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Developing of graphene oxide (GO) bio-filter for pathogenic bacterial control in farmed Asian clam, *Corbicula fluminea*

Y Akrimah¹, F A Mohd Zin², M N An'amt^{2,4}, S W Lee¹ and E R Aweng³

¹Faculty of Agro-based Industry, Universiti Malaysia Kelantan, Locked bag No. 100, 17600 Jeli Kelantan, Malaysia.

²Faculty of Bio-engineering and Technology, Universiti Malaysia Kelantan, Locked bag No. 100, 17600 Jeli Kelantan, Malaysia.

³Faculty of Earth Science, Universiti Malaysia Kelantan, Locked bag No. 100, 17600 Jeli Kelantan, Malaysia.

anamt@umk.edu.my

Abstract: Graphene oxide (GO) possess a potential as an antimicrobial agent in the aquaculture sector to substitute the role of antibiotic. Meanwhile, the consumption of Asian clam *Corbicula fluminea* has been gaining popularity throughout Malaysia in a line with the increasing cases of food poisoning caused by pathogenic bacteria resulted from the clam consumption. Thus, this experiment was conducted to investigate the potential of graphene oxide (GO) as an antibacterial filter in controlling pathogenic bacteria i.e. *Escherichia coli*, *Salmonella* spp., *Vibrio* spp. and coliform bacteria. A total of 180 clams were reared in both systems i.e. system installed with graphene oxide (GO system) and system with normal filter (Control system) for 2 weeks. Then, the bacteria were screened and enumerated from Asian clam tissue and farm water samples using total plate count (TPC) method and most probable number (MPN) method by comparing these two rearing systems. The results showed all isolated bacteria were 100 % reduced from farm water while coliform bacteria with 94% reduction in GO system. Conversely, bacteria reduction in clam tissue sample in GO system was varied and relatively low for coliform bacteria, *Salmonella* spp., total bacteria, *Vibrio* spp. as it reduced by 60.71 %, 31.38 %, 18.16 % and 1.34 %, respectively, while *E. coli* showed increasing bacteria count by 12.52 %. Therefore, it can be concluded that GO system is effective in reducing pathogenic bacteria in water compared to Asian clam tissue sample.

Keyword: Graphene oxide, antibacterial filter, Asian clam, *Corbicula fluminea*



1. Introduction

Graphene oxide (GO) is another derivative of graphene under oxide-functionalization. The involvement of graphene oxide in water purification is a great idea since it demonstrated effective removal of pollutants at any concentrations [2] by acting as separation and sorbent material due to their high porosity, large surface area and high hydrophobicity [8]. The wrinkled surface of graphene can disrupt bacterial viability directly by trapping bacteria within the GO surface thus damaging their cell membrane [17]. Furthermore, researchers have studied the potential of GO in the aquaculture field where it showed positive results when applied as an antibacterial agent [15], immunostimulator [14] and immunosensor [20].

Asian clam or *Corbicula fluminea* is a famous traditional snack among Kelantanese but now consumed by people throughout Malaysia. However, food poisoning cases are common with the consumption of this clam that shown by the symptoms like diarrhoea, fever, nausea, abdominal cramping, dehydration, headache, and vomiting [12]. Polluted environment especially water and the clams' natural behaviour that tend to filter all the micro-particles including pathogenic bacteria such as *E. coli*, *Salmonella* spp., *Vibrio* spp. and coliform bacteria [13] are the main reason of this issue. Therefore, by combining the water purification and antibacterial properties of GO, this study aims to evaluate the effectiveness of GO in reducing pathogenic bacteria concentration in Asian clam and water in the system by comparing the system fixed with graphene oxide (GO system) and the system with normal filter system (Control system).

