20 *Egypt. J. Chem.* **57**, No.4, pp.327–341 (2014)

A Study on The Effect of Type of Solvent on Chitosan Efficiency for Treatment of Drinking Water Contaminations

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IN this research, chitosan (Ch) a natural polymer, was employed in drinking raw water treatment of Nile River against *Escherichia coli* via simple method, it is dissolution of chitosan in three different solvents;acetic acid (AC),malonic acid (MA) and N carboxymethyl N,N diethylbenzene ammonium chloride (CMEBAC) to produce three different solutions; Ch-AC, Ch-MA and Ch-CMEBAC, respectively. These solutions were characterized by FTIR and elemental analysis. The investigations of their effect against total coliform bacteria of raw water were carried out according to method of Most Probable Number (MPN) at different contact times (30, 60 and 120 min). The results showed that the full inhibition of total coliform were achieved after 30 min as contact time by 7 gm L^{-1} ml of Ch-Ac , 5 gm L^{-1} of Ch-Ma and 0.75 gm L^{-1} ml of Ch-CMEBAC. Otherwise, by increasing of contact time, the volumes of inhibitors (chitosan solutions) decrease.The minimum inhibitory concentration (MIC) values of those solutions were detected as 2.75, 2 and 0.2 µ/ml , respectively.

Keywords: Chitosan, *E.coli*, Biological treatment of water and Malonic acid .

Water is one of nature's most important gifts to mankind which is essential to life. Water is a major component of all organisms, the matrix of all processes in living organism that serves as a lubricant in digestion and almost all other bodyprocesses, lubricates our joints and cartilages,moves within our cellular systems transporting vital blood plasma and functioning in regulation of body temperature[(1)] . Body dehydration can lead to death.

Fecal contamination of water is a serious problem due to the potential risk for contracting diseases from pathogens and here exists the need of testing for indicator organisms such as coliform bacteria. To get rid of this problem, disinfection is applied to prevent the growth of pathogenic organisms and to protect public health[(2)]. Different methods of disinfection were handled beginning with sedimentation to chlorination, ozone, UV, chlorine dioxide, potassium

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permanganate, nanofiltration, hydrogen peroxide, solar disinfection, organic acids and biological treatment. The most common disinfection method involves some form of chlorine or its compounds such as chloramine or chlorine dioxide. Upon using chlorine certain forms of chlorine react with organic material, naturally present in many water sources, to form harmful chemical by-products. Application of a less or no harm disinfection methods paved to use biopolymers such as chitosan.

The Gram-negative bacterial (*E. coli*) outer membrane contains polyanionic lipopolysaccharide stabilized by divalent cations, such as Mg^{2+} and Ca^{2+} [3] . The OM serves as an effective permeability barrier to restrict macromolecules and hydrophobic substances from entering or leaving bacterial cells[4]. The cation-binding sites of lipopolysaccharide are critical to outer membrane integrity. However, cationic substances, known as membrane permeabilizers, *e.g.*, polymyxin and aminoglycosides (chitosan), can compete with divalent cations to bind with lipopolysaccharide, therebydisorganizing the outer membrane structure[5] .

Organic acids are preferable to use in water treatment, it's more safe than mineral acids to inhibit the germination of the spores in water[6] and not react with metals. However, the mineral acids are highly reactive with metals, and they have corrosive ability than the organic acids.

Chitosan has a number of unique properties such as antimicrobial activity, nontoxicity, and biodegradability[7]. It has a broad spectrum of antimicrobial activities[8], high bactericidal rates[9] and low toxicity toward mammalian cells,making it a potential biocide in food preserving[10] which reflects its administration through oral route. As result of all this we came up with using chitosan as an antimicrobial in water treatment. The antimicrobial activity of chitosan against a variety of bacteria and fungi coming from its polycationicnature is well known[11] . However, this activity is limited to acidic conditions due to its poor solubility above pH6.5[12]. Non-chemically modified chitosan must be dissolved in acid solvents to activate its antibacterial activity[10] .

As a result we prepared three solutions of chitosan using acetic acid 2%, N-(carboxymethyl)-N,Ndiethyl benzene ammonium chloride 2% and malonic acid 1% as solvents creating acidic media that favour its antimicrobial activity. Potential applications of chitosan as a bactericide in water treatment were examined recently[13]. Raw water samples were collected, physically, chemically and bacteriologicaly examined then treated with different consequent amounts of each solution separately and tested for the effect they had on bacterial growth of total coliform group.

