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A Novel Strategy for Removal of Pathogenic Bacteria for Wastewater Treatment

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This study aims to apply a novel strategy to explore the possibilities of utilizing rubber nanocomposite borne bacteriophages for removal of pathogenic bacteria in hospital wastewater. Samples of wastewater were collected from different hospitals in Benha city, Egypt. The collected samples had acceptable pH level (within WHO standards) but high chemical oxygen demand (COD) and very low of dissolved oxygen (DO) levels (out WHO standard). The treated wastewater with nanocomposite or/and phages had physicochemical characters within WHO standard; as well as reduced total viable and spore forming bacteria(CFU); total coliform (TC); fecal coliform (FC) and fecal Streptococci (FS) population. They were reduced from 12x10 ^{6}; 15x10^{3} ; 13X10^{5} ; 12X10^{3} and 2.5x10^{2} to zero CFU/ml^{-1} for water sample respectively. The isolated bacteria *Escherichia* spp.; *Pseudomonas* spp., *Salmonella* spp. and *Staphylococcus* spp. were reduced to 110, 80, 50 and 30 CFU/ml^{-1} of wastewater respectively. *Escherichia* sp. has the highest frequency of antibiotic resistance followed by *Pseudomonas* sp., *Salmonella* sp. and *Staphylococcus* sp. The specific lysate phages against isolated pathogen bacteria were isolated from hospital waste water. Phages treatments have the potential to eliminate isolated bacteria. They have been standardized as 20X10^{1}, 5.2X10^{1}, 3X10^{1}and 2.2 X10^{1} PFU/ml^{-1} respectively. The application of nanocomposite borne specific phage lysate resulted in 100% removal of pathogens from hospital wastewater after 20 hours of phage treatment.

Key words: Multidrug bacteria, Pathogenic bacteria, Phages, Rubber nanocomposite, Wastewater.

Bacteriophages are viruses which range from 24-200 nm; infect bacterial cells[5] these are the obligate intracellular parasites which infect bacteria, seize their replication machinery, replicate into thousands of new progenies and lyse the cell for escape[17].

Fecal coliforms are natural inhabitants of the gastrointestinal tract of humans and other warm-blooded animals. These bacteria in general cause no harm. However, because they are eliminated with faces, they are sometimes associated with pathogens that can transmit human diseases such as *Escherichia coli, Pseudomonas aeruginosa, Salmonella* spp*, Staphylococcus, Shigellosis, Vibrio cholera* and *gastroenteritis (Campylobacter jejuni, Escherichia coli* and *Gardia Lamblia*). Threat of such diseases transmission becomes more serious as the population density increase and more sewage pollutes public water supplies. Fecal coliform bacteria, members of the family Enterobacteriaceae, include all coliforms that can ferment lactose with the production of gas (CO_2) at 44°C within 24hr. This group comprises bacteria such as *Escherichia coli and klebsiella pneumoni*. The several countries embarked on programs to reduce water borne multidrug resistant bugs (MDR). A major cause of hospital borne infection was R. plasmid carrying bacteria.

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Indiscriminate releasing of hospital wastewater in the sewage system leads to entry of multidrug resistant bacteria in the sewage. Recently, the ability of bacteriophages has extended to control plant pathogenic bacteria[13], Kill *P. aeruginosa* in wastewater[12], *E. coli* O157; H7 in manure[35], and remove pathogen from carcasses and food preparation areas is already underway[35].

Rubber nanocomposite is a matrix with added nanoparticles to improve a particular feature of the material. The size of the added particles is in the (nanoscale) defined as having one or more of its dimensions in the order of 100 nm or less. The properties of nanocomposite raised the possibility of its use in several fields[15]

This study has been performed to explore the possibilities of utilizing the rubber nanocomposite borne specific bacteriophages to remove the pathogenic bacteria from hospital wastewater

MATERIALS AND METHODS

Assessment of physicochemical properties of hospital wastewater

Wastewater samples were collected from Hospitals in Benha city, Qualybia Governorate according to methods of water and wastewater examination[6]. Samples were collected from the outer most terminals before flow of the drainage to the municipal sewage. Polypropylene containers of two liters capacity were used for chemical analyses. All collected samples for physicochemical and bacteriological analyses were stored in an ice cooler box and delivered immediately to the laboratory for analysis within through 6 hrs. from collection. Field parameters, temperature, pH, electric conductivity, dissolved oxygen (DO), Biochemical oxygen demand (BOD); Chemical oxygen demand (COD) and total dissolved solids (TDS) were assayed using the multiprobe system according to[6].