2. Methodology

2.1 Preparation of graphene oxide

The oxidation of graphite was started by mixing sulphuric acid, phosphoric acid and potassium permanganate (Merck, USA) before subjected to continuous stirring for 3 consecutive days. The changing colour of the mixture from dark purplish hue to dark brown indicated oxidation process. Then, hydrogen peroxide (Merck, USA) was added to stop the oxidation process and the colour of the mixture immediately changed to bright yellow signifying graphite oxide production. Later, the graphite oxide was washed thrice with hydrochloric acid (Merck, USA) aqueous solution and deionized water until pH of 4-5 is achieved resulted to the thickening of graphene solution and formation of GO solution [9]. The absorption feature of the rGO-Ag nanocomposite was characterized using EvolutionTM 600, a UV-visible spectrophotometer within the range of 200 - 800 nm. XRD pattern of the nanocomposite was recorded on a D5000 Siemen X-ray diffractometer in a 2θ range of $5 - 80^\circ$ with monochromatic Cu K α source ($\lambda=1.5406 \text{ \AA}$). Next, cotton wools were dipped in GO solution on both sides before being dried and dipped in GO and distilled water. Then, the beaker was heated in an oven. Lastly, the cotton was rinsed with distilled water thrice and dried at room temperature to complete the process.

2.2 Testing graphene oxide as an antibacterial filter

Asian clams *Corbicula fluminea* (n=180) with shell length ranged from 11 mm to 17 mm was collected from Rantau Panjang river, Kelantan. The clams were placed in two aquariums. One aquarium was installed with GO filter while another aquarium was installed with normal filter. The clams were reared in both systems for 2 weeks. During that, water parameter i.e. dissolved oxygen, temperature, pH and salinity were also measured with multiparameter (YSI, USA) and recorded as 7.06 mg/L, 27.0 °C, 7.88 and 0.03, respectively. At the end of the testing period, the clams and water samples were collected from both systems. The water was directly proceeded with bacteriological analysis while for the clams, their flesh was taken out from the shell and homogenised in a sterilized container [4].

2.3 Bacteriological analysis

In order to enumerate *E. coli*, *Salmonella* spp., *Vibrio* spp. and total bacteria, TPC method was applied. Three selective agars i.e. Xylose Lysine Deoxycholate agar (XLD), Eosine Methylene Blue (EMB) agar, Thiosulphate-Citrate-Bile Salt-Sucrose (TCBS) agar (Oxoid, England) and a universal agar Trypticase soy agar (Himedia, India) were used in this study. After the incubation period was over, the formed bacteria colonies were counted and confirmed by using BBL crystal kit (BBL, USA). Colonies count below than 25 was categorized as TFTC that stand for too few to count [18]. The colonies formed were expressed as colony forming units (CFU/ml) by using the following formula [7]:

$$\frac{\text{Number of CFU}}{\text{ml}} = \frac{\text{Number of colonies counted} \times \text{Dilution factor}}{\text{Volume of sample taken}} \quad (1)$$

Meanwhile, MPN method was used in the enumeration of coliform bacteria. The number of test tube that showed positive result was counted and the bacteria concentration was found out by referring to MPN index table [13]. The percentage of the effect of GO antibacterial filter on bacterial reduction was calculated as follows:

$$I\% = (N1 - N2) / N1 \times 100\% \quad (2)$$

where N1 is the number of surviving bacterial colonies from Control system and N2 is the number of surviving bacterial colonies from GO system [21]. The bacteria concentration was converted to log function before calculating the percentage of bacteria reduction

3. Results

The UV-visible absorption spectra of GO are shown in Figure 1a. As revealed by the spectrum, two characteristic peaks of GO were observed at 227 and 299 nm, which were assigned to the $\pi \rightarrow \pi^*$ transition of aromatic C-C bond and the $n \rightarrow \pi^*$ transition of C=O group, respectively while Figure 1b displays the XRD patterns of GO. It was seen that the characteristic peak of GO centered at 10.0°, which was assigned to the (0 0 1) reflection of GO [5] and the morphology of the GO was demonstrate in the

FESEM image (Figure 1c). It is clearly shown that, large surface graphene oxide with transparent thin film-like structure was synthesized.

