygiene may significantly decrease the hazard of contamination *via* infected food handlers [12].

3.2.5 *Listeria* spp.

L. monocytogenes is widely diffused in the environment and caused the contaminating of vegetables during growing, harvesting, post-harvesting, handling or distribution. In this experiment, the result showed positive growth of *Listeria*, 1-2 mm diameters black-grey colonies with the black halo were observed on PALCAM agar. The medium is a differential diagnostic medium utilizing two indicator systems, as aesculin and ferric citrate and mannitol and phenol red. *Listeria monocytogenes* have the ability of growth and multiplication in packed vegetables that stored in the modified atmosphere. There could be a high chance of transmission by cross-contamination during processing fresh cut produce. From the survey that conducted in Florida shows that, from 63 salads, only one was contaminated by *Listeria monocytogenes* and about 4.8% were analysed from ready-to-eat salads. Furthermore, another estimation of the spread of *L. monocytogenes* shows that in 512 packages of fresh cut vegetables checked, 3.1% was a positive result.

3.3 Biofilm formation by different types of bacteria

3.3.1 *E. coli*

Biofilm of *E. coli* was determined using the microtiter plate assay. From the observation, *E. coli* was able to form biofilms, but it is falls under weak biofilm producer classification where the OD reading was recorded as 0.112. The adhesion ability of *E. coli* was depended on the flagellar motility that initiate the attachment of *E. coli* to the surface of the food and forming biofilm. The chemotaxis was unnecessary and motility was crucial for *E. coli* to initiate the biofilm formation. Thus flagella have an important role in initial adhesion of *E. coli* [6].

3.3.2 *Salmonella* spp.

Salmonella did not have any effect on biofilm formation throughout this study. The OD reading of *Salmonella* was less than reading of the ODc. These bacteria are reported to be capable in adhere and form biofilm on metal, rubber or glass surfaces [13].

Salmonella also has curli fimbriae but it usually associated with the cellulose. The biofilm produced by *Salmonella* depends on the environment. A study has mention, biofilm of *Salmonella* produces more when in low nutrient condition compare to high nutrient condition [14]. *Salmonella* can form varied types of biofilms in response to environmental conditions [15]. For instant, the biofilm developed at room temperature in a rich medium that induce the formation of fimbriae and cellulose, can be represented as a pellicle at the air-liquid interface that contain a strong bacterial network [16].

3.3.3 *Shigella* spp.

Shigella did not show the ability of biofilm formation in this study. It is categorized as non-producer. *Shigella* infections have been found in fruits and vegetables, and several large outbreaks were associated with contaminated raw vegetables. The conventional culture methods that used for isolation were not so efficient. *Shigella* was reported to survive on foods in sufficient amount to developed illness but lacking for laboratory methods. For the better detection, rapid methods have been developed. The polymerase chain reaction (PCR) was effectively used to detect *Shigella* for the sequences of nucleotide that encode the invasion plasmid antigen H was amplified and utilized for screening of *Shigella* from food and clinical samples [11].

3.3.4 *Listeria* spp.

The growth of *Listeria* was observed in the PALCAM agar, but no biofilm was observed in a microtiter plate. The OD reading was 0.023 where it is less than ODc and fall under category non-producer biofilm. *Listeria* is a ubiquitous organism and is found on raw fruits and vegetables. The biofilms represented about 80% from total population of bacterial on plant surfaces. *Listeria* biofilms play a critical role in protection against antimicrobials and sanitizers, leading to a persistent contamination of food processing area. Many environmental factors, like temperature, pH, osmolarity, static and dynamic growth conditions, and the nature of the colonized surfaces can affect the level of biofilm formation. In food processing conditions, biotic factors can affect biofilm formation by *L. monocytogenes* as well [17].

4. Conclusion

In conclusion, all the four bacteria naturally have the ability to form biofilm. Yet in this study, only *E.coli* bacteria show the ability to form biofilm on winged bean at room temperature. The other bacteria; *Listeria* spp., *Salmonella* spp., *and Shigella* spp., have not shown the biofilm formation. This could be due to the environment, pH, temperature, rheological and adhesive properties of biofilms, or the sample size. However, there is an urgent need to ensure the microbiological safety in food industries, especially in processed foods, like fresh-cut vegetables, in such a way that the lack of heat treatment does not promote the growth of potentially pathogenic microorganisms.

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