Assessment of bacteriological parameters for hospital wastewater

Bacteriological parameters ,total viable bacteria counts (TVBC), total spore forming bacteria counts (TSFBC), total coliform (TC), fecal coliform (FC), and fecal streptococci (FS) were assayed by the membrane filter technique using spread plate method by spreading 100 ml of 10^{-1} to 10^{-10} dilution prepared in sterile saline over the nutrient agar plate. The incubated plates were divided into two sets, one set was incubated at 37°C for 24hrs and the other set was incubated at 22°C for 48 hrs. according to standard methods of wastewater examination[6].

Identification of isolated bacteria

The pathogenic bacteria was isolated and purified according to[6] by using membrane filter technique. One hundred ml of water sample was filtrated by membrane filter system and the membrane was placed on specific media to isolate the potentially dead fault pathogenic bacteria[11] such as *E.coli* on Mac-conkey agar, *P. aerugonosa* on MPAC agar; *Salmonella* spp. (Brilliant green agar), *Staphylococcus* spp. on Bared parker agar and subjected to further characterization to identify the isolates as per the standard procedures[20, 21] and confirmed by VETIC system .

Multidrug bacteria

Antibiotic resistance (table 4) of bacteria isolates were tested using disk diffusion test according to[9]. For the estimation of the multidrug bacteria (MDR), diluted bacteria isolate were spread over agar plates supplemented with antibiotic disk saturated with 10 to 30 mg (Table 4).

Bacteriophage isolates

Phages specific *Escherichia* spp., *Pseudomonas* spp., *Salmonella* spp. and *Staphylococcus* spp. isolates were detected in hospital waste water by spot test . Crude phages suspension was assayed quantitatively by plaque assay[1].

Clear-plaque producing lysate phages specific for each of the bacterial isolates Phage of each bacterial isolate was produced by inoculating log phage cultures (approximately 10^8 CFU/ml) in nutrient broth with a multiplicity of infection varying between 0.01 and 1.0. The mixture of isolated bacteria and their phage isolate were shacked for a minimum of 9 hours to overnight at 28°C. The four phage isolates produced different plaque types were mixed for using as biological control (4.5 x 10^{10} PFU/ml). Bacterial debris and survivors were removed by centrifugation at 6000 rpm for 10 min.. Appropriate phage mixtures were sterilized through a 0.45 mm microbiological filter. The phage mixtures consisted of 4 to 3 different phage isolates for each bacterial isolate and had

an approximate final titer of $4.5X10^8$ PFU/ml were stored at 4°C.

Phages morphology

Transmission electron microscope (TEM) was used to detected phages mixing of *Escherichia* spp., *Pseudomonas* spp., *Salmonella* spp. and *Staphylococcus* spp. The phages were visualized using negative staining method with 1% aqueous uranyl acetate. The grids were air dried and were examined by TEM (JEOL – JEM – 1010 Electron microscope) in (The Regional Center for Mycology Al-Azhar Univ.) according to[16].

