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FULL PAPER

Assessment of *Pseudomonas aeruginosa* biofilm-forming capacities from drinking water in water vending machine

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Abstract

The establishment of *P. aeruginosa* with biofilm formation in water vending machines may cause serious health issues to the consumers and its emergence has led to the public's concern. This study aimed to assess the quality of water vending machines and to evaluate the biological contaminant *P. aeruginosa* in biofilm capacities. The qualities of the drinking water from a total of fifteen (n = 15) water vending machines at Kota Samarahan were evaluated based on physical and chemical parameters including pH value, turbidity, total of carbon (TOC), total dissolved solid (TDS) and total suspended solid (TSS). The colonies *Enterobacteriaceae* has been morphology characterized through biochemical tests and *P. aeruginosa* bacteria was identify through the PCR method. The results of the physical and chemical parameters complies with the authority standard including turbidity values found in conformance with values were lower than 0.1 NTU. Morphological analysis with a total of 66.7% (n = 10) was detected with the presence of *Enterobacteriaceae*, and a total of 40% (n = 6) of the isolates were found to be *P. aeruginosa*. This study extended by assessing the potential strength of biofilm formation. The microtiter assay performed in a 96-well polystyrene microtiter plate showed that 83.33% (n = 5) of the bacterial isolates have moderate potential as biofilm producers, while only 16.67% (n = 1) isolates were non-adherent and showed no potential in producing biofilm. The highest OD isolates found occupying moderate biofilm strength was (mean = 0.217) and the lowest moderate biofilm strength was (mean = 0.136). In conclusion, the significance and impact of the study displayed the qualities of water vending machines complies with Food Act 1983, Regulation 360C and Malaysian Drinking Water Quality, Ministry of Health 1983. However, the presence of biological contaminants may raise consumer concerns. This study had successfully assessed the potential strength of *P. aeruginosa* biofilm collected from water vending machines. Further microbiological assessments should be perform continuously to predict and eliminate any future risks related to water vending machines.

1. Introduction

In this modern era, there has been an increase in the availability of water vending machines in populated urban areas. This trending demand is due to several reasons such as public infrastructure, modern working and lifestyle changes (Schillinger and Du-Vall Knorr, 2015). A water vending machine is an electronic machine that consists of a filtration system that dispenses filtered drinking water when inserted with the appropriate amount of money. Due to its convenience and affordable price, consumers gradually gravitate towards using this machine as a source of clean drinking water for household use as opposed to boiling tap water which has an undesirable nickel-like taste to it and the belief that drinking tap water possesses a health risk because of its uncleanliness.

Pseudomonas aeruginosa is an aerobic Gram-negative bacterium and motile, non-spore-forming rods that are oxidase-positive and lactose non-fermenters. They cause urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, bacteremia, bone and joint infections, gastrointestinal infections and

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a variety of systemic infections. It is also a bacterial species, which is particularly good at forming biofilms. The formation of biofilm in drinking water can cause problems with colour, taste, odour and turbidity if found in high numbers. Once established, biofilms can be difficult to be eradicated from manmade water systems because chemicals are required to break down the biofilm formation or physical removal is necessary prior to disinfection. *Pseudomonas aeruginosa* is very difficult to get rid of once established in a vending machine, because of the inaccessibility of many of the components (Hall and Mah, 2017). In recent studies, *P. aeruginosa* has been found to have an adaptive ability to develop high resistance against broad-spectrum antibiotics through the formation of biofilm (Drenkard and Ausubel, 2002; Owlia *et al.*, 2014). In addition, findings by Gu *et al.* (2019) reported that cells present in the biofilm are more susceptible to ampicillin as compared to cells within clusters or a larger colony. These findings provide more insights into the physiology of biofilm susceptibility patterns.

There are a few cases involving water-vending machines that have been reported. On 19 August 2014, The Star Online reported a case in which almost all drinking water samples taken around the Klang Valley area were contaminated with harmful microbes (Aruna and Camoens, 2014). Another case reported by The Star Online on 2 September 2015 mentioned that 786 water samples have been collected nationwide with a total number of 22 water vending machines confiscated, where 4 of which had low levels of hygiene and 10 were positive for the presence of microbes (Bernama, 2015). The incidents resulted from poor maintenance and hygiene conditions of the water vending machines. New Strait Times has reported in a media statement on June 12, 2019, in regards to Singapore Food Agency (SFA) the bacterium *P. aeruginosa* was detected in Malaysia's bottled drinking water during a routine sampling of the product.

