N-halamines from rice straw

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Abstract The cellulosic part of rice straw was modified to develop N-halamine derivatives for disinfection. The process involved cross-linking of the cellulosic material with amino/amide/imide containing compounds; cyclic and acyclic. The structures of the prepared materials were identified using FTIR and solid state ^{13}CNMR. The modified materials were halogenated to form N-halamines and the antimicrobial activity of each evaluated against examples of Gram-positive (*Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli*) using a variety of methods; agar plate, blended agar, stirred flask and in columns. One of the N-halamines achieved a 9 log reduction against both *E. coli* and *S. aureus* in 4 hours. In addition, no *S. aureus* growth was recorded on agar plates blended with 0.5g of this same material.

Keywords: Bioactive materials, Cellulose, N-halamine, Bacteria, Cross-linking.

Introduction

The generation of straw waste constitutes a difficult environmental issue associated with rice production. Despite some suggested uses to reduce the overall waste, such as paper manufacturing, charcoal production, and conversion to animal food, this remains an issue (Mansour et al., 2007; Suramaythangkoor and Gheewala 2010). As a result, most rice-straw waste is burnt, producing significant air pollution associated with fume and smoke generation; the obvious carbon footprint derived from this activity. In rice producing countries, such as Egypt, this problem is evident each year during the rice production season (Abou Zeid et al., 2008; Garas et al., 2008). Air pollution affects not only the population's health but other sectors of the economy, such as tourism, and cultural heritage (damage to buildings and artifacts). We herein propose the use of waste rice cellulosic materials for water disinfection purposes; both as a means of dealing with the large amounts of rice waste, as well as, tackling access to healthy sources of water for the affected rural communities.

Cellulosic material from rice straw was extracted and cross-linked with amino/amide/imide containing compounds; cyclic and acyclic (AAICC; C/A), such as urea, barbituric acid and cynuric acid, followed by halogenation to form N-halamine biocidal materials. This method can be applied to any agricultural waste or food remains containing cellulosic material.

N-halamine polymers stabilize attached halogen moieties and deliver them to microorganisms that approach the polymers (Ahmed et al., 2008a, b, 2009, 2010, 2011a and b). They are usually prepared by loading heterocyclic rings that contain amide, imide or amino function groups which can be halogenated to the polymer backbone (Ahmed et

al., 2008a). When the bacterial cells come in contact or close association with the polymer, the halide ion can be exchanged between the polymer and the cell (Ahmed et al., 2009 and 2010). These polymers exchange halide ions with the cells by contact, release, and may change the nature of medium around the cells (Ahmed et al., 2009 and 2010).

In the literature there are reports of trials to prepare biocidal polymers by loading acyclic moieties to a polymer backbone that contains amide function groups, followed by halogenation, to form N-halamine textiles (Liu and Sun, 2006). We have developed N-halamine derived polymeric materials for biocidal applications (Ahmed et al., 2008a, b, 2009, 2010, 2011a and b) and now this expertise has been transferred to the halogenation of cellulosic material.

In this work we have used both cyclic and acyclic moieties, example in Fig. 1, in grafting to cellulose extracted from rice straw; to compare the reactivity of both of types. In addition, two further factors were considered; the associated costs of each moiety and their stabilization of the resulting halogen ion. The cross-linking agents, that would carry the halogen, were attached using one-step chemical transformations. Moieties containing more than two amino, amide or imide protons were selected for this job, example in Fig. 1. Two of the groups were used in the cross-linking process while the rest were left to carry the halide ion, Fig. 1. The novel modified cross-linking process will be supported by the presence of epichlorhydrin in a reaction through the cellulose hydroxyl groups as reported in the literature (Zhou et al., 2007; Simkovic, 1999; Nada and Hassan, 2005).

The antimicrobial activity of the halogenated form of these products was examined by challenge against bacteria to investigate the potential of such materials as

disinfectants. This was achieved by different methods; such as agar plate (Ahmed et al., 2008a), blended agar, a stirred flask and column methods (Ahmed et al., 2008b, 2009 and 2010). The stirred flask method was used to determine the effect of one of the prepared materials on bacterial viability and growth (Ahmed et al., 2008b). The prepared materials were also evaluated as potential disinfecting water filters using a column method, (Ahmed et al., 2008b).

Experimental

Materials

Rice straw was supplied from a farm in Sharkia Governorate, a region in the east Nile delta, Egypt. Sodium hydroxide, sodium hypochlorite (10% w/v) and cyanuric acid were supplied by Fisher Chemicals, UK. Epichlorohydrin, barbituric acid, semicarbazide, chloroacetamide and urea were supplied by Sigma-Aldrich Chemicals, UK.

Growth and maintenance of stock cultures

Staphylococcus aureus and *Escherichia coli* were obtained from the University of Surrey culture collection. Primary cultures were maintained on nutrient agar slopes stored at 4^{o}C. Experimental stocks of the bacteria were prepared by making subcultures of the primary culture on nutrient agar plates. Subcultures were grown at 37^{o}C for 24 hours and then stored at 4^{o}C.