The three solutions were proved to have an inhibitory effect on total coliform bacteria that varied from one solution to another and that effects were studied at different contact times proving that increasing contact time could increase the antimicrobial effect.

Materials and Methods

Materials

Chitosan was prepared from shrimp shells that were commercially obtained. Molecular weight of chitosan was equal to 109.050 kDa. NaOH, acetone, ethyl alcohol and acetic acid were purchased from Adwic, while HCl was from Panreac. N,N diethyl aniline, monochloro acetic acid and malonic acid were purchased from Aldrich. Agar media was purchased from EDM but Lauryl sulfate broth media was from Merck KGaA. Brilliant green Bile 2% broth media was a product of Lab M Limited while both EC broth media and M endo agar media were purchased from Becton, Dickinson and Company. Sterillized segmented membrane filter papers were produced by PALL Corporation, while cellulose filtration papers by Ahlstorm.

Method

i. Preparation of chitin and chitosan from shrimp shells

Chitin and chitosan were prepared from shrimp shells according to the following references[14, 15].

ii. Preparation of N carboxymethyl N,N diethyl benzene ammonium chloride (CMEBAC).

CMEBAC was prepared and characterized as in the reference [16] .

iii. Preparation of chitosan solutions

Three solutions of chitosan were prepared by dissolving 2 gm of grinded chitosan in each of acetic acid 2 %,malonic acid 1% and CMEBAC 2% solvents, respectively to give solutions Ch-Ac, Ch-Ma and Ch-CMEBAC.

Characterization of prepared compounds

i. FTIR

Chitosan and its derivatives sample (2 mg), which was dried overnight at 60°C under reduced pressure, was mechanically well-blended with 100 mg of KBr. The thickness of the KBr disk was 0.5 mm. The KBr disk of the mixed powder was desiccated for 24 hr at 110°C under reduced pressure and then its IR spectrum was recorded with a Shimadzu FTIR-4200 spectrometer using a disk of 100 mg KBr as a reference. The intensity of maxima of the IR absorption band was determined by the baseline method.

ii. Elemental analysis of the three solutions

Chitosan and its derivatives sample were dried at 60^{o}C and subjected to the elemental analysis at the National Research Centre in the Organic Microanalyses Section via Vario Elementar that is manufactured in Germany.

Characterization of raw water samples

The characterization of collected raw water samples were 6.98NTU as turbidity value at 25˚C, pH 7.44, conductivity 323 µS/cm, TDS 203 mg/L,

alkalinity 132 mg/L, total hardness (TH)132 mg/L, calcium hardness (CaH) 88 mg/L, magnesium hardness (MgH) 44 mg/L, chloride 14 mg/L, Fe concentration 0.46 mg/L, Mn concentration 0.05 mg/L, $NH_3$0.05 mg/L and 0.12 mg/L as phosphates concentration value.

Estimation of bacterial density of the raw water samples as a Most Probable Number (MPN) value

Bacterial density in raw water samples after and before treatment ware performed through a series of decimal dilutions (five 10 ml, five 1 ml and five 0.1 ml volumes of samples). MPN value was determined through the combinations of positive and negative results.It was detected as 8400 MPN/100 ml for raw water before treatment.

Treatmentof raw water samples by chitosan solutions

One liter of raw water, which was previously tested for its Most Probable Number (MPN) value, is treated with different concentrations (mg L^{-1}) from three chitosan solutions at 30, 60 and 120 min.

Determination of MIC of chitosan solutions against total coliform bacteria and E.coli

Minimum inhibitory concentration (MIC) is the lowest concentration to cause an inhibition of a certain organism or a group of microorganisms. It was performed according to Kirby-Bauer disk-diffusion method[17] using small filter paper discs labeled in a manner that each labeled disc represented a certain concentration. As for test against total coliform bacteria, mEndo agar media was used as the cultured media and the discs were placed over a 45 µ dimensioned filter paper on which a positive sample was filtered.