Bio-sanitation of Hospital wastewater

Rubber nanocomposite granule ($0.01m^2$) was obtained from physics department, faculty of science, Benha University. It was activated by 75% ethanol and then washed with sterilized water according to[30]. It was carried out with mixed isolated phages solutions with 10^{10} PFU ml^{-1} concentration for about 2 hours at room temperature. The activated rubber nanocomposite borne phages mixtures were added to the tested Hospital wastewater samples by 10 and 20% W/V for 24 h with aeration using air supply. Since hospital wastewater is going to end up with drain system, collected water was applied in this study as follow:-

T1 = Wastewater (control)
T2 = Wastewater + nanocomposite
T3 = Wastewater +Specific phages.
T4 = Wastewater + nanocomposite + phages

Assessment of phage population

The nanocomposite granules treated with phages were collected into a portable plastic freezer bags. Each bag was empty weighted and then dionized water was poured into each bag with 20% w/v and weighted. The bags were shacked for 15 min and 1ml of the rinse was transferred into a microcentrifuge tube then 100 µl of chloroform was added to each tube. The tubes were incubated on a rotary shaker for 30 minutes. The tubes were centrifuged for 15 min at 14000 rpm in order to remove cellular debris. The supernatant was used for enumeration of the phage titer by single plaque assay according to[1]. The plaques were counted and phage titer was expressed as a number of plaque forming units (PFU) per gram of nanocomposite by the following equations:

Y= Plaque number X 1.000 (since 100 µL of the original, 100ml volume was plated dilution ratio / sample bag weight – empty bag weight.

Assessment of survival pathogenic bacteria

After 24 hours from hospital wastewater treatment with nanocomposite borne phages, viable bacterial cells count (*Escherichia spp., Pseudomonas* spp., *Salmonella* spp. and *Staphylococcus* spp.) were assayed to test phages efficacy. This helps to fix the phage concentration during the scale up process. If the colony forming units exceeded 300, it is denoted as uncountable number (UC). Wastewater was collected and centrifuged at 6000 rpm for 5 min. The serial dilutions of supernatant were carried out up to 10 dilutions. From the serially diluted samples, 0.1ml was added to sterile plates containing LB and 0.1 mL of tap water as control and incubated at 37°C for 24hrs. The pathogens survival was assayed every 1 hour interval for 6 hours and up to 24 hrs.

Determination of protein leakage on treated nanocomposite

Protein leakage was determined by measuring the protein concentration of cell free culture broth as described by[24]. Rubber nanocomposites treated with wastewater were washed with sterilized distilled water. The supernatant was assayed for protein using Bradford method. The concentration of protein was determined from bovine serum albumin standard curve according to[10].

RESULTS

The present study was done to evaluate microbiological quality of hospital wastewater treated with a new approach by rubbernanocomposite borne phages.

Physicochemical characters

The obtained results in Table 1 showed that the physicochemical characters of wastewater, temperature and PH were lowered non significantly, but BOD; COD; OD and TDS were recorded significant reduction in wastewater treated with nanocomposite or/and phages compared with untreated ones. Physiochemical parameters studied revealed that the hospital wastewater through show some parameters within the WHO standards. Other parameters, whose values are higher than the WHO acceptable limits for the hospital wastewater.

Bacteriological characters

Bacteriological characters were used as indicators for sanitary quality of water. Untreated wastewater contains numerous pathogenic bacteria that reside in the human intestine may contaminate the soil or water body where hospital waste is released. The results in table(2) indicated that the values of TC, FS,TVBCs at 22°C and 37°C TSFBC were reduced in nanocomposite or/and phages treated water samples compared with untreated ones due to its adsorbed on the surface of nanocomposite (Table 2).

Identification of bacterial isolates

Qualitative analyses were used to determine the sanitary condition of the waters.

One hundred and ten bacterial colonies were isolated from hospital wastewater. The isolated colonies were plated in specific medium to isolate the potentially default pathogens using specific media. The isolated bacteria were identified according to Bergey's Manual of Systematic Bacteriological, 1994 and confirmed by VETIC system. The identified bacterial isolates were included in 8 genera belonging to four main bacterial families (Enterobacteriacea, Pseudomonadaceae, Staphylococcaceae and Enterococcaceae). These genera included 40 *Escherichia spp*, 25 *Pseudomonas* spp., 15 *Staphylococcus* sp., 8 *Enterococcus* sp., 5 *Kliebsiella* sp.,3 *Serratia* sp., 4 *Streptococcus* sp. and 10 *Salmonella* sp. The total number and frequency percentage of identified genera from hospital wastewater are shown in Table 3.