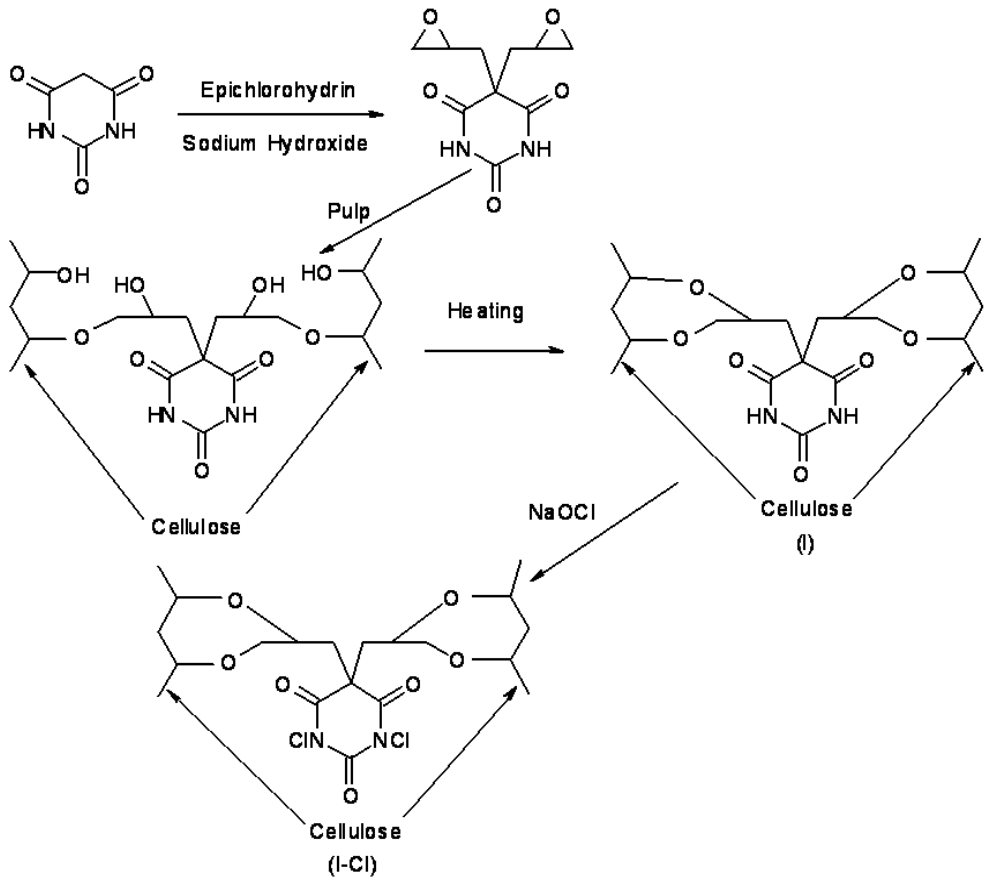


Fig. 1 Cross-linking with Barbituric acid

Cellulose extraction (pulping and bleaching)

Dry rice-straw (10g) was ground and soaked in sodium hydroxide solution (17.5%, 100ml) in a 250 ml round bottom flask. The suspension was refluxed for 1hr and the resulting material filtered, washed with distilled water to neutralization and dried at 100^{o}C for 24hrs. The cellulosic material was bleached using sodium hypochlorite (single stage bleaching) (Helmy and Abou-State, 1993). Chemical analysis of raw material showed that the ratios of α-cellulose, hemicellulose and lignin were 37.2, 24.7 and 16.2% respectively while after pulping they were 89.3, 8.6 and 0.8% respectively. After

bleaching the ratios of α-cellulose and hemicellulose were 90.3 and 0.6% respectively while the degree of polymerization was 762.6 and brightness 87.3%.

General method for cross-linking with Barbituric acid, Cyanuric acid, urea and semicarbazide

Amino/amide/imide containing compound was dissolved in sodium hydroxide solution (100ml, 0.7% w/v). Epichlorhydrin (1.7g) was added and the reaction heated at 60^{o}C for 30 min. The cellulosic material (1g) was added and the reaction continued for an extra 2 hours. The resulting grafted product was filtered, washed with distilled water and dried at 95^{o}C for 1 hour; then cured at 140^{o}C for another 1 hour to increase the cross-linking possibilities, Fig. 1. The amounts of AAICC; C/A (barbituric acid, cyanuric acid, urea and semicarbazide) were 1.2, 1.9, 0.6 and 0.7 respectively to produce samples I, II, III and IV respectively. FTIR and solid state ^{13}CNMR of the produced samples are given in Table 1.