The formation of biofilm in water vending machines poses a significant health risk to the consumers (Chaidez *et al.,* 1999). Bacterial biofilm is formed when free-swimming planktonic cells change into a multi-complex bacterial community that is enfolded by extracellular polymeric substances adhered to a solid substance (Olsen, 2015). According to Olsen (2015), health risk caused by biofilm is mainly attributed to its potential strength, especially biofilm that is formed by pathogenic bacteria, which causes it to be extremely difficult to remove once it causes biofilm-related infections. In addition to its strength, biofilm can prevail in harsh conditions due to the existence of inter microbial species that exhibits complex community and cooperation directing to emergent properties that allows it to survive in unfavourable environments (Wilson *et al*., 2017).

This study focused on the safety and security of drinking water around Kota Samarahan, Sarawak as this location is categorized as a district with a high-density population, especially among students who are one of the main consumers of the water vending machine. The objectives of this study are to assess the quality of drinking water from the water vending machine and to validate the finding with the sanitary standard of water vending machines. This study will contribute to a more efficient approach in compliance with the Malaysian Food Act 1983, Malaysia Drinking Water Quality Standard 2010 and National Water Quality Standards (NWQS).

2. Materials and methods

2.1 Sampling of drinking water from water vending machine

A total of fifteen (n = 15) samples were collected at every water vending machine at different locations around Kota Samarahan. The samples were collected for three months (September-November 2019), twice each month, respectively, to evaluate the consistency of maintenance of the water vending machine (Douterelo *et al.*, 2017). The samples were collected using a sterilized sampling bottle, then preserved in nitric acid (Merk, 65 wt. % in D_2O) solution with ratio 1:1 and rinsed with Mili-Q ultra-pure water (Merk) for 48 hrs. The samples were placed in an icebox with a temperature of 4-6°C. After 2 days, the samples were filtered through membrane filters (0.45 µm pore size) to trap and concentrate the organisms. To avoid any changes in the samples collected, all the samples are processed in the laboratory within 5 days from the day of collection (Hashim and Yusop, 2016).

2.2 Identification of Enterobacteriaceae using selective media

A total volume of 100 µL of each drinking water sample was cultured in violet red bile glucose agar (VRBGA, (Sigma-Aldrich) and incubated for 24 hrs. The colour of the colonies and the number of colonies present on the VRBGA were observed to determine the presence of *Enterobacteriaceae.* The gram staining method was carried out for further morphological identification. A loop-full of the sample was taken and smeared on a clean slide and crystal violet was poured on it. The sample was allowed to stain for about 30 s to 1 min before it was rinsed with water. Then, the sample was stained with Gram's iodine (Sigma-Aldrich) for 1 min and rinsed with water. Next, 95% alcohol or acetone was used to wash the sample for about 10-20 s before it was washed with

water. Lastly, the sample was flooded with safranin (Sigma-Aldrich) for about 1 min and washed with water.

2.3 Water quality analysis using physical and chemical parameters

The drinking water samples were evaluated based on physical and chemical parameters, which are pH, turbidity, total dissolved solid (TDS), total suspended solid (TSS) and total organic carbon (TOC). The pH of the water samples was measured by using a calibrated pH meter. The turbidity of the drinking water sample was examined using a spectrophotometer. Meanwhile, the total dissolved solid meter was used to measure the TDS. The drinking water samples and tap water were filled in two different glass bottles for the readings to be compared. The TDS of the water sample should be in the range of 10-30% of the tap water reading. Lastly, the TSS was measured by filtering and weighing the water samples.

2.4 DNA extraction of Pseudomonas aeruginosa

Genomic deoxyribonucleic acid (DNA) was extracted using the boil cell method. The cultures from the purified colony were enriched in 5 mL of LB broth at 37°C for 18 to 24 hrs and centrifuged at 10,000 rpm for 5 mins. The supernatant was then removed and 500 µL of the sterile distilled water was used to re-suspend the pellets. It was then boiled for 10 mins and immediately incubated in ice for 5 mins. The mixture was then centrifuged at 10,000 rpm for 10 mins and the supernatant was then transferred into a new sterile microcentrifuge tube and kept at -20°C.