As for *E.coli*, nutrient agar was used and a freshly prepared plate was swapped with a fresh lobe loaded with *E.coli*. Impregnated discs were placed on and the test was proceeded as a normal Kirby-Bauer disk-diffusion technique giving the first inhibition zone around the disc representing the MIC value.

Results and Discussion

FTIR data

Figure 1 shows the IR data of chitosan virgin, Ch-AC, Ch-MA and Ch-CMEBAC. The spectrum of chitosan virgin show the broad and strong band ranging from 3200 to 3600 cm^{-1} regards to the presence of OH and NH_2 groups, which is consistent with the peaks at 1074 and 1152 cm^{-1} assigned to alcoholic C-O and C-N-H stretching vibration. The peaks at 2920 and 2875 cm^{-1} assigned to asymmetric and symmetric CH_2 groups. The peaks at 1590 and 1430 cm^{-1} are characteristic of NH_2 and CH_2 deformation , respectively. The small peak at 1235 cm^{-1} attributed to the C-O-C stretching[18] .

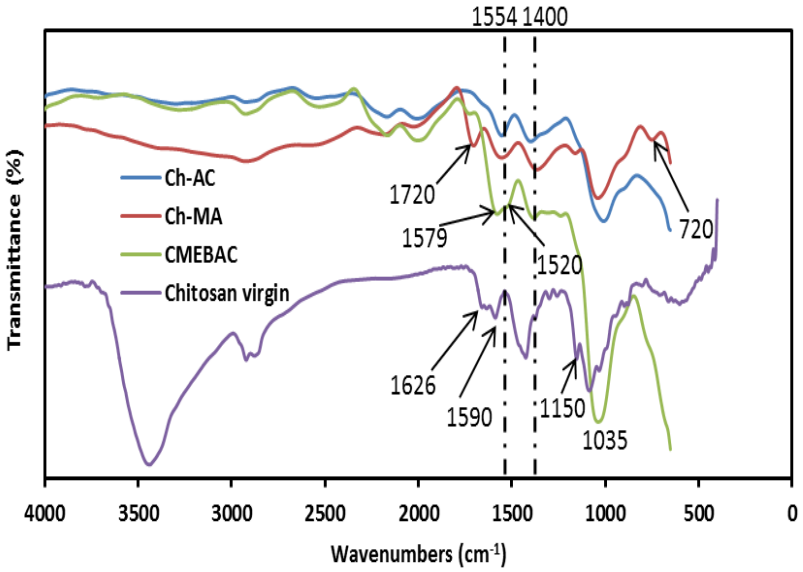


Fig. 1. FT-IR of chitosan and its solutions.

In the spectrum of Ch-AC, the peaks of NH_2, NH_2 deformation and C-N-H groups at 3450, 1590 and 1152 cm^{-1} , respectively disappeared, attributed to the protonation of all NH_2 groups to (NH_3^+) groups. While the strong peak at 1550cm^{-1} and a weak peak near 1400 cm^{-1} region appeared which are attributed to asymmetric and symmetric carboxylate anion stretching. These peaks provide evidence of the conversion of chitosan to chitosan acetate[19,20] .

While the Ch-MA spectrum show the same change of peaks of the Ch-AC spectrum, in addition, appearance of two peaks, at 1720 cm^{-1} due to the ester carbonyl (C=O) stretching vibrations, which is related to the two carboxylic groups of MA in the specimen[21] , and the peak at 720 cm^{-1} regarding to CH_2 rocking of MA[22,23] .

As for spectrum of Ch-CMEBAC also present the similar changes of Ch-AC, as well as, the appearance of broad peak between 1600 and 1500 cm^{-1} which are attributed to two peaks of C=C-C of aromatic ring stretch at 1579 and 1520 cm^{-1}[24] , and the peak of the asymmetric carboxylate anion stretching at 1550 cm^{-1}. Also, the increasing of the intensity of peak at 1007 cm^{-1}, due to the C-C stretching of diethyl groups[25] .

Elemental analysis of the three solutions

Table 1 shows the Elemental analysis of all prepared compounds, the results illustrate that the percentages of the elements vary from compound to an other, indicating to the formation of the compounds.

TABLE 1. The elemental analysis of chitosan and its solutions.