Cross-linking with chloroacetamide

Dried cellulosic material (1g) was suspended in distilled water (100ml) and sodium hydroxide (0.7g) was added. The solution was cooled, chloroactamide (0.9g) added and the reaction stirred at 40^{o}C for 1hr. Epichlorohydrin (0.9 g) was added and the stirring continued for an extra 1hr. The temperature was then raised gradually to 60^{o}C and then to 80^{o}C over 2hrs. The resulting product was filtered, washed with distilled water, dried at 95^{o}C for 1hr and then cured for an extra 1 hour at 145 ^{o}C. FTIR and solid state ^{13}C NMR analysis for the product are in Table 1.

Table 1 FTIR and Solid state ^{13}C NMR analysis of different cross-linked pulps.

Sample	FTIR, υ (cm^{-1})	Solid state ^{13}C NMR
I	1666, 1697 and 3124	32, 41, 44, 62, 75, 84, 88, 105, 107, 117, 168 and 173
II	1670 (broad band) and 3126	31, 42, 47, 62, 75, 84, 87, 105, 107, 179 and 182
III	1692 and 3176	38, 40-55 (broad band), 65, 77, 85, 90, 108 and 163
IV	1660, 1597, 3187 and 3282	35, 45, 52, 65, 78, 82, 85, 87, 109 and 170
V	1618 and 3149	36, 45-55 (broad band), 65, 68, 69, 77, 82 90,108 and 164

Where: I = pulp cross-linked with barbituric acid, II = pulp cross-linked with cynuric acid, III = pulp cross-linked with urea, IV = pulp cross-linked with semicarbazide and V = pulp cross-linked with chloroactamide.

Chlorination

Prepared samples of different products (I-V) were halogenated by soaking 1g of each sample in sodium hypochlorite (10 ml, 10%) for 1hr at ambient temperature. The resulting samples were filtered, washed copiously with distilled water and dried at 40^{o}C overnight. The halogen content was determined using iodometric titration, Table 2

(Ahmed et al., 2008a; Chen and Sun 2006), and the halogenation process was followed by FTIR, Table 2 (Ahmed wt al., 2008a; El-Masry et al., 2004a and b).

Table 2 Halogen content and FTIR characterization of each chlorinated sample

Sample number	Halogen content (ppm)	υ_{max} (cm^{-1}) of N-Cl in each halogenated material
I-Cl	112 ±10	805
II-Cl	123 ± 14	812
III-Cl	164 ± 12	775
IV-Cl	56 ± 11	760
V-Cl	60 ± 9	763

Biological activity

Agar plate method

Nutrient agar (Oxoid) was prepared (250 ml), held molten at 50^{o}C and 1.0 ml of a 17 h nutrient broth culture of either *S. aureus* or *E. coli* was added. The seeded agar was poured into Petri dishes; 3 dishes for each sample (triplicates) per bacterium type (*E. coli* or *S. aureus*). Wells (5 mm) were cut into the agar in the middle of each dish. Small amounts of each sample (0.05 g) were placed in the corresponding well. The dishes were incubated for 24 hr at 37^{o}C and the inhibition zones around the sample were recorded, Table 3 (Ahmed et al., 2008a; El-Masry et al., 2004a and b).

Blended agar method

Molten nutrient agar was cooled to around 40^{o}C and blended (15 ml each) with different amounts of III-Cl; 0.0, 0.25, 0.5, 1.0, 1.5 and 2 g before pouring in plates. Bacterial suspensions of *E. coli* and *S. aureus* were prepared by inoculating 10 ml nutrient broth with a single colony and incubating for 17 hours at 37^{o}C. The viability of each bacterium was determined on the blended plates using the 'Miles and Misra' method (Miles and Misra, 1938). Two controls were applied; counting on non-blended agar plates and agar plates blended with the non-halogenated form of the material (III), Figs 2 and 3.

Table 3 Inhibition zones diameters (mm) around different grafted samples on agar dishes containing Gram-positive or Gram-negative bacteria.

Sample number	*E. coli*	*S. aureus*
I-Cl	6 ±0	9 ±1
II-Cl	6 ±1	8 ±1
III-Cl	10 ±2	12 ±0
IV-Cl	ND	ND
V-Cl	ND	ND

Fig. 2 Counts on agar blended with III-Cl compared to counts on normal agar and agar without any cells.