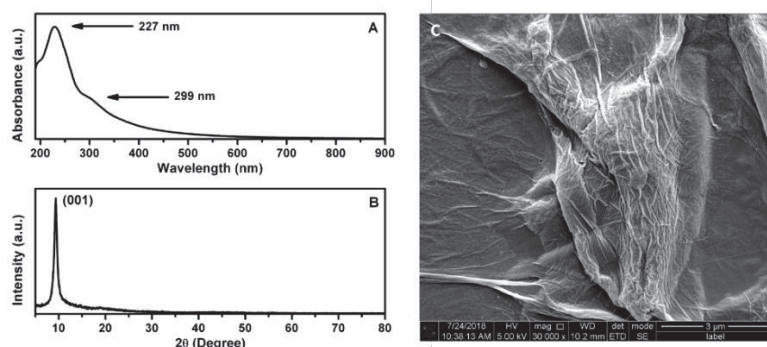


Figure 1: a) UV-vis absorption, b) XRD pattern and c) FESEM of GO.

According to Table 1, the number of isolated bacteria (CFU/mL), i.e., total aerobic bacteria and *Salmonella* spp. from farm water sample in GO system was too few to be counted while *Vibrio* spp. was not existing in the system. Thus, the bacteria reduction for these bacteria was 100 % reduced. Similarly, the MPN level of coliform bacteria in GO system for farm water sample was 14.6 MPN/100mL that the bacteria reduction became 94 % when compared to Control system. In contrary, the result for GO system in Asian clam tissue sample was varied between the tested bacteria. *Salmonella* spp., total aerobic bacteria, *Vibrio* spp. and coliform bacteria presented lower bacteria count in GO system compared to Control system. This has resulted to the positive of bacteria reduction percentage for *Salmonella* spp., total aerobic bacteria, *Vibrio* spp. and coliform bacteria i.e. 31.38 %, 18.16 %, 1.34 % and 60.71 %, respectively. In contrary, the current result of isolated *E. coli* in GO system was higher than the Control system. This has made the percentage of bacteria reduction to be in negative value or in a simpler word, *E. coli* was increased in GO system by 12.52 %.

In this study, the isolated bacteria of *E. coli* on EMB agar appeared metallic sheen green but some of the bacteria colony appeared purplish white in terms of colour. Meanwhile, *Salmonella* spp. showed a combination colour of black, red, pink and colourless bacteria colonies on XLD agar. On the other hand, yellow colonies of *Vibrio* spp. were observed in the TCBS agar. At some parts of the agar, colour changes occurred where the initial green agar turned into yellow. In this study, only metallic green sheen colony for *E. coli*, red, pink and colourless colony for *Salmonella* spp. and yellow colony for *Vibrio* spp. were selected for CFU/mL reading. Plus, BBL crystal kit results showed all the selected colonies belonged to the correct bacteria strain.

Table 1: Number of isolated bacteria (CFU/mL) i.e. total aerobic bacteria, *Escherichia coli*., *Salmonella* spp., *Vibrio* spp. and presumptive value of coliform bacteria from both farm water samples and Asian clam tissue samples in GO system and Control system.