2.5 PCR amplification of Pseudomonas aeruginosa

Polymerase chain reaction (PCR) master mix was prepared by adding 1.8 µL of DNA boil lysate to the PCR mixture in a total volume of 15 µL reaction mixture. The PCR mixture consisted of 0.6 µM of each universal forward (27F) and reverse primers (1492R), 4.5 µL ddH_2O and 7.5 µL 2× GeneTech PCR master mix. Agarose gel electrophoresis was then conducted on the PCR products on 1% (w/v) agarose gel in 1× Tris/ Borate/EDTA (TBE) buffer for 45 mins at 90 V and observed under ultraviolet light.

2.6 Pseudomonas aeruginosa biofilm in vitro formation

Six positive strains of *P. aeruginosa* and *P. aeruginosa* ATCC® 15442™ were assessed for the *in vitro* biofilm formation by using pre-sterilized, polystyrene flat-bottomed 96-well microtiter plates as described by Elexson *et al.* (2014). The wells of microtiter plates were filled with 100 µL of tryptic soy broth (TSB) prepared with 0.5 McFarland Standard. *Pseudomonas aeruginosa* ATCC® 15442™ (1.0×10^6 CFU/mL) served as positive control and fresh medium was used as a negative control. A total volume of 100 µL of each standard inoculum was pipetted into the selected wells of the microtiter plates. The plates were then covered and sealed with parafilm and incubated at 37°C without agitation for 24 hrs. After incubation, the medium in the plate was discarded, and non-adherent cells were removed by thoroughly washing the biofilm thrice with sterile phosphate-buffered saline (PBS). The plates were inverted and drained by blotting them with paper towels to remove any residual medium. Biofilms were then ready to be assessed for their biofilm-forming capacity.

2.7 Quantification of Pseudomonas aeruginosa biofilm

Biofilm formation was quantified by a crystal violet (CV) assay as described by Djordjevic *et al.* (2002). Briefly, the biofilm-coated wells of microtiter plates as described above were vigorously shaken in order to remove all non-adherent bacteria. The remaining attached bacteria were washed twice with 200 µL of 50 mmol PBS (pH 7) and air-dried for 45 mins. Then, each of the washed wells was stained with 110 µL of 0.4% aqueous crystal violet solution for 45 mins. Afterwards, each well was washed twice with 350 µL of sterile distilled water and immediately de-stained with 200 µL of 95% ethanol. After 45 mins of de-staining, 100 µL of the de-staining solution was transferred to a new well, and the concentration of the crystal violet stain in the de-staining solution was measured in the form of absorbance with the microplate reader (VERSAMAX, Sunnyvale, CA, USA) at optical density (OD) 590 nm.

2.8 Pseudomonas aeruginosa biofilm-forming capacities assessment

The cutoff value (OD_c) was calculated to categorize the degree of strength of biofilm produced ranging from non-adherence, weakest, moderate to strongest biofilm. The OD_c and $OD_{isolates}$ were calculated according to the formula in Table 1 with scale in Table 2 (Kırmusaoğlu, 2019).

Table 1. Formula for OD_c and $OD_{isolates}$ calculation

OD_c = Average OD of negative control + (3 × standard deviation (SD) of negative control)
$OD_{isolates}$ = Average $OD_{isolates}$ – ODc

Table 2. *P. aeruginosa* biofilm-forming capacities.

Result of calculation	Biofilm capacities
$OD \leq OD_C$	No biofilm
$OD_C < OD \leq 2OD$	Weak
$2OD_C < OD \leq 4OD$	Moderate
$4OD_C < OD$	Strong

2.9 Statistical analysis

All measurements were carried out in triplicates and reported as mean ± standard deviation with p-value ($p>0.05$) were considered statistically non-significant on the response variable.

3. Results

3.1 Enterobacteriaceae identification

From the total of fifteen (n = 15) water vending machines, ten (n = 10) were found to be contaminated with *Enterobacteriaceae*. Upon Gram staining, all of the samples showed pink colouration with rod-like morphology. This indicates that the isolates are Gram-negative bacteria with a total of ten *Enterobacteriaceae.*

3.2 Physical and chemical parameters for water quality analysis

The result of water quality analysis using physical and chemical parameters were tabulated in Table 3.