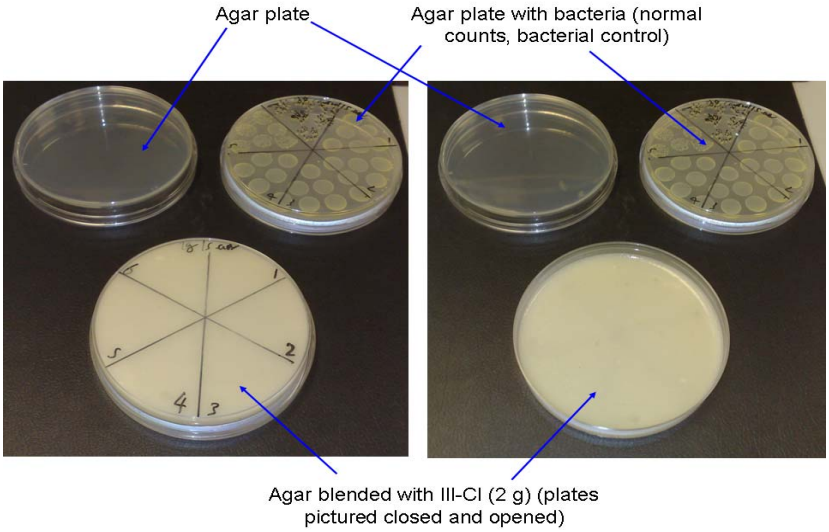
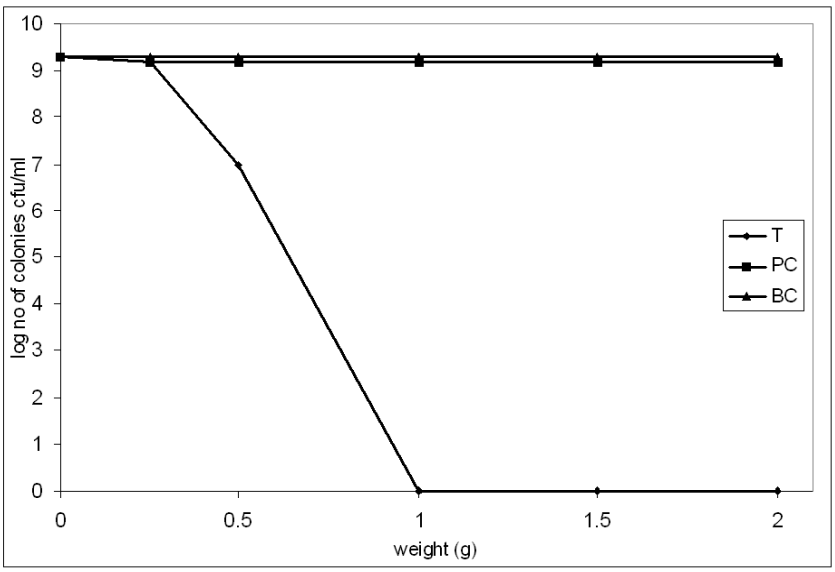
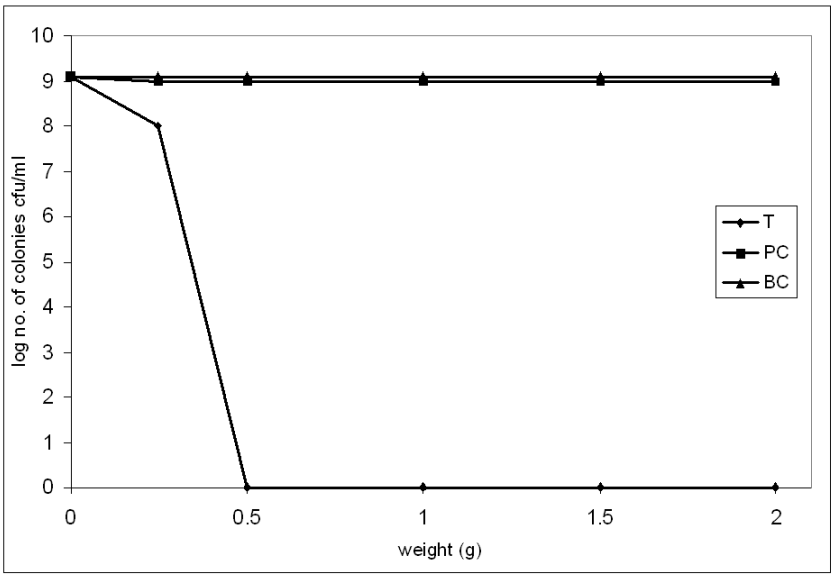


Fig. 3 Effect of blended agar with III-Cl on the viability of *E. coli* (a) *and S.aureus* (b). Where T = plates blended with III-Cl, PC = III (non-halogenated material as a control) and BC = bacterial control (no material). Error bars have been removed as the error is too small to show on a log scale.



(a)



(b)