Type of isolated bacteria	FARM WATER SAMPLE				ASIAN CLAM TISSUE SAMPLE				
	GO system		Control system		GO system		Control system		
	CFU/mL	Log (CFU/mL)	CFU/mL	Log (CFU/mL)	CFU/mL	Log (CFU/mL)	CFU/mL	Log (CFU/mL)	
<i>Salmonella</i> spp.	TFTC ^b	None	3.55 x 10 ³ CFU/ml	2.6±0.061 ^a	100 %	2.35 x 10 ⁴ CFU/ml	3.0±0.37 ^a	1.75 x 10 ⁶ CFU/ml	4.4±1.48 ^a
Total aerobic bacteria	TFTC ^b	None	6.1 x 10 ³ CFU/mL	3.6±0.24 ^a	100 %	3.67 x 10 ⁴ CFU/ml	5.1±0.15 ^a	1.22 x 10 ⁶ CFU/ml	6.3±0.40 ^a
<i>Vibrio</i> spp.	0 CFU/ml	None	2 x 10 ² CFU/ml	2.3±0.21 ^a	100 %	3.75 x 10 ³ CFU/ml	3.5±0.01 ^a	4.7 x 10 ³ CFU/ml	3.6±0.21 ^a
<i>E. coli</i>	TFTC ^b	None	3.23 x 10 ² CFU/ml	2.3±0.72 ^a	100 %	7.19 x 10 ⁵ CFU/ml	4.4±0.69 ^a	4.41 x 10 ⁴ CFU/ml	3.9±0.69 ^a
Coliform bacteria	14.6 MPN/100 mL	-	253 MPN/100m L	-	94 %	103.33 MPN/100m L	-	263 MPN/100m L	-

^a Values are means ± SD of log CFU per mL

^b TFTC stand for too few to count

4. Discussion

The results from this study revealed that GO system is effective in reducing pathogenic bacteria in farm water sample compared to Asian clam tissue samples. The results also showed that GO system can reduce *Salmonella* spp., *Vibrio* spp. and total bacteria in Asian clam tissue samples. The current findings are consistent with a study by [11] who found that graphene is predominant in inhibiting *Salmonella* bacteria, even better than the antibiotic kanamycin. The mechanism of graphene against pathogenic bacteria was discovered by [1] who discovered that cell wall of both Gram-positive and Gram-negative bacteria were damaged due to direct contact with extremely sharp graphene nanowalls. Plus, [17] added on the antibacterial activity of GO for the nanomaterial disrupting cell membrane of targeted bacteria i.e. bacteria cell deposited on graphene nanosheets resulting in the damage of bacterial membrane due to direct contact with the nanosheets. On the other hand, high number of bacteria in clams' tissue sample than in the water during the study can be explained by the prolonged contamination time [10] that increased the accumulation of bacteria within the flesh of the shellfish species. It is a valid explanation since the substrate of the rearing system was collected from Asian clams' natural habitat, thus increasing the possibility for the clams to gather bacteria from the substrate into their tissue. Being adapted to have another way of acquiring food instead of filter-feeding, i.e. deposit feeding [23] has allowed the Asian clam to feed on all edible particles in the sediments, including bacteria [6]. The most notable results in the present study are the high number of CFU/mL for *E. coli* in Asian clam tissue samples from GO system. The result is contradicted to the *E. coli* results from the farm water sample since the bacteria were completely eliminated from the system. A study found that *E. coli* was slowly eliminated than non-*E. coli* coliform species after undergoing depuration process [18]. Not only that, there is also a significant difference in bacteria inhibiting rates between mussels and infaunal bivalve (naturally burrowing edible bivalve) [18]. On the contrary, [11] reported that graphene can inhibit *E. coli* effectively. The real reason of unsuccessful elimination of *E. coli* is still undetermined and the further studies must be carried out in this area. According to [16], the presence of some purplish white colonies of *E. coli* may due to the lack of sheen production that is caused by interference by the composition of samples. Meanwhile, *Salmonella* spp. can produce various colour of colony on XLD selective agar such as pink and colourless colonies instead of red colony that can be explained by its characteristic as hydrogen sulphide negative [22]. These results were in line with the present study where bacteria colonies in pink and colourless in colour were selected to be counted. Meanwhile, yellow colonies produced on XLD agar in this study were believed to be *E. coli*. [3].

5. Conclusion

GO antibacterial filter possess potential in reducing pathogenic bacteria in farm water compared to Asian clam tissue sample that the GO need to be upgraded to improve its function as antibacterial filter such as incorporating with other compounds of nanomaterials.

6. References

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