3.3 Molecular identification of Pseudomonas aeruginosa

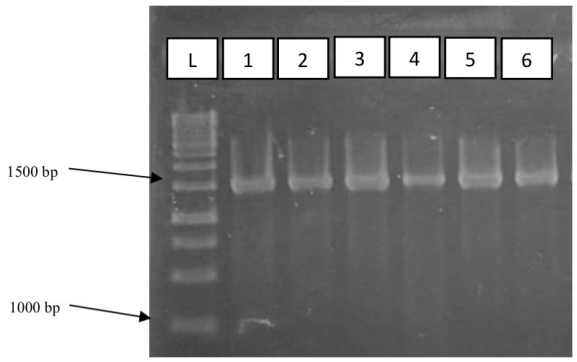

Figure 1. Agarose gel electrophoresis for Lane 1: *P. aeruginosa* ATCC® 15442™ (positive control), Lanes 2- 5: *P. aeruginosa,* Lane *L*: 1000 bp DNA ladder

According to Figure 1, the PCR analysis showed the presence of *P. aeruginosa* isolates from the water samples. DNA amplification of the samples produced PCR products at the size of 1500 bp.

3.4 Biofilm-forming capacities of Pseudomonas aeruginosa

The highest biofilm-forming capacity of *P. aeruginosa* is at the average mean OD_c: ±0.217, while the lowest was at OD_c: ± 0.136. By referring to Table 2, there are no weak or strong positive *P. aeruginosa* biofilm producers were discovered as tabulated in Table 4.

4. Discussion

Based on the growth result on violet red bile glucose agar (VRBGA), *Enterobacteriaceae* can have two types of morphology, which is a pink colony with red bile precipitate and a colourless to grey colony. The morphology of the bacteria was observed using a microscope with 40× magnification and the shape observed was a rod shape (Figure 2). According to Neogen Corporation (2009), bacteria species in the pink colony with red bile precipitate are expected to be *Enterobacter aerogenes, Escherichia coli* or *Salmonella*

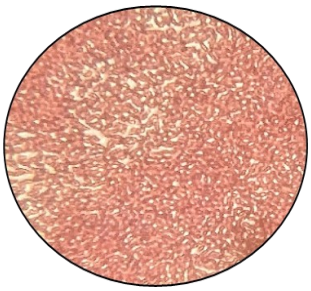

Figure 2. *P. aeruginosa* Observation under 40× magnification

Table 3. Mean of physical and chemical parameters of drinking water from vending machine.

No. of water vending machine	Physical and chemical parameters					Mean (Total average)
	pH	Turbidity (NTU)	TDS (mg/L)	TSS (mg/L)	TOC (mg/L)	
1	6.8±0.3	0.05±0.006	97.1±5.2	-	-	117.83
2	7.3±0.3	0.06±0.005	103.5±8.1	-	-	20.50
3	7.8±0.2	0.06±0.008	91.0±2.9	-	-	154.00
4	6.5 ±0.3	0.060±0.004	85.13±0.5	-	-	61.17
5	6.7±0.3	0.05±0.006	94.8±4.5	-	-	36.83
6	7.2±0.3	0.04±0.008	90.1±2.7	-	-	39.67
7	7.0±0.4	0.04±0.007	88.3±5.3	-	-	47.83
8	6.8±0.4	0.03±0.006	86.0±7.6	-	-	115.00
9	7.0 ±0.3	0.07±0.005	96.0±7.2	-	-	47.50
10	7.2±0.3	0.05±0.007	92.5±3.9	-	-	60.00
11	6.8±0.3	0.06±0.004	85.3±4.3	-	-	75.67
12	7.0±0.4	0.05±0.007	88.0±4.3	-	-	86.67
13	7.2±0.3	0.05±0.003	84.7±7.1	-	-	168.00
14	7.0±0.3	0.07+0.003	94.0±6.2	-	-	69.00
15	6.7±0.3	0.06+0.003	104.0±8.6	-	-	79.33

-, No Reading values

Table 4. Summary of biofilm-forming capacities of *P. aeruginosa.*

P. aeruginosa	OD_c value	Degree of biofilm-forming capacities
P. aeruginosa ATCC® 15442™	3.00±0.200	Moderate ($2OD_C < OD \leq 4OD$)
P. aeruginosa 003	3.00±0.217	Moderate ($2OD_C < OD \leq 4OD$)
P. aeruginosa 006	3.00±0.194	Moderate($2OD_C < OD \leq 4OD$)
P. aeruginosa 002	3.00±0.163	Moderate($2OD_C < OD \leq 4OD$)
P. aeruginosa 001	3.00±0.138	Moderate($2OD_C < OD \leq 4OD$)
P. aeruginosa 004	3.00±0.136	Moderate($2OD_C < OD \leq 4OD$)
P. aeruginosa 005	3.00±0.034	Non adherent ($OD \leq ODC$)