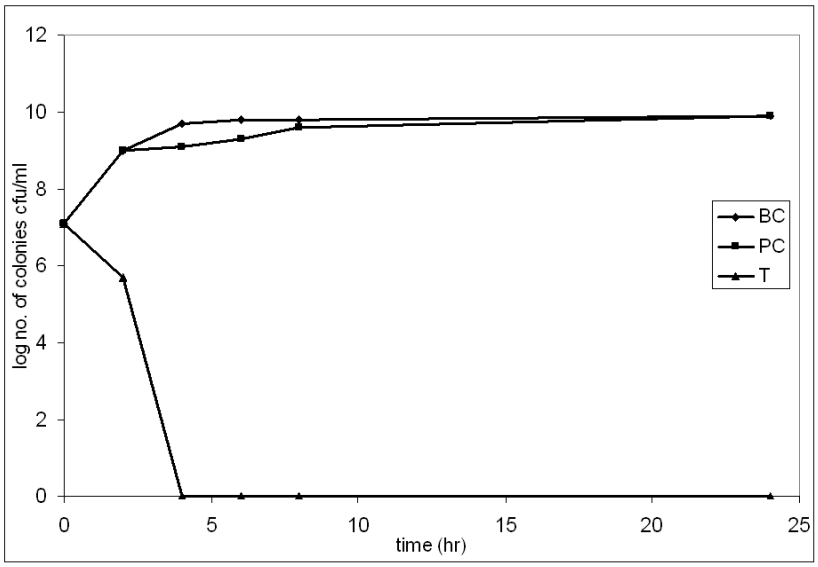
Stirred flask method

A culture of *E. coli* was prepared by inoculating one bacterial colony into 20 ml of nutrient broth in a Universal bottle and incubating for 17 hr at 37^{o}C. From the bacterial suspension, 0.1 ml was transferred to a 20 ml Universal bottle containing 10 ml of fresh medium. Six Universals were prepared; three used in testing the effect of the halogenated material on bacterial growth and the other three to test the effect on bacterial viability.

To study the effect on *E. coli* growth rate, 0.5 g of the halogenated material (III-Cl) was added to the first bottle of broth while 0.5 g of the control material (non-halogenated, III) was added to the second bottle, and the third was left as a bacterial control without any material. The three bottles were stirred at 37^{o}C and sampled at timed intervals for viable count, employing the 'Miles and Misra' technique, Fig. 4 (Miles and Misra, 1938)

To investigate the effect of III-Cl on *E. coli* viability, the other three bottles, inoculated as above, were incubated for 17 hr at 37^{o}C, and the number of bacteria determined by viable count. At this time 0.5 g of III-Cl was added to one bottle; 0.5 g of the control material (non-halogenated, III) was added to the second, and the third vessel was left as a bacterial control. The three bottles were stirred at ambient temperature, and samples from each culture taken for viable count at regular time intervals, as previously, Fig 5. The procedure was repeated to test the effect of III-Cl on a Gram-positive bacterium (*S. aureus*) (Ahmed et al., 2008b and 2009).

Fig. 4 Effect of III-Cl on the growth rate of *E. coli* (a) and *S. aureus* (b). Where T = plates blended with III-Cl, PC = III (non-halogenated material as a control) and BC = bacterial control (no material). Error bars have been removed as the error is too small to show on a log scale.