Compound name	C%	H%	N%	Cl%
Chitosan virgin	39.88	4.63	7.26	-
Ch-AC	63.1	8.25	5.2	-
Ch-MA	37.4	7.89	4.47	-
Ch-CMEBAC	33.15	7.76	4.88	9.1

Inhibition mechanism of chitosan solutions

The solutions under this study possess two parts, chitosan and organic acids. Chitosan free (powder or flaks) has antibacterial activity for *E. coli* involve disrupt the barrier properties of the outer membrane[26,27] through the free amino groups which interact against waterborne pathogens. Otherwise the introduction of a large amount of –OH from chitosan may chelate metal elements such as Ca in cell membrane[28] .

While the organic acids possess two functions as antimicrobial agents. The primary action is through pH depressions, which give the ability of organic acids to change from undissociated to dissociated form, makes them effective antimicrobial agents[29,30] . When an organic acid is in the undissociated form, it can freely diffuse through the semi-permeable cell wall of microorganisms into their cell cytoplasm. Once inside the cell, where the pH is maintained near 7, the acid will dissociate and pH will decrease, thus disturbing and finally killing the microorganism[31] . The secondary action is the anionic part of the organic acids (carboxylic groups) that cannot escape the bacteria in its dissociated form will accumulate within the bacteria and disrupt many metabolic functions, leading to osmotic pressure increase, incompatible with the survival of the bacteria[32- 34] .

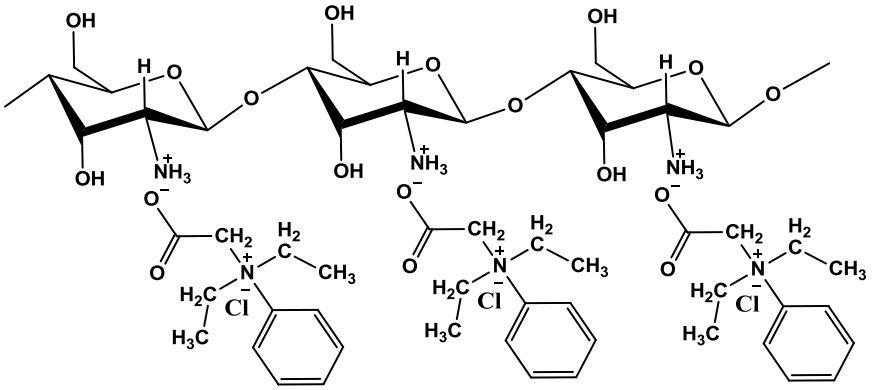
When, chitosan mix with organic acid solutions a several quaternary ammonium groups are produced in the backbone of chitosan which are responsible for the antimicrobial action as shown in Schemes (1-3). There were two possibilities explaining the antibacterial action of chitosan solutions[35] , (1) chitosan solutions might interact with and interactive some contents in the broth (media) by the method of ionic interaction[36] and (2) the bacteria could adhere to each other through the positive charges on chitosan chains which adsorbed on their surfaces, caused disruption of cell membranes[35, 37, 38] .

The adsorbed positively charged of chitosan solutions (quaternary ammonium groups) interacts and forms polyelectrolyte complexes with negatively charged of acidic polymers (polyanionic lipopolysaccharide) produced at the bacterial cell surfaces causing the leakage of proteinaceous and other intracellular constituents[39] . The mechanism of this interaction could be explained in the following sequence, (i) adsorption and penetration of the quaternary ammonium groups into the cell

wall; (ii) reaction with the cytoplasmic membrane (lipid or protein) followed by membrane disorganization; (iii) leakage of intracellular low-molecular-weight material; (iv) degradation of proteins and nucleic acids; and (v) wall lysis caused by autolytic enzymes[40-42] . There is thus a loss of structural organization and integrity of the cytoplasmic membrane in bacteria, together with other damaging effects to the bacterial cell.