Table 1. Physicochemical charactersof hospital wastewater treatment with nanocomposite borne phages

Treatment	Physicochemical characters					
	Tm(°c)	pH	ODmg/L	CODmg/ L	BOD	TDS
Wastewater (control)	27	7.2	5.20	725.7	152.7	135.25
Wastewater + nanocomposite	28	7.0	3.75	300.5	85.5	70.25
Wastewater + specific phages	27	7.0	4.25	275.3	50.3	125.5
Wastewater + nanocomposite +specific phages	28	7.0	2.72	250.4	45.2	50.5
WHO standard	27	7.0	3.75	350	75	85

Tm: temperature. OD: oxygen demand. COD: Chemical oxygen demand. BOD: Biochemical oxygen demand. TDS: Total dissolved solids.

Table 2. Bacteriological characters of hospital wastewater treatment with nanocomposite borne phages

Treatment	Bacteriological characters				
	TVBCs	TSFBC	TC	FC	FS
Waste water (control)	$12X10^6$	$2.5X10^2$	$13X10^5$	$12x10^3$	$2.5x10^2$
Waste water +nanocomposite	250	120	275	0	420
Waste water + phages	150	250	230	50	50
Waste water +nanocomposite +phages	0	0	0	0	0

TVBCs: Total viable bacteria counts. TSFBC: Total spore forming bacteria counts. TC: Total coliform. FC: Fecal coliform. FS: Fecal streptococci

Table 3. Total number and frequency percentage of identified bacteria hospital wastewater sample.

Bacterial isolates	Collection numbers of isolates	% frequency
Escherichia spp.	40	36.4
Enterococcus spp.	8	7.3
Klebsiella sp.	5	4. 6
Pseudomonas spp.	25	22.7
Salmonella spp.	10	9.1
Serratia spp.	3	2.7
Staphylococcus spp.	15	13.6
Streptococcus spp.	4	3.6
Total	110	100

Multidrug resistant (MDR) bacteria

MDR problem encountered in wastewater mainly due to gram-negative bacteria. Whereas ,the bacterial isolates were tested for the antibiotic sensitivity using disk diffusion technique for determination of MDR bacteria. It was found *most* isolates of *Escherichia* spp. were found to be resistant to tested antibiotics fallowed by *Pseudomonas* spp. *Salmonella* spp. and *Staphylococcus* spp. isolates. The majority of isolates were resistant to four or more antibiotics thus, indicating multiple antibiotics resistance (MAR). Moreover Amoxicillin; Ampicillin , Streptomycin, Erythromycin, Cephalosporin, Gentamycin and chloramphenicol formed the common MDR pattern (Table 4).

Bacteriophage isolates

Clear-plaque producing lysate phages specific for each of the bacterial isolates (*E. coli, Pseudomonas* spp*., Salmonell*a spp. and *Staphylococcus* spp.), were isolated from hospital wastewaters. The four phage isolates produced different plaque types [Clear confluent lyses, turbid confluent with large and regular, irregular circular form, clear center and turbidity center with size 3 to 5 nm, distinct translucent spreading halo, small circular with halo and without halo, (fig. 1A) . Electron microscope revealed phage particles have long, short, curled, non-contractile tail. The phage particles have an isometric head with different diameter size 65.2 to 75.5 nm and the tail with 200.3 to 245.5 nm in length and 15.4 to 18.5 nm in width (Fig.1B).