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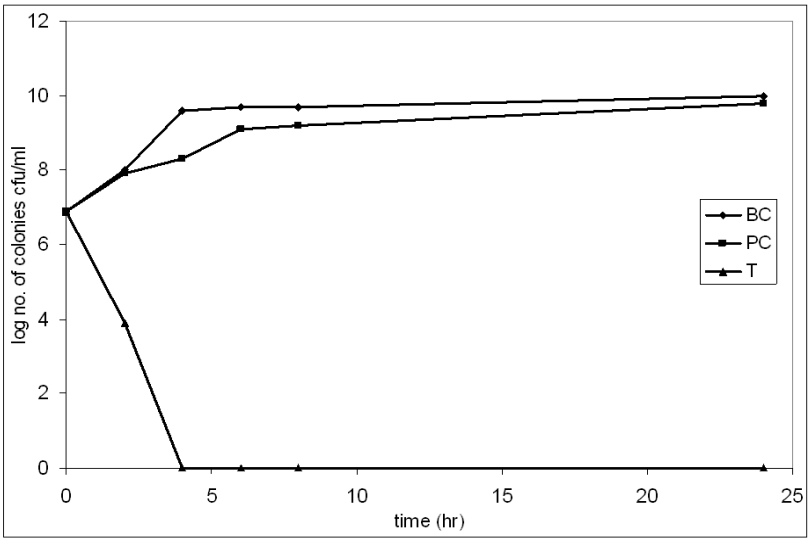
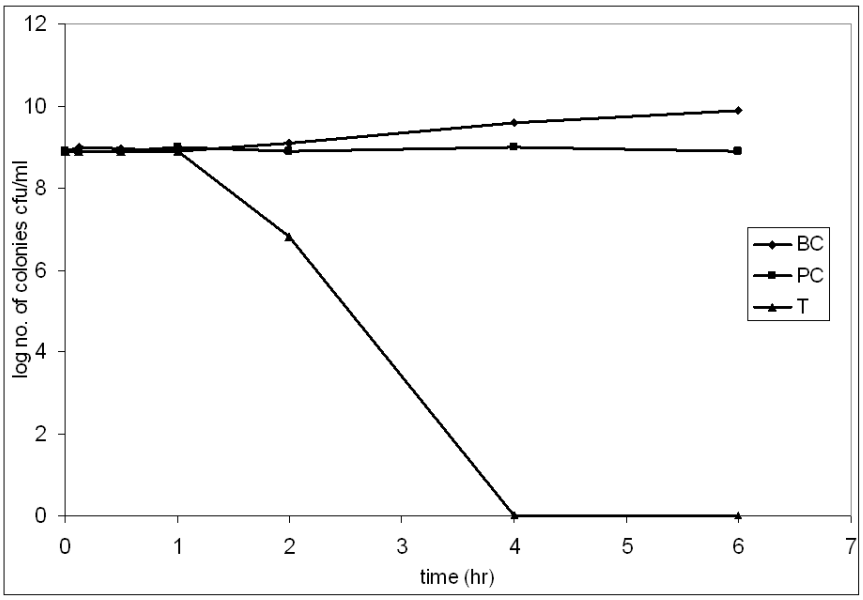
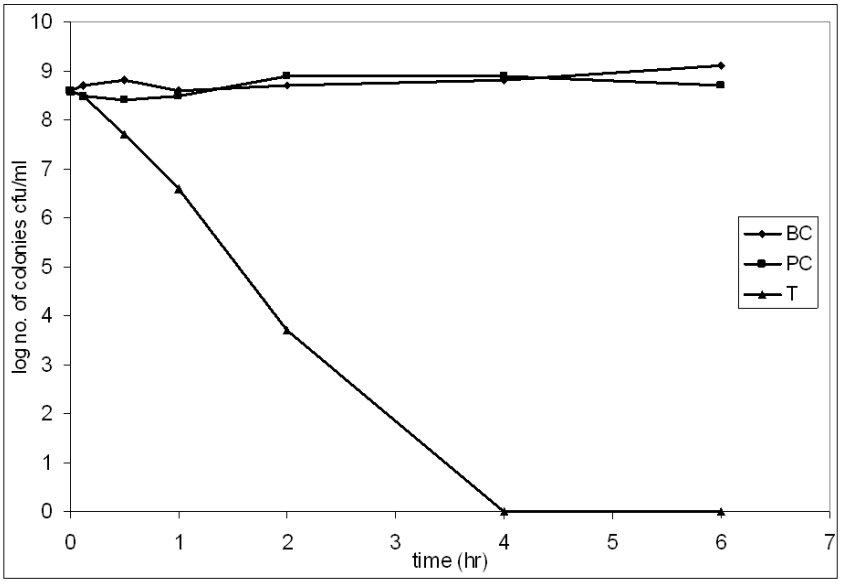


(b)

Fig. 5 Effect of III-Cl on the viability of *E. coli* (a) and *S. aureus* (b). Where T = III-Cl, PC = III (non-halogenated material as a control) and BC = bacterial control (no material). Error bars have been removed as previously.



(a)



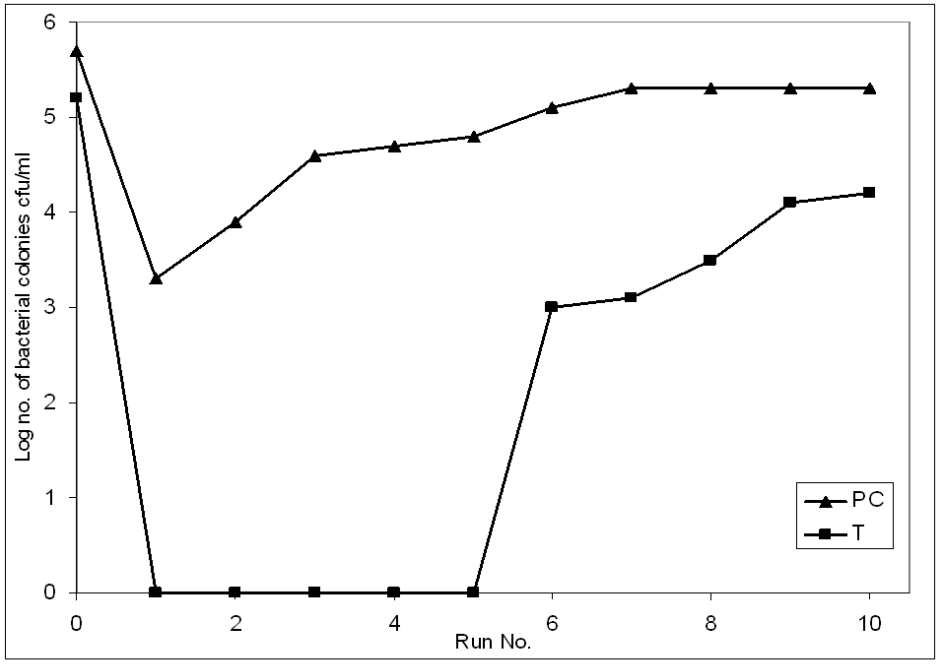
(b)

Column method

Two glass tubes (10 cm length X 2.5 cm diameter) were loaded with III-Cl**,** 5.0 g each (columns C1 and C2), while two similar columns were filled with III to be used as controls (columns C3 and C4). Bacterial suspensions, either *E. coli* or *S. aureus,* were prepared by inoculating 100 ml of liquid medium followed by incubation for 17 hours at 37^{o}C. *E. coli* suspension (concentration up to 2.4 X 10^{7}cfu/ml) was passed through columns C1 and C3 (50 ml each) and the output from the column recycled through it again up to 10 times (recycling the same suspension through the same column). Before recycling, 0.1 ml from the passed liquid was sampled for viable count. The same protocol was followed for *S. aureus* (concentration up to 1.3 X 10^{7}cfu/ml) using columns C2 and C4 (Ahmed et al., 2008b).