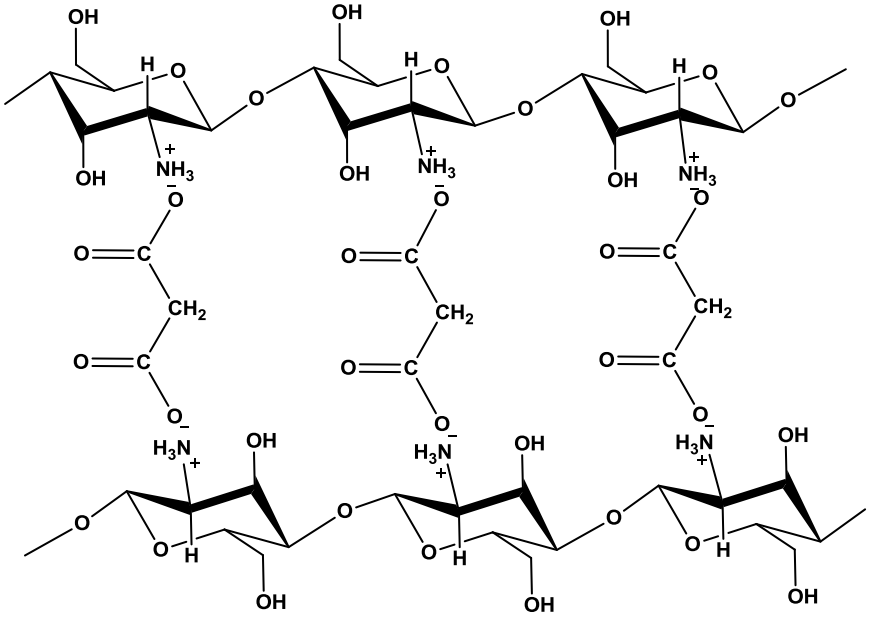
Effect of solvents

The antimicrobial activity of chitosan solutions with different contact times (30, 60 and 120) are present in the Fig. 2, 3, 4, respectively, it's clear from this figures that the antimicrobial activity of Ch-CMEBAC is higher about Ch-AC and Ch-MA, and gave the full inhibition of *E. coli* at 1.25 gm L^{-1} after 30 min, this attributed to its chemical structure which contains many influencing groups possess antimicrobial activity as can be seen in Scheme (1), such as, benzene ring, carboxylic group, chlorine ions and alkyl chains (two ethyl groups) which could form a layer of film around cells to affect the absorption of nutrients, and make the CMEBAC in more proximity to the cell membrane[28] , as well as, two quaternary ammonium (N^+) groups that has cationic nature to facilitate the bonding to negative sites on the cell wall of bacteria, and also it has mycobacteriostatic action[43,44] , as mentioned above.These groups support and increase the efficiency of Ch-CMEBAC to *E. coli*.



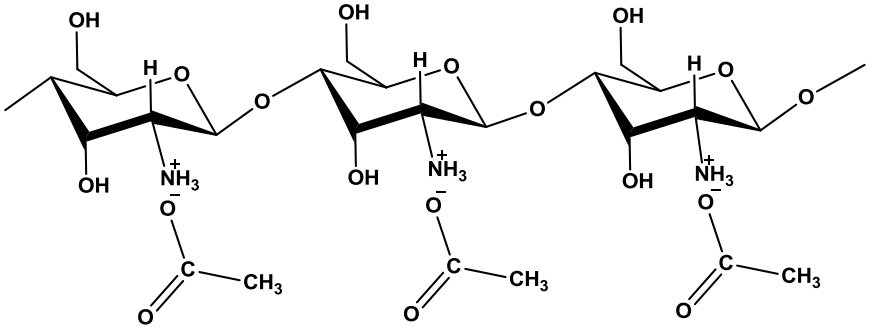
Scheme 1. Chitosan in N-(Carboxy methyl)-N,N-diethyl benzen ammonium chloride (CMEBAC).

While the Ch-MA has a medium antimicrobial effect, it exhibits the full inhibition at 5 gm L^{-1} after 30 min, due to the bi-functional organic acids (two carboxylic groups) which give a strong dissociation of the solution, and appear to exert their disinfectant action in the manner of themineral acids, mostly through the hydrogen ion[30]. Moreover, malonic acid binds between chitosan chains as can be seen in Scheme (2) to allow the attack of *E.coli* to be by two NH_3^+ groups in one time[45] .



Scheme 2 . Chitosan in malonic acid.

However, the Ch-AC exhibit a lower antimicrobial affect about Ch-CMEBAC and Ch-MA, it gives a full inhibition at 7 gm L^{-1} after 30 min, due to containing a one NH_3^+ group and one carboxylate ion and also lower degree of disposition, as can be seen in Scheme (3).