ODc = Cut off value

enterica serovar Typhimurium. While the colourless to grey colony is *P. aeruginosa.*

Out of the total of fifteen (n = 15) drinking water samples from water vending machines, 66.7% (n = 10) were found to contain *Enterobacteriaceae*. There are several medically significant bacterial species belonging to the *Enterobacteriaceae* family which can cause a broad range of infections in humans and animals. Among them is the Enterobacter species which can cause infections in the eyes, skin, blood, gastrointestinal and urinary tract infections (Roger, 2020). Another example is *E. coli* which commonly lives in human and animal intestines. Some strains of *E. coli* are the cause of intestinal infection, bloody diarrhoea, dehydration and kidney failure (Pietrangelo, 2017). *Pseudomonas aeruginosa* is also a type of harmful bacteria that possesses a high risk to human health by causing dermatitis, urinary tract infection, gastrointestinal infection, bacteremia, and others (Mena and Gerba, 2009).

The mean of the results for physical and chemical parameters was tabulated in Table 3. In comparison with the Malaysian Food Regulation, Regulation 360c (Vended Water) and according to Malaysian Food Act 1983 and Malaysian Drinking Water Quality Standard, the recommended standard value of pH for drinking water is ranging from 6.5 to 8.5 (Ministry of Health Malaysia, 2006, Ministry of Health Malaysia, 2010). Based on the tabulated result in Table 3, the highest mean pH of vending machine 3 was slightly higher than the others, which was at 7.8±0.2 but was still in the acceptable range of the standard pH level. All the drinking water samples were found to follow the standard pH required. A low pH value would indicate that the water is contaminated with pollutants and is unsafe to drink. It can also corrode the metal pipes because of its acidic property. While the pH levels are above 8.5, it would cause the water to have a bitter taste and mineral incrustations can occur (Malaysian Drinking Water Quality Standard, 2010).

According to the Malaysian Food Regulation (1983), the maximum acceptable turbidity value for drinking water set by the Malaysian Drinking Water Quality Standard is below 5 NTU, while the Malaysian Food Regulation 1983 set the standard value of turbidity at 0.1 NTU. The turbidity is an indicator of the presence of both suspended matter and other microorganisms. The vending water is expected to show a turbidity concentration less than raw pipe water, which normally has a turbidity of 5 NTU. This is because vending machine water undergoes reverse osmosis (RO) system that is specially designed to efficiently remove particles at a size of 0.1 nm and larger depending on the filter type. The turbidity values of all water samples (as tabulated in Table 1) are in the range of 0.03-0.07 NTU. Based on Malaysian Food Regulation 1983 standards, if the values exceeded the standard values, there might be the presence of microorganisms in the water filtration RO system. Its ineffective filtration might be due to the possible defects in the RO filtration membrane or caused by poor maintenance of the vending machine. The high turbidity indicates that there are a lot of particles suspended in the water, and this can lead to gastrointestinal illness (Tinker *et al*., 2008).

According to the Malaysian Drinking Water Standard, total dissolved solids (TDS) concentrations should be below 1000 mg/L. However, no specific value has been provided for TDS in the Malaysian Food Regulation. In this study, all the water samples had TDS values lower than 1000 mg/L, therefore, met the standard drinking water quality requirement. The total suspended solids (TSS) are defined as particles found in the water column that is larger than 2 µm. Based on Table 1, no suspended solid was determined. TSS is one of the significant factors when observing water clarity. For the total of carbon (TOC) observation, the TOC values are not regulated to any standard values. However, the Malaysian Drinking Water Standard determined the TOC of a drinking water sample can be present at less than 100 g/L and can be higher than 25,000 g/L. The TOC concentrations indicate the presence of organic matter in the water samples. There was no reading for TOC concentrations indicating the presence of organic matter in the water samples was absent.

Vending machines usually have a sticker patch to state the period of time for the maintenance schedule.