The same method was followed to identify the useful lifetime of III-Cl in water filters. In this case the above experiment was repeated up to 10 times (runs) and in each run a fresh bacterial suspension with concentrations up to 10^{5}cfu/ml of *E. coli* was used. In addition, each run includes 10 recycling times for the same suspension before changing to the next run using a fresh suspension. Viable counts were performed at the end of each run, example in Figure 6 (Ahmed et al., 2011b). At the end of the 10 runs, and to investigate the regeneration possibilities of the column, the column was washed copiously with sodium hypochlorite (10%) followed by washing with distilled water. The column was then closed, filled with sodium hypochlorite and left for 6 hours followed by washing copiously with distilled water. The lifetime determination experiment was repeated, as described above, up to 3 times (Ahmed et al., 2011b).

Fig. 6 Different runs of *E. coli* through III-Cl column. Where T = III-Cl, PC = III (non-halogenated material as a control). Error bars have been omitted as the error is too small to show on a log scale.



Results and discussion

Burning rice straw results in many health problems to the people who live in those countries where rice is a major crop such as Egypt. Many applications (such as production of paper, animal food, fertilizers and bio-fuel) have been suggested as uses for such waste but the amount of straw produced remains too large (Navaee-Ardeh et al., 2004; Prasad et al., 1998; Sallam et al., 2007; Toor and Beri, 1991; Chou et al., 2009).

In our trials to produce low cost disinfectants, mainly for water treatment (Ahmed et al., 2011b), we have found that rice straw waste can be a good source for cellulose that can be modified to produce polymers with antimicrobial properties. Their commercial

value starts from the fact that rice straw is currently freely available from farming communities. In addition, most of the rice producing countries, especially in the third world, have problems with inadequate supplies of clean drinking water. Therefore, converting rice straw into a product with potential for use in water purification systems results in an especially suitable way to address the problem of rice straw waste; thereby reducing air pollution and providing a means of disinfecting drinking water in affected communities.

As a result of this idea, cellulosic material was extracted from rice straw and modified as N-halamine polymeric materials. The cellulose was extracted using sodium hydroxide for one hour followed by bleaching using sodium hypochlorite. The resulting material was cross-linked with AAICC; C/A that contained more than 2 amide or imide function groups. Two of these function groups were used to achieve cross-linking to cellulose in the presence of epichlorohydrin while the rest of the function groups were left for halogenation. Compounds with an active methylene group such as barbituric acid can be used to achieve cross-linking in a similar manner to the amide or imide groups.

At the same time, the process was performed using cyclic and acyclic compounds to compare reactivity and production costs. Other factors, such as halogen stability were also taken into consideration, as cyclic structures usually stabilize the halide ions attached to it more than those of acyclic (Sun et al., 1996). Cynuric acid and barbituric acid, that contain more than two amide/imide groups, were used as examples for the cyclic compounds. Barbituric acid has an active methylene group with 2 hydrogens which can facilitate cross-linking leaving the other 2 imide groups free for halogenation, Fig. 1. Cynuric acid has 3 imide groups; two of them can perform the cross-linking leaving the

third for halogenation. Cross-linking was performed usually in the presence of epichlorhydrin; acting as a connector between the rings and the cellulose hydroxyl groups.

FTIR and solid state ^{13}C NMR were used to investigate the success of the grafting process, Table 1. From the FTIR data it was noticed that for all samples the NH amide or imide band appears in the region 3100-3200 cm^{-1}, while the C=O bands usually appear in the region from 1600-1700 cm^{-1}. Solid state $^{13}CNMR$ showed that the cellulosic ring carbons usually appear in the region from 60-110 ppm while the carbons of the epichlorhydrin part appear in the region 30-55 ppm, Table 1. In addition, the carbonyl carbons usually appear in the region form 160-180 ppm.