Bio-reduction of pathogenic bacteria

The used nanocomposite; phages and nanocomposite borne phages had been reported to significantly reduce bacterial load in hospital wastewater (table 5) due to its rubber nanocomposite as antimicrobial properties and

Table 4. Multidrug resistance profile of pathogenic bacteria against individual antibiotics

Antibiotics	*Escherichia*			*Pseudomonas*			*Staphylococcus*			*Salmonella*		
	R	I	S	R	I	S	R	I	S	R	I	S
Amoxicillin(10mcg)	10*	8	22	2	2	21	2	3	5	2	2	6
Ampicillin (10mcg)	12	10	18	1	3	21	1	1	8	1	4	5
Corbenicillin(10mcg)	3	17	20	3	4	18	0	0	12	4	3	3
Ceproflaxin (10 mcg)	5	15	20	1	3	21	0	0	3	0	0	6
Tetracycline (30 mcg)	5	12	13	4	0	21	4	1	5	1	2	7
Streptomycin (10 mcg)	3	15	21	3	1	21	3	2	5	2	0	8
Kanamycin (10 mcg)	4	16	20	0	3	22	2	3	10	0	0	5
Erythromycin (15mcg)	5	13	22	1	2	22	2	3	15	0	2	8
Penicillin (10 mcg)	2	16	22	2	5	18	0	0	5	0	2	10
Cephalosporin(30 mcg)	3	15	22	3	3	19	1	0	3	7	0	9
Gentamycin (20 mcg)	5	13	22	5	1	19	2	0	8	3	0	7
Chloramphenicol(30 mcg)	8	7	25	7	3	15	7	4	4	3	0	7

R: Resistant. I: Intermediate sensitive. S: Sensitive.* Mean diameter of inhibition zone (mm) included antibiotic disk for tested isolates

Table 5. Total count of bacteria in hospital wastewater treated with nanocomposite borne phages

Treatment	Total count of bacteria						
	0 time	2hr	4hr	8hr	12hr	16hr	20hr
Wastewater (control)	$28X10^7$	$2X10^7$	$12X10^5$	$25X10^4$	$12x10^2$	1252	1175
BM+Wastewater (control)	$28X10^7$	$2X10^7$	$12X10^5$	$25X10^3$	1210	9275	9210
Wastewter+nanocomposite	$25X10^7$	$19X10^6$	$8X10^3$	$13X10^2$	75	-	-
Wastewater + phages	$25X10^7$	$23X10^5$	$17X10^2$	121	-	-	-
Wastewater +nanocomposite +phages	$25X10^7$	$13X10^4$	132	25	-	-	-

BM: broth media

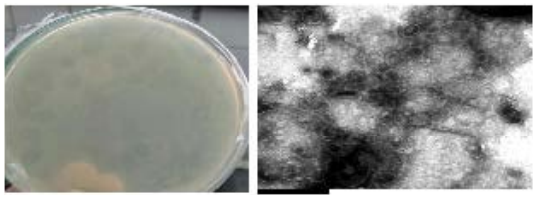
Table 6: Total count of Pathogenic bacteria in hospital wastewater treated with nanocomposite borne their phages

spp.	*Escherichia* spp.			*Salmonella* spp.			*Pseudomonas* spp.			*Staphylococcus*
Treatment	0 time	10hr	20hr	0 time	10hr	20hr	0 time	10hr	20hr	0 time 1 0 h r
BM+ pathogenic	25×10^8	3×10^8	14×10^8	15×10^8	18×10^8	25×10^8	12×10^7	14×10^7	27×10^7	3×10^7 12×10^7 21×10^7
Wastewater	14×10^7	12×10^5	25×10^3	8×10^6	3×10^5	11×10^3	17×10^6	11×10^4	21×10^2	5×10^6 18×10^5 17×10^4
Wastewater + nanocomposite	8×10^2	8×10^6	4×10^2	17×10^6	4×10^2	5×10^6	7×10^2	14×10^7	125	75 128 175
Wastewater+ phages	14×10^7	17×10^2	93	8×10^6	7×10^2	83	17×10^6	3×10^2	75	5×10^6 8×10^2 25
Waste water + nano Composite borne phages	14×10^7	171	-	8×10^6	213	-	11×10^6	427	-	5×10^6 125 -

BM: broth media.