Scheme 3. Chitosan in acetic acid.

Effect of contact time

Figures 2-4 show the antimicrobial activity of chitosan solutions with contact time, it's clear that by increasing contact time the antimicrobial activity of chitosan solutions, increase as in the literature[46-47] .

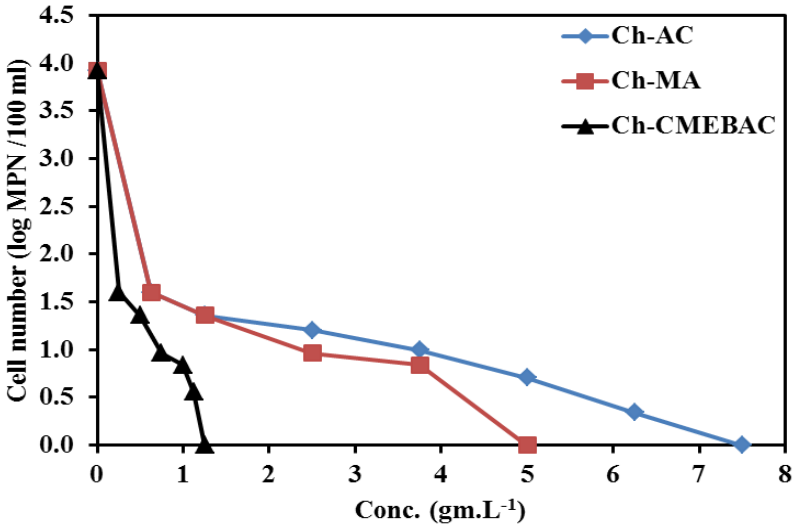


Fig. 2. Inhibition of *E.coli* by chitosan solutions at contact time 30 min.

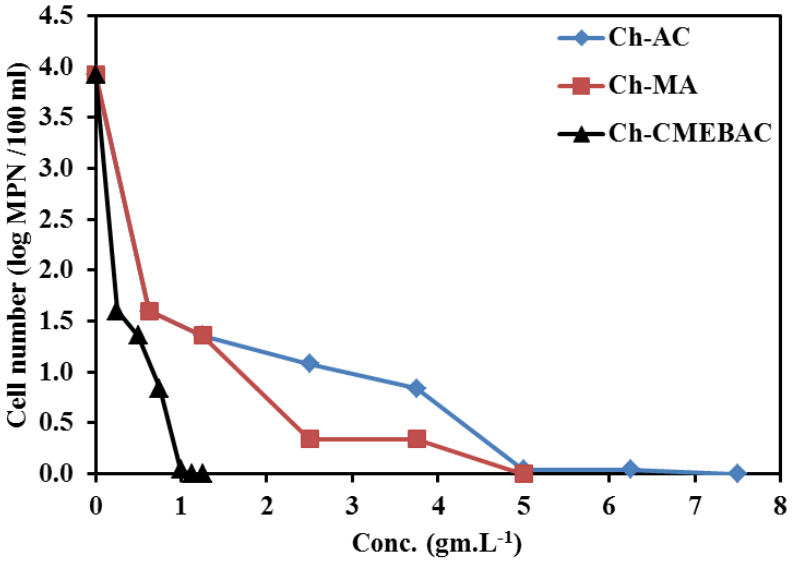


Fig. 3. Inhibition of *E.coli* by chitosan solutions at contact time 60 min.