Based on the sticker patch on the vending machine, the maintenance is only implemented once a month without consistent monitoring. The inconsistent maintenance could further increase the presence of the bacteria in the drinking water, thus, causing the physical and chemical parameter data to be inconsistent for every sampling. A reduction in the quality of the drinking water will cause infection, and diseases and can cause toxic effects on the consumers (Tinker *et al.,* 2008). Other than maintenance and inspection, consideration must be given to the location where the drinking water vending machine is installed. According to a study by Al Moosa *et al.* (2015), placing a water dispenser in a display area with exposure to direct sunlight was found to be high in total coliform because of the high ambient temperature. The consumers should also be responsible for handling the vending machine. The machine's door should be closed at all times especially after using the machine to prevent any cross-contamination from particulate matter in the air or animals such as stray dogs or cats. The consumers should also refrain from touching the nozzle of the vending machine as this could cause the unwanted transfer of bacteria to the dispensed water.

The presence of *P. aeruginosa* was further carried out in this investigation and as a result, 40% (n = 4) of the isolated bacteria were identified as *P. aeruginosa*. Their presence in the drinking water is quite alarming due to its opportunistic characteristic which can pose significant health risks to immunocompromised individuals and this bacterium is also known to cause acute infections in healthy individuals (Chaidez *et al.*, 1999).

Table 4 shows the isolates of *P. aeruginosa* were the non-adherent and moderate biofilm producers. Although the biofilm present was low as shown in Figure 3, the risk for it to progress is alarming which may cause further waterborne diseases outbreaks in future. The strength of this biofilm can potentially change and be affected by abiotic factors such as pH, temperature, and water flow rate, while its composition can be influenced by the existing biofilm community that is already present within the surrounding pipes (Douterelo *et al.,* 2017). The optical density measurement used for the quantification of the biofilm assay provides high accuracy measurement for the total number of live, dead, and non-culturable cells, as well as the cell sizes (Larimer *et al.,* 2016). In addition, crystal violet dye that was used to visualize and identify *P. aeruginosa* in the microplate titer served as an indirect method for quantification. This is because the amount of Crystal Violets dye absorbed is proportional to the number of bacterial cells in the biofilm (Wilson *et al*., 2017).

The quantification using the microtiter assay was selected as the procedure to enumerate the characteristics of the biofilm produced by *P. aeruginosa* because it is quick, general and inexpensive. In addition, the microtiter assay and UV/Vis spectrophotometer are able to provide information regarding the strength of the biofilm (Douterelo *et al.,* 2017). However, more advanced assessment methods can be used to study it in a more detailed characteristic and its population size. Approaches such as electron microscopy imaging (EMI) and fluorescence microscopy (FM) with imaging of DNA, RNA, Encapsulated PostScript and epifluorescence have been widely used to study biofilm (Liu *et al*., 2016). More advance, reproducible, and specific instruments can provide better information in predicting the biofilm potential capacity in drinking water machines (Chan *et al.* 2019).

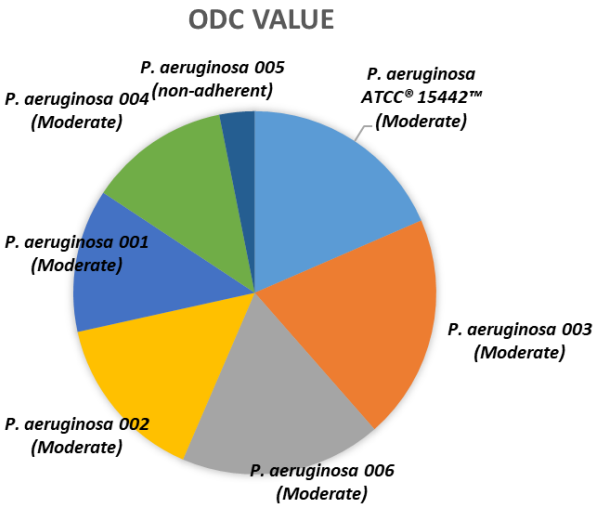

Figure 3. Summary of biofilm-forming capacities of *P. aeruginosa*.

5. Conclusion

In conclusion, this study has successfully assessed the potential strength of *P. aeruginosa* biofilm collected from water vending machines. The emergence of biological contamination in the form of biofilm from *P. aeruginosa* is alarming, and this condition may trigger a waterborne outbreak in the future. Although the physical and chemical parameters were found to comply with the legal requirement standards set, the findings of this study suggest that proper routine maintenance should be administered from time to time on the water vending machines to ensure safety, hygiene, and consumer satisfaction meet appropriate health standards.

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