All prepared materials and the corresponding N-halamine polymeric material reacted with halide, Table 2. It can be seen from Table 2 that the lowest charged material with halogen was the pulp cross-linked with semicarbazide (IV) and with chloroacetamide (V). We believe that this is because both of them have free amide (not sharing in the cross-linking process) that may be broken during halogenation or curing. In the case of chloroacetamide (V), epichlorohydrin addition was performed while the cellulosic material was in the reaction container. This may affect the reaction because there is a possibility that epichlorhydrin may perform cross-linking to the cellulose itself without including the amide of chloroacetamide. This may have resulted in the presence of free amide in compound (V) similar to that of (IV) which can be decomposed during the halogenation, resulting in low halogen load and low biological activity. Currently we are exploring connecting epichlorohydrin to chloroacetamide before reaction with cellulosic material. This has affected the antimicrobial activity of compounds IV and V,

as can be seen from the agar plate method to determine the antimicrobial activity, Table 3. All prepared materials showed antimicrobial activity against both Gram-positive and Gram-negative bacteria except the materials cross-linked with chloroacetamide and semicarbazide, Table 3. From Table 3 it can be seen that cross-linked material III has the highest biological power, as it was carrying the highest chlorine content, Table 2; acyclic amides can release halogen more readily than cyclic amides. Cyclic amides (as in I and II) can stabilize the halogen more than acyclic amides. Moreover, connecting AAICC; C/A to cellulose through a carbon chain may help in stabilizing the halogen attached to the compound due to the electron donating action of the methyl groups.

Further investigations were performed to determine the antimicrobial activity of one of the prepared materials; III-Cl. This particular compound was selected due to its high antimicrobial power in the agar plate experiment and also due to its expected low production costs.

III-Cl was blended with nutrient agar before pouring as plates. The blending was achieved with different amounts 0.0, 0.25, 0.5, 1.0, 1.5 and 2 g per 15ml nutrient agar. Bacterial counting using the 'Miles and Misra' method was performed on the surface of the blended agar, Fig. 2. From Fig. 3, it can be seen, as expected, that increasing the amount of III-Cl increased the antimicrobial action. One gram per 15ml nutrient agar was enough to inhibit the growth of *E. coli*, Fig. 3a, while 0.5 g/15ml nutrient agar achieved the same goal for *S. aureus*, Fig. 3b. These results are encouraging and indicate that there may be other applications for this product - blended in paints and surface coatings to confer antimicrobial properties.

Figs 5a and 5b shows the effect of III-Cl on the viability of grown cultures of *E. coli* and *S. aureus* respectively. III-Cl achieved a 9 log reduction for both types of bacteria in 4 hours. At the same time no growth from either was detected in the presence of III-Cl, Figs 4a and 4b.

A column method was used to show the ability of using such material in one of its suggested applications; water filters. Bacterial suspensions (either *E. coli* or *S. aureus*) were perfused through a column contains III-Cl and the outlet was recycled for up to 10 times through the same column. Viable counts after each perfusion through the column indicated no detection. These results show that the column succeeded in disinfecting bacterial suspensions with concentrations up to 10^7 cfu/ml for both *E. coli* and *S. aureus*. The same method was repeated for several runs (10 runs) to indicate the useful lifetime of the column. The column stayed active for more than 5 runs, Figure 6, indicating that it must be reloaded with halogen at this stage to be regenerated. To study the regeneration possibilities the column was charged with halogen after the end of its lifetime and the experiment of identifying the lifetime was repeated 3 times. It was found that the regeneration process was successful and the column was regenerated successfully without significant loss in its antibacterial action during those 3 repeats.

The above results showed that preparation of cross-linked materials was successful and some of them have antimicrobial activity. Moreover, demonstrating that the material has antimicrobial action, using agar plate and stirred flask methods, before testing the material in columns indicated that the material is bactericidal; the effect in columns is not due to a filtration effect only. Connecting AAICC; C/A to cellulose through a carbon chain may aid stabilizing the halogen attached to the amide or imide

groups. Moreover, stability of halogen attached to cyclic moieties is greater than that to acyclic. However, acyclic moieties can be charged with more halogen and subsequently release it more readily than cyclic ones. The compounds with free amide groups have a low halogen content and consequently low biological activity.

Considering the economics of the processes, despite the evidence that cyanuric and barbituric acids confer greater stability on the halogen, employing urea as the cross-linker is more cost effective. Cyanuric acid can be used in the second stage as it could be prepared from urea (El-Masry et al., 2004b). For future development of these materials for field use, equilibrium has to be struck between stability and cost.

The above results encourage the development of such materials for a wide range of applications where disinfection is important; such as surface coats and in water filters. The environment is served in two important aspects: by reducing microbial growth and contamination of water systems and by reducing the amount of atmospheric pollution due to the current practice of burning-off rice-straw in the fields.

Conclusion

Novel N-halamine cellulosic materials were prepared and their structure elucidated using FTIR and solid state ^{13}CNMR. Some of the prepared materials achieved good antimicrobial action in an agar plate method. Further antimicrobial investigations were followed for one of the prepared materials (III-Cl). This compound succeeded in inhibiting bacterial growth when both blended into agar and also in a stirred flask method; achieving a 9 log reduction in viable bacterial count in 4 hours. III-Cl was used successfully in columns as model water filters and its functional lifetime and regeneration possibilities determined.

Acknowledgments

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