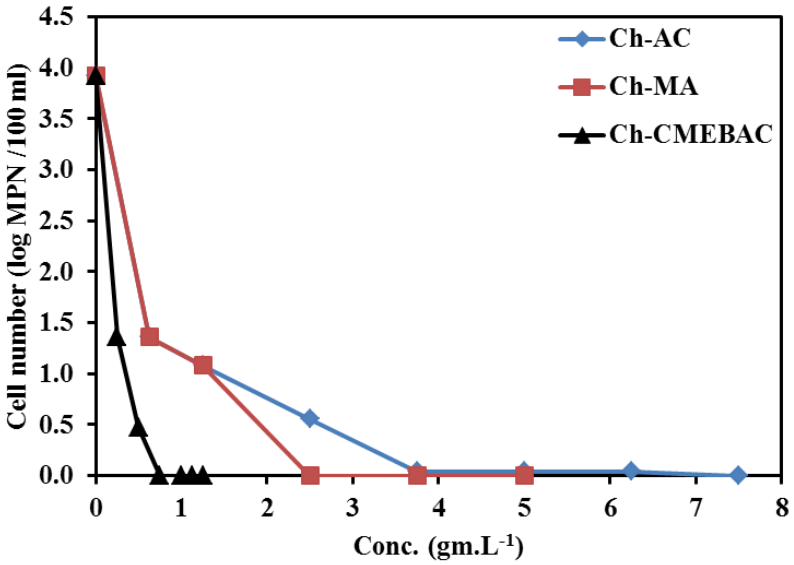


Fig. 4. Inhibition of *E.coli* by chitosan solutions at contact time 120 min.

The MIC of chitosan solutions against total coliform group of bacteria

For Ch-Ac, the MIC was 2.75 µg/ml, while Ch-CMEBAC the MIC was much less than for Ch-Ac declaring a better effect and inhibited at 0.2µg/ml. Ch-Ma had a concentration about 2 µg/ml as its MIC.

The MIC of chitosan solutions against E.coli

E.coli, which is the major organism in the total coliform group, was inhibited at MIC values that are the same as those for the total coliform group. The MIC values of *E.coli* are as the following, 2.75 µg/ml for Ch-AC, 2.0 µg/ml for Ch-MA and 0.2 µg/ml for Ch-CMEBAC.

Conclusion

In this work, the drinking raw water of Nile River contains *E. coli* (8400 MPN/100 ml), were full killed by using ecofriendly materials (chitosan solutions) after contact time 30 min by 7 gm L^{-1} of Ch-AC, 5 gm L^{-1} of Ch-MA and 1.25 gmL^{-1} of Ch-CMEBAC.

The higher antimicrobial activity of Ch-CMEBAC than other two solutions, regards to the influencing groups and atoms in its structure such as, benzene ring, carboxylic group, chlorine ions, alkyl chains and two quaternary ammonium groups (N^{+}) other solutions. These solutions depend on their structures. While the lower activity of Ch-AC regards to the poverty in the influencing groups in its structure.

Acknowledgments : We are grateful for the help of Egyptian Petroleum Research Institute and the Holding Company for Water and Wastewater in Menoufia.

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(*Received* 29/10/2014 ;
accepted 10/11/2014)

دراسة عن تأثير المذيبات المختلفة على كفاءة الكيتوزان فى معالجة ملوثات مياه الشرب

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تم من خلال هذا البحث استخدام الكيتوزان فى معالجة تلوث المياه ببكتريا الايشريشيا كولاى باستخدام طريقة مبسط وذلك عن طريق اذابة الكيتوزان فى ثلاثة مذيبـات مختلفة وهـى حمض الأسيتيك ، حمض المالونيك و ان كاربوكسى ميثيل ان و ان داى ايثيل بنزين أمونيوم كلوريد على الترتيب. تم توصيف هذه المركبات باستخدام الاشعة تحت الحمراء (FTIR) والتحليل العنصرى.تمت متابعة تأثير هذه المحاليل على البكتربا القولونية عن طريق طريقة العد الاكثر احتمالا(MPN) وذلك عند قيم مختلفة من اوقات التلامس وهى 30 ، 60 و120 دقيقة. اوضخت النتائج انــه قـد تــم تثبيط نشاط البكتريــا بعد 30 دقيقة من وقت التلامس باستخدام 7 جم/لتر من محلول الكيتوزان مع حمض الاستيك و 5 جم/لتر من محلول الكيتوزان مع حمض المالونيك و0,75 جم/لتر من محلول الكيتوزان مع ان كاربوكسى ميثيل ان و ان داى ايثيل بنزين أمونيوم كلوريد كما اوضحت الدراسة أنه بزيادة وقت التلامس تقل هذه الكميات المستخدمة من المحاليل الثلاثة. تم ايضا تحديد (MIC) للمحاليل الثلاثة وهو أقل تركيز من المادة يمكنه تثبيط البكتريا وقد كان 2,75 و 2 و 0,2ميكرو/مل وذلك للمحاليل الثلاثة على الترتيب.