specific phages for tested bacteria. Application of rubber nanocomposite or its borne phages as bio-reduction pathogenic bacteria was done in hospital wastewater. It was found steady decrease in total bacterial count in all treatments after 4hrs of inoculation. While in case of treatment broth medium inoculated with isolate pathogenic bacteria and non-inoculated with specific isolated phages there was steady decrease in the population after 12 hrs. (Table 5).The effect on pathogenic bacteria population was more pronounced in wastewater treated with rubber nanocomposite borne specific phages after 2hrs, while after 12hrs no detected bacteria cells , and after 20hrs no detected any cells of pathogenic bacteria (*Escherichia*, *Pseudomonas*, *Salmonella* and *Staphylococcus*) as shown in Table(5,6). Also data show the specificity Phages inoculated treatment drastic reduction in population was observed after 8,10 hrs.of inoculation. After 8hrs of inoculation itself the reduction was high and after 20hrs the pathogenic bacteria population is completely vanished (Table 5, 6).

At the same time the increasing the incubation period the bacteria population was also increased in broth medium where as in other treatments not much increase was observed. This may be due to adsorption of phage particles and may change the metabolic rate of bacteria pathogens. Based on the single step growth experiment, the phage population reached the maximum level within 7 and 8 hours. So, the incubation time in this experiment was maintained up to 14 hours._The amount of protein leakage was determined to the four bacterial (*Escherichia*, *Pseudomonas*, *Salmonella* and *Staphylococcus*). The used rubber nanocomposite caused damage to the cell wall of the treated cell, which led to



(A) plaque assay showing different type of plaque morphology and (B) TEM showing different phage typing for *E.coli, Staph. Aurues, Salmonella* spp. and *P.aerugonosa* different type

Fig. 1. Photogram of mixing phages for *E.coli, Staph. aurues, Salmonella* spp and *P.aerugonosa*

leakage considerable amount of proteins. The amount of protein leakage from the tested bacterial cell was increased by cell suggesting cell wall disruption mechanism. These results demonstrated that the amount of protein leakage of *Escherichia, Salmonella* and *Pseudomonas* cells was lower than that of *Staphylococcus* cells. So, *Staphylococcus* cells were more sensitive to the antibacterial property of the rubber nanocomposite than the other three species. It could be provided that the rubber nanocomposite to reuse it in water treatment by washing with 75% ethanol solution to kill any bacteria on the surface and then washed with sterilized distilled water to remove any remaining ethanol solution. So water treatment with rubber nanocomposite become at reasonable costs. To minimize pollution from their sources encourage low cost treatment processes and force regulating laws.

DISCUSSION

The main aim of the bacteriological analyses is to assess the microbial pollution, which is of a paramount importance in assessing the related health problems. Among the three primary bacterial indicators, total coliforms represented the highest values followed by fecal coliforms and fecal Streptococci. Also, TVBCs and 22°C recorded higher density that at 37°C in untreated wastewater . Nanocomposite treatment reduced the four primary bacterial indicator. The reduction of the bacterial count in untreated water sample and in the nanocomposite treatment water samples by spread plate count method. Hospital wastewater contained numerous pathogenic bacteria that dwell in the human intestinal tract and may contaminate water body where hospital waste is disposed. The wastewater samples were plated in specific media to isolate the potentially default pathogens. According to scheme of biochemical tests [8] VITEC kit and using specific media, the pathogenic bacteria were isolated and characterized. *Escherichia*, *Pseudomonas*, *Salmonella* and *Staphylococcus,* were the most frequently distributed and isolated in hospital wastewater. Physicochemical characters of hospital wastewater samples had acceptable level of temperature and pH, but low of TDS, BOD, COD and high of OD.

The discharged of physicochemical characters due to contamination of the receiving environment (water, soil, air) which could probably be hazardous to human health. The improper management of water systems may cause serious problems in availability and quality of water[32]. Further studies [4,28], and investigated the physicochemical and bacteriological quality of hospital wastewater and observed the same results as that of the present study. The multidrug resistant problem encountered in hospital is mainly due to Gram-negative bacteria. The multidrug resistant bacteria were estimated on specific agar plates supplemented with antibiotic drugs (gentamicin. ampicillin, Penicillin and Chloramphenicol) because they have greater *in vitro* stability and commonly used over the last twenty years. The similar colonies morphology were selected individually and identified by standard biochemical methods and subject to drug susceptibility by the disk diffusion technique[7]. The antimicrobial selective pressure through indiscriminate use of antibiotics has played a significant role in enriching the MDR strains in the hospital wastewater. A sizeable number of hospitals trains has become resistant simultaneously to most of the available antibiotics[29].

Host specificity is central to selection of suitable phages for wastewater treatment applications[33, 3]. The clear plaque variant was purified several times and on further infection of the host cells[25]. [26] isolated two bacterial strains (*E coli* and *Salmonella* spp.) from wastewater of Poona hospital. Titer for phage against isolate 1 was determined to be 2.5 x 10^6 PFU /ml and for phage against isolate 2 was determined to be 1.9 x10^4 PFU/ml by soft agar overlay method. TVC for the wastewater sample was found to be 6.87 x 10^6 CFU/ml, same water was treated with cocktail of phages for 14 hours and significant reduction in TVC was found to be 5 x 10^5 CFU/ml.

Development of multidrug resistant bacteria and emergence of multiple antibiotic companies necessitates searching for novel approaches to get rid of these multidrug resistant bacteria. Phage therapy is an alternative to overcome these threatening organisms, it is essential for the success of phage therapy. Proper phage must be isolated and enriched to produce adequate numbers for application. The number of phages to be used should be 3 to 10 times

greater than bacteria_[19,27] noticed that insufficient concentration of host cells may also contribute for phage decline. Phage enrichment normally includes the inoculation of mixed environmental samples and growth media with single host strain. Phages decay and loss of infectivity may decreased its efficacy for wastewater treatment[34]. Reduction in phage population may occur due to adsorption of phage particles to sludge blocks and may reduce the efficacy of phage treatment[23]. So the host and phage ratio showed be maintained for success of the treatment.

Rubber nanocomposite benefits were mechanical properties, Improvement, increase stiffness without loss of flexibility, increased dimensional stability, chemical and thermal stability, easy processing and recycling. The used nanocomposite had been reported to significantly reduce the bacterial load in water due to its antimicrobial properties. This occurred by bacterial adhesion on the surface and then bacterial cells disrupted and shrunk. These results were in agreement with those reported by[22,14]

The lysate phages of *Escherichia*, *Pseudomonas*, *Salmonella* and *Staphylococcu*s were mixed and used for the treatment. After 12 hours of incubation, there was no *Escherichia*, *Pseudomonas*, *Salmonella* or *Staphylococcus* population in wastewater[18] stated that almost 10% of isolated phages from activated sludge were polyvalent in nature. Many sewage waste treatment systems are aiming for complete removal of pathogen which require search for approaches that does not harm the environment. One such novel approach is exploring the possibilities of rubber nanocomposite bacteriaphages[31,18] for pathogen removal. The inoculation resulted in 100% removal of pathogens from sewage water of incubation.

Results demonstrated that the amount of protein leakage of bacterial cells was higher on nanocomposite phage than nanocomposite only. So, *staphylococcus* cells were more sensitive to the antibacterial property of the rubber nanocomposites than the other three bacteria species. This was due to the difference in the structure of cell walls between Gram negative and Gram positive bacteria. Whereas Gram positive have a thick cell wall consisting mainly of peptidoglycan covering the cytoplasmic membrane, while Gram negative bacteria have an outer membrane covering an inner thin layer of peptidoglycan[2]. It seems likely that the difference of cell wall structure between *Escherichia coli* and *Staphylococcus aureus* bacteria was an important reason. A most important function of the outer membrane is to serve as protective barrier which hinders the entrance of bactericidal agents and other toxic substances that might kill or injure the bacteria These findings agree with a previous report that also revealed Gram negative bacteria were more resistant than Gram positive bacteria[30], The obtained results in this study demonstrated that the mechanism of antibacterial activities of the rubber nanocomposite were by way of physical damage to bacterial cells which led to protein leakage from the bacterial cells and shrunk cells. Protein leakage and inhibition of cell wall biosynthesis led to bacterial death